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Ο ρόλος του υαλουρονικού οξέος στην ενεργοποίηση των κερατινοκυττάρων από αλλεργιογόνα επαφής.

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The role of Hyaluronic acid in keratinocyte activation by contact allergens

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2. Kavasi, R.M., Berdiaki, A., Spyridaki, I., Corsini, E., Tsatsakis, A., Tzanakakis, G., Nikitovic, D., 2017. HA metabolism in skin homeostasis and inflammatory disease. Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association 101, 128-138.

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Ευχαριστίες

Ολοκληρώνοντας τη διδακτορική μου διατριβή θα ήθελα να ευχαριστήσω όλους όσοι με βοήθησαν, καθένας με τον δικό του τρόπο. Πρωτίστως, την επιβλέπουσά μου επίκουρη καθηγήτρια Ντράγκανα Νικίτοβιτς για τη συνεργασία όλο αυτό το διάστημα, καθώς μου έδωσε την ευκαιρία να ασχοληθώ με μία ενδιαφέρουσα μελέτη και να εξελιχθώ σε ερευνητικό επίπεδο. Τον καθηγητή Γεώργιο Τζανακάκη που ως επιβλέπων της πτυχιακής μου με δέχτηκε στο εργαστήριο Ιστολογίας-Εμβρυολογίας. Στη συνέχεια, θα ευχαριστήσω τον καθηγητή Αριστείδη Τσατσάκη για την υποστήριξη, καθώς και την καθηγήτρια Emanuela Corsini μέλη της τριμελούς συμβουλευτικής επιτροπής, που βοήθησαν στην εξέλιξη και ολοκλήρωση της παρούσας διατριβής. Επιπλέον, θα ήθελα να ευχαριστήσω τα υπόλοιπα μέλη της επταμελούς εξεταστικής επιτροπής που συμμετείχαν στην αξιολόγησή μου: τους Καθηγητές Κωνσταντίνο Κρασαγάκη και Μαρία Τζαρδή, την Αναπληρώτρια Καθηγήτρια Μαρία-Ελένη Καμπά και τον Επίκουρο Καθηγητή Μανώλη Τζατζαράκη.

Ακολούθως, θα ήθελα να ευχαριστήσω τα μέλη του εργαστηρίου με πρώτη την Δρ Αικατερίνη Μπερδιάκη για την καθοδήγησή της. Ιδιαίτερες ευχαριστίες και στην κολλητή μου φίλη Δρ Βουδούρη Καλλιρρόη, από τους πρώτους ανθρώπους που γνώρισα μόλις ξεκίνησα τις σπουδές μου στο Ηράκλειο, για τις συμβουλές της όλα αυτά τα χρόνια. Δε θα μπορούσα να παραλείψω της διδακτορικές φοιτήτριες Ειρήνη-Μαρία Γιαταγάνα και Γεωργία Φανουράκη και την προπτυχιακή φοιτήτρια Εμμανουέλλα Περισυνάκη με τις οποίες πέρα από την αλληλεπίδραση σε επαγγελματικό επίπεδο, δημιουργήσαμε μία όμορφη παρέα που έκανε την καθημερινότητα στο εργαστήριο πιο ευχάριστη. Επίσης, ευχαριστώ και τα παλαιότερα μέλη του εργαστηρίου και φίλες μου Ειρήνη-Μαρία (Ρενάτα) Μωραΐτη και Κωνσταντίνα Αντωνιάδου για τη στήριξή τους ακόμα και μετά την αποχώρησή τους από το εργαστήριο. Στα παλαιότερα μέλη του εργαστηρίου που θα ήθελα να ευχαριστήσω ανήκουν και η Δρ Κατερίνα Κουβίδη και Δρ Μαρία Μυτιληναίου. Ιδιαίτερες ευχαριστίες και στους φοιτητές Ιατρικής Θεόδωρο Κρασανάκη, Αντώνιο Κωστούρο, Αγγελική Βουϊδάσκη και Γεώργιο Σαριδάκη, για την εθελοντική συμμετοχή τους στο

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Φυσικά το μεγαλύτερο «ευχαριστώ» το οφείλω στους γονείς μου Θεοχάρη και Όλγα στους οποίους οφείλω τα πάντα. Όλα τα χρόνια των σπουδών μου με στήριζαν ψυχολογικά και οικονομικά και ήταν «δίπλα» μου, παρόλο που βρίσκονταν στην άλλη άκρη της Ελλάδας. Οι λέξεις και ένα απλό «ευχαριστώ» θα ήταν πολύ λίγο.

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Abstract

Allergic contact dermatitis (ACD) is the most common dermatopathy and is caused by topical exposure to chemical allergens. ACD is a type IV (delayed) hypersensitivity response resulting in sterile inflammation (Tan et al., 2014). The term “sterile” characterizes inflammatory responses not caused by pathogens/ microbes (Chen and Nunez, 2010; Kurbet et al., 2016). Importantly, almost 20% of the general population presents ACD to at least one or more contact allergens (Peiser et al., 2012). The symptoms include erythema, vesiculation, scaling and itchiness. Contact allergens are haptens, low molecular weight chemicals (<1000Da) (Martin, 2012) normally unable to trigger immune responses (Corsini and Kimber, 2007). Hence, binding of haptens to self- macromolecules is crucial for the initiation of inflammation (Murphy et al., 2019). The complexity of the immune system may sometimes result in a “mistaken” response to haptens (McFadden et al., 2012). P-phenylenediamine (PPD) and 2,4 dinitrochlorobenxene (DNCB) are common contact sensitizers, responsible for many cases of ACD (Milam et al., 2019; Saarnilehto et al., 2014). They were previously shown to trigger the production of IL-18, a reliable marker of keratinocyte sensitization (Galbiati et al., 2014; Nikitovic et al., 2015). Epidermal keratinocytes play a key role in innate immunity, affect the skin inflammatory responses and are crucial for ACD progression (Corsini et al., 2013). The extracellular matrix (ECM) that provides tissue structure, specificity and organization, regulates as well cellular functions and responses, such as inflammation by triggering specific signaling pathways (Nikitovic et al., 2013; Kavasi et al., 2017). Hyaluronic acid or Hyaluronan (HA), an important ECM component, is a linear non-sulfated glycosaminoglycan, composed of repeating units of N-acetylglucosamine (GlcNAc) and glucuronic acid. HA is well established to participate in a wide variety of biological functions. The metabolism of HA has been demonstrated to play an important role in sterile inflammation (Kavasi et al., 2017). Indeed, HA deposition has been demonstrated to be altered, upon keratinocyte treatment with PPD and DNCB contact sensitizers. Notably, Low molecular weight Hyaluronan (LWMHA) is able to trigger IL-18 production (Nikitovic et al., 2015). HA is synthesized by specific enzymes called Hyaluronan

Synthases (HAS1, HAS2 and HA3). The process takes place on the plasma membrane where these enzymes are located, with simultaneous secretion of the HA chain to the ECM. HA degradation is performed by enzymes denominated, Hyaluronidases (HYAL1 and HYAL2). In the skin HA is synthesized mainly by dermal fibroblasts, but also by epidermal keratinocytes (Kavasi et al., 2017). Toll- like receptors (TLRs) are a family of transmembrane receptors, widely known for their roles in innate immunity. Toll-like receptor 4 (TLR4), is strongly implicated in skin inflammation with the ability to bind Damage Associated Molecular Patterns (DAMPs), including LMWHA (Termeer et al., 2002; Kuzmich et al., 2017). DAMPs are endogenous molecules, with the ability to act as “danger signals,” that trigger cellular responses and they have strongly been correlated to the process of sterile inflammation (Chen and Nunez, 2010; Hernandez et al., 2016). A category of DAMPs are the Reactive Oxygen Species (ROS), chemically active small molecules, resulting from incomplete reduction of oxygen (Kubert et al., 2016; Nastase et al., 2017). Indeed, ROS have been characterized as mediators of inflammation (Mittal et al., 2014) and noteworthy they can degrade HA chemically. TLR4 activation results in the downstream triggering of nuclear factor kappa-beta (NF-κB), the transcription factor, established to regulate the transcription of pro- inflammatory cytokines (Kaplan et al., 2012). In its inactive form this transcription factor is located to the cytoplasm, while in its activated form, translocates to the nucleus engaged in the transcription of several genes (Akira and Takeda, 2004). NF-κB has been strongly correlated to innate immunity and inflammation (Kaplan et al., 2012). In fact, several therapeutic approaches for inflammation target the inhibition of TLR4/ NF-κB signaling (Huang et al., 2014; Gomes et al., 2015). In the present study, the putative co- operation of HA and TLR4 in the process of contact allergen-induced keratinocyte activation was investigated. Specifically, contact sensitizers PPD and DNCB were shown, in NCTC2544 human keratinocytes, to significantly increase the expression of the TLR4 receptor in a concentration dependant manner, as demonstrated by western blot and Real-time PCR. The contact allergens were also demonstrated to induce HAS1, HAS2 and HAS3 overexpression at both the protein and mRNA level. These data suggest that the contact sensitizers enhance HMWHA production by the

upregulation of HAS isoforms. Thus, the previously shown contact sensitizer mediated HYALs upregulation (Nikitovic et al., 2015) in combination to ROS action, will increase endogenous LMWHA release. Furthermore, LMWHA treatment of keratinocytes induced increased expression of not only the TLR4 receptor, but also of the HAS1 and HAS3 enzymes. This observation implies that the increased release of LMWHA by the activated keratinocytes will result in enhanced HMWHA deposition, available for degradation to LMWHA and the subsequent TLR4 signaling, forming a loop for the sustaining of inflammation. Moreover, downregulation of TLR4, upon RNA interference utilization, resulted in an attenuation of HAS1 and HAS3 basal levels, suggesting a direct correlation between HA synthesis and TLR4 signaling. PPD and DNCB effects on HA metabolism, were shown to be partly executed through the TLR4 downstream signaling. Indeed, blocking of the TLR4 receptor with a neutralizing antibody, resulted in attenuated contact allergen mediated HYAL and HAS upregulation. Furthermore, PPD and DNCB stimulated the activation of the TLR4 downstream mediator NF-κB as well as its translocation to the nuclei of keratinocytes. Blocking of TLR4 activities reduced NF-κB activation. Additionally, LMWHA treatment enhanced NF-κB activation, whereas blocking HYALs' action with an inhibitor (aristolochic acid) attenuated the contact allergen mediated NF-κB activation. In conclusion, keratinocyte sensitization by the PPD and DNCB contact allergens is partly mediated *via* a LMWHA/ TLR4/ NF-κB signaling axis.

Περίληψη

Η αλλεργική δερματίτιδα εξ επαφής (ACD) είναι η πιο συχνή δερματοπάθεια και προκαλείται από την επαφή του δέρματος με χημικά αλλεργιογόνα. Αποτελεί μία αντίδραση υπερευαισθησίας τύπου IV (επιβραδυνόμενη), της οποίας το αποτέλεσμα είναι η «στείρα» φλεγμονή (Tan et al., 2014). Ο όρος «στείρα» αντιπροσωπεύει φλεγμονώδεις αποκρίσεις που δεν προκαλούνται από παθογόνα/ μικρόβια (Chen and Nunez, 2010; Kurbet et al., 2016). Σχεδόν το 20% του γενικού πληθυσμού παρουσιάζει αλλεργική δερματίτιδα σε τουλάχιστον ένα αλλεργιογόνο, ή ακόμη και σε περισσότερα (Peiser et al., 2012). Στα συμπτώματα συγκαταλέγονται ερυθρότητα, φυσαλιδώδες δέρμα, απολέπιση και κνησμός. Τα αλλεργιογόνα που προκαλούν δερματίτιδα εξ επαφής αποτελούν απτένια, δηλαδή ατελή αντιγόνα που είναι χημικά χαμηλού μοριακού βάρους (<1000Da) (Martin,2012) τα οποία υπό φυσιολογικές συνθήκες αδυνατούν να προκαλέσουν ανοσολογικές αποκρίσεις (ατελή αντιγόνα) (Corsini and Kimber, 2007). Επομένως, για να πυροδοτηθεί η διαδικασία της φλεγμονής, είναι απαραίτητη η πρόσδεση των απτενίων με μακρομόρια του εαυτού (Murphy et al., 2019). Η πολυπλοκότητα του ανοσοποιητικού συστήματος είναι πολύ πιθανό να οδηγήσει σε «λανθασμένες» αποκρίσεις στα απτένια (McFadden et al., 2012). Δύο συνηθισμένα αλλεργιογόνα υπεύθυνα για πολλές περιπτώσεις δερματίτιδας εξ επαφής είναι η παραφενυλενεδιαμίνη (PPD) και το 2,4 δινιτροχλωροβενζένιο (DNCB) (Milam et al., 2019; Saarnilehto et al., 2014). Σε προηγούμενες μελέτες έχει δειχθεί η ιδιότητά τους να προκαλούν στα κερατινοκύτταρα την παραγωγή ιντερλευκίνης- 18 (IL-18), η οποία αποτελεί αποδεκτό δείκτη για το χαρακτηρισμό ουσιών που προκαλούν «υπερευαισθησία εξ επαφής» (Galbiati et al., 2014; Nikitovic et al., 2015). Τα κερατινοκύτταρα της επιδερμίδας διαδραματίζουν σημαντικό ρόλο στην έμφυτη ανοσία, επηρεάζουν φλεγμονώδεις αποκρίσεις, επομένως, είναι εξαιρετικής σημασίας για την εξέλιξη της αλλεργικής δερματίτιδας εξ επαφής (Corsini et al., 2013). Η εξωκυττάρια θεμέλια ουσία (ECM) η οποία καθορίζει στους ιστούς τη δομή τους, την ιστοειδικότητα και την οργάνωση, ρυθμίζει πολλές κυτταρικές λειτουργίες και αποκρίσεις, για παράδειγμα πρόκληση φλεγμονής, καθώς πυροδοτεί την ενεργοποίηση συγκεκριμένων σηματοδοτικών μονοπατιών

(Nikitovic et al., 2013; Kavasi et al., 2017). Ένα ιδιαίτερα σημαντικό συστατικό της εξωκυττάριας θεμέλιας ουσίας είναι το υαλουρονικό οξύ, δηλαδή μία γραμμική μη θειωμένη γλυκοζαμινογλυκάνη. Συντίθεται από επαναλαμβανόμενες μονάδες Ν-ακετυλ-γλυκοζαμίνης (GlcNAc) και γλυκουρονικού οξέος. Το υαλουρονικό οξύ είναι γνωστό για την πληθώρα των βιολογικών λειτουργιών που επηρεάζει και μάλιστα, συχνά αυτές οι λειτουργίες είναι αντίθετες μεταξύ τους, καθώς εξαρτώνται από το μοριακό βάρος του υαλουρονικού. Ο μεταβολισμός του υαλουρονικού οξέος διαδραματίζει σημαντικό ρόλο στη στείρα φλεγμονή (Kavasi et al., 2017). Επιπρόσθετα, η επώαση κερατινοκυτταρων με τα αλλεργιογόνα PPD και DNCB είναι υπεύθυνη για αλλαγές στην τοποθέτηση του υαλουρονικού. Επιπλέον, το χαμηλού μοριακού βάρους υαλουρονικό (LWMHA) προκαλεί παραγωγή ιντερλευκίνης- 18 (Nikitovic et al., 2015). Η σύνθεση του υαλουρονικού οξέος γίνεται από κατάλληλα ένζυμα που ονομάζονται συνθάσες του υαλουρονικού (HAS1, HAS2 and HA3). Η σύνθεση πραγματοποιείται στην κυτταρική μεμβράνη όπου εδράζονται τα συγκεκριμένα ένζυμα, με ταυτόχρονη έκκριση του μακρομορίου στην εξωκυττάρια θεμέλια ουσία. Η αποικοδόμησή του γίνεται από μία άλλη κατηγορία ενζύμων τις υαλουρονιδάσες (HYAL1 και HYAL2). Η σύνθεση του υαλουρονικού στο δέρμα γίνεται κυρίως από τους ινοβλάστες της δερμίδας, αλλά και από τα κερατινοκύτταρα της επιδερμίδας (Kavasi et al., 2017). Οι Toll- like υποδοχείς (TLRs) αποτελούν μία οικογένεια διαμεμβρανικών υποδοχέων, ευρέως γνωστών για τους πολλαπλούς ρόλους τους στην έμφυτη ανοσία. Μεταξύ των μελών αυτής της κατηγορίας υποδοχέων, ιδιαίτερη σημασία έχει ο TLR4, ο οποίος έχει συσχετιστεί με τους μηχανισμούς φλεγμονής του δέρματος. Χαρακτηριστικό του είναι η ιδιότητά του να προσδένει μοριακά μοτίβα που σχετίζονται με βλάβες (DAMPs), όπως είναι το χαμηλού μοριακού βάρους υαλουρονικό οξύ (Termeer et al., 2002; Kuzmich et al., 2017). Τα DAMPs είναι ενδογενή μόρια που δρουν ως «σήματα κινδύνου» και πυροδοτούν διάφορες κυτταρικές λειτουργίες και μάλιστα έχουν συσχετιστεί με ασθένειες στείρας φλεγμονής (Chen and Nunez, 2010; Hernandez et al., 2016). Μία συγκεκριμένη κατηγορία DAMPs είναι μικρά χημικά ενεργά μόρια που προκύπτουν από ατελή αναγωγή του οξυγόνου και ονομάζονται δραστικές μορφές οξυγόνου (ROS) (Kubert et al., 2016; Nastase et al., 2017). Μάλιστα,

έχουν χαρακτηριστεί ως ρυθμιστές της φλεγμονής (Mittal et al., 2014) και έχουν την ικανότητα να αποικοδομούν χημικά το υαλουρονικό οξύ. Η ενεργοποίηση του υποδοχέα TLR4, έχει ως συνέπεια την ενεργοποίηση ενός μεταγραφικού παράγοντα που ονομάζεται Πυρηνικός παράγοντας- κάππα βήτα (NF-κB), και είναι γνωστός για τη μεταγραφή προ- φλεγμονωδών κυτοκινών (Kaplan et al., 2012). Η ανενεργή μορφή του απαντάται στο κυτταρόπλασμα, ενώ στον πυρήνα βρίσκεται σε ενεργοποιημένη μορφή, επάγοντας μεταγραφή πολλών γονιδίων (Akira and Takeda, 2004). Ο NF-κB έχει συσχετιστεί με μηχανισμούς έμφυτης ανοσίας και φλεγμονής (Kaplan et al., 2012). Μάλιστα, ορισμένες θεραπευτικές προσεγγίσεις κατά της φλεγμονής, στοχεύουν την αναστολή της σηματοδότησης TLR4/ NF-κB (Huang et al., 2014; Gomes et al., 2015). Στην παρούσα διατριβή μελετήθηκε η πιθανή συνεργασία του υαλουρονικού οξέος με τον υποδοχέα TLR4 στη διαδικασία ενεργοποίησης των κερατινοκυττάρων από αλλεργιογόνα επαφής. Πιο συγκεκριμένα, τα αλλεργιογόνα PPD και DNCB προκάλεσαν σημαντική αύξηση του υποδοχέα TLR4 με τρόπο εξαρτώμενο από τη συγκέντρωσή τους, στην κυτταρική σειρά ανθρώπινων κερατινοκυττάρων NCTC2544, όπως φάνηκε με τις τεχνικές της ανοσοαποτύπωσης κατά western και της αλυσιδωτής αντίδρασης πολυμεράσης πραγματικού χρόνου (Real- time PCR). Επιπλέον, τα αλλεργιογόνα οδήγησαν σε αυξημένη έκφραση των συνθασών του υαλουρονικού HAS1, HAS2 and HAS3, επίσης σε επίπεδο πρωτεΐνης και mRNA. Τα δεδομένα αυτά υποδεικνύουν ότι τα αλλεργιογόνα εξ επαφής επάγουν αύξηση της σύνθεσης του υψηλού μοριακού βάρους υαλουρονικού από τις αυξημένες συνθάσες του. Επιπλέον, η επαγόμενη από αλλεργιογόνα αύξηση των υαλουρονιδασών (Nikitovic et al., 2015) σε συνδυασμό με τη δράση των δραστικών μορφών οξυγόνου, οδηγεί στην αύξηση των ενδογενών επιπέδων του χαμηλού μοριακού βάρους υαλουρονικού. Επιπρόσθετα, η επώαση των κερατινοκυττάρων με υαλουρονικό οξύ χαμηλού μοριακού βάρους, προκάλεσε αύξηση της έκφρασης του υποδοχέα TLR4, όπως επίσης και των συνθασών HAS1 και HAS3. Αυτό καταδεικνύει πως η αύξηση του χαμηλού μοριακού βάρους υαλουρονικό στα ενεργοποιημένα κερατινοκύτταρα, θα οδηγήσει σε επιπλέον υψηλού μοριακού βάρους υαλουρονικό, διαθέσιμο για αποικοδόμηση σε μικρότερα τμήματα και περεταίρω ενεργοποίηση της σηματοδότησης του

TLR4, σαν μία «λούπα» διατήρησης της φλεγμονής. Ακόμα, σε συνθήκες έλλειψης TLR4, με τη μέθοδο RNA σίγησης, μειώθηκαν τα ενδογενή επίπεδα των συνθασών HAS1 και HAS3, δείχνοντας μία άμεση συσχέτιση μεταξύ σύνθεσης του υαλουρονικού και της σηματοδότησης του TLR4. Οι επιπτώσεις των αλλεργιογόνων PPD and DNCB στο μεταβολισμό του υαλουρονικού οξέος, φάνηκε να εκτελούνται έστω μερικώς από τη σηματοδότηση του TLR4. Αυτό προέκυψε από το γεγονός ότι αναστολή του υποδοχέα TLR4 με ειδικό αντίσωμα, οδήγησε σε μείωση της επαγόμενης από αλλεργιογόνα αύξησης των υαλουρονιδασών και των συνθασών του υαλουρονικού. Ιδιαίτερα σημαντικό ήταν το γεγονός ότι τα αλλεργιογόνα PPD και DNCB επηρέασαν την ενεργοποίηση του NF-κB, όπως επίσης και τη μετατόπισή του στον πυρήνα. Αναστολή της δράσης του TLR4 προκάλεσε μείωση στην ενεργοποίηση του NF-κB. Επιπλέον, επώαση με χαμηλού μοριακού βάρους υαλουρονικό οξύ ενίσχυσε την ενεργοποίηση του NF-κB στα κερατινοκύτταρα. Η αναστολή της δράσης των υαλουρονιδασών με κατάλληλο αναστολέα (αριστολοχικό οξύ), είχε ως συνέπεια τη μείωση της επαγόμενης από αλλεργιογόνα ενεργοποίησης του NF-κB. Συμπερασματικά, η ενεργοποίηση των κερατινοκυττάρων καθορίζεται μερικώς από τον σηματοδοτικό άξονα χαμηλού μοριακού βάρους υαλουρονικό οξύ/ TLR4/ NF-κB.

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1. Introduction

1.1 Contact dermatitis (Irritant and Allergic)

Contact Dermatitis is a type of inflammation of the skin, caused by environmental factors. The symptoms, not always occurring all together, are itchiness, burning, stinging and pain. Clinical presentation includes erythema, vesiculation and scaling. Usually, but not in all cases, contact dermatitis occurs only at the region of the skin exposed to the irritant or allergen (1). Unfortunately, clinical presentation varies hence, exact diagnosis is sometimes difficult (2). A systemic method for contact dermatitis diagnosis includes four steps; (i) elimination where non-allergic or non-dermatitis cases must be eliminated or included, (ii) perception, as a pre-patch test diagnosis is necessary, before the utilization of suitable patch test series, to use as a guide; (iii) detection where the appropriate patch test series, with highest possible sensitivity and specificity is determined, (iv) finally, deduction phase, where all information including allergen, concentration and time points should be combined for reaching a correct diagnosis (3).

Contact dermatitis is classified into two discrete subtypes, Irritant and Allergic Contact dermatitis. Irritant contact dermatitis (ICD) occurs as a direct damage of the epidermis without prior activation of immune system, or skin sensitization. A strong irritant is able to induce ICD through a single exposure of the skin to it, while weaker irritants may require multiple exposures. On the other hand, allergic contact dermatitis (ACD) is a delayed type IV hypersensitivity reaction to various chemicals upon exposure and subsequent sensitization of the individual to them (1). These chemicals are denominated haptens and are characterized by their low molecular weight (<1000Da) (4). Haptens are unable to be recognized alone by the immune system, but need to be bound in with protein carriers to form a complete antigen (5). ICD and ACD are difficult to distinguish based only on their presentation symptoms, hence, histological analysis of the lesion, during the acute phase of the disease, is required. One of the important histological distinctions is the

presence of necrotic keratinocytes in ICD lesions without the intense spongiosis observed in respective ACD lesions (6).

Importantly, almost 15-20% of the general population globally, presents allergic reaction to one or more contact allergens and the percentage seems to increase over the years (3). Indeed, in Europe, epidemiological studies indicate that nearly 20% of the population experiences ACD (7). Moreover, 40% of occupational diseases are skin related and 90% of this incidence is attributed to contact dermatitis (irritant or allergic) (2). Risk factors for ACD can be genes with higher susceptibility for the presentation of ACD, sex, age, ethnicity or occurrence of other skin pathologies, such as atopic eczema, ICD or Stasis dermatitis as well as the duration and localization of the exposure (3, 8). Noteworthy, the presenting lesions may occur patchy or diffuse, in a manner dependent on the chemical structure and specificities of each respective allergen. Moreover, autosensitization has been determined, as regarding the occurrence of an ACD lesion in a different part of the body, most frequently the skin near the eyes (1). This subtype of ACD is also known as ectopic contact dermatitis (6).

Crucially, sensitization is the first phase in the progression of ACD. After the allergen contacts the skin, occurs penetration *via* the stratum corneum and the creation of covalent bonds, or if it is a metal ion, complexes with self macromolecules (9). The binding of the hapten to a self macromolecule, will lead to the formation of a complete antigen, a procedure assisted by enzymes from epidermal keratinocytes. Upon, processing of the antigens by skin dendritic cells, likewise denominated Langerhan's cells, they are displayed on these cells' surfaces and the cells migrate towards the regional lymph nodes (10). More accurately, during the migration process, Langerhan's cells differentiate from antigen- processing to antigen- presenting cells (mature immunostimulatory dendritic cells), with an effective ability to present the antigen to responsive T-lympocytes in the draining lymph nodes. Importantly, the maturation and migration of Langerhan's cells is mediated by keratinocytes *via* production and secretion of cytokines including TNF-α, IL-1β and IL-18 (11). In continuation, adjacent T-lymphocytes will come in contact with Langerhan's cells and antigen specific T-cells are generated. The latter

ones, subsequently, have the ability to be transferred to the epidermis *via* blood circulation (10). It should be mentioned, that often, repetitive exposure to the allergen is needed for sensitization. At this point, allergen-specific T-cells clones are strongly expanded. The clinical presentation of ACD will occur after additional exposure to the allergen in question, and this second phase in disease progression is characterized as elicitation (7, 10). In most cases, the symptoms of ACD occur approximately 48h after skin contact with the allergen (3). Thus, Langerhan's cells presenting the allergen antigens interact with the corresponding specific T-cells whereupon an inflammatory response is initiated with massive cytokine production (10). It should be mentioned that keratinocytes contribute significantly to the elicitation phase by likewise, acting as antigen presenting cells, assisting activation of specific T-cells (9).

1.2 Patch test

The standard clinical procedure used for the diagnosis of ACD is the utilization of the so called "patch" test. In this procedure, patients with symptoms or history of the disease are exposed to suspected allergens in order to topically induce ACD correlated lesions and thus, verify the diagnosis and the causative agent. Often, a patch test is performed in the case of patients presenting with separate dermatopathies, such as various types of eczema, in order to detect potential allergies to prescribed medications. For the correct performance of the test in question, it is important that the allergens tested are properly stored and used in appropriate concentrations with carefully chosen vehicles (12).

1.3 Contact Allergens

Allergens are synthesized or natural substances usually commonly utilized in everyday activities. It is noteworthy, that more than half of ACD cases are attributed to various cosmetics ingredients (1). Contact allergens are quite often components of products utilized for personal care or health issues, jewelry and can have either organic or inorganic origin (13). For instance, widely used in cosmetics are the preservatives methylchloroisothiazolinone

(MCI) and methylisothiazolinone (1) with the latter being assigned the allergen of the year for 2013 (14). Earlier, nickel was determined as the most common allergen positive in patch tests and designated allergen of the year in 2008. This metal is present in innumerable products of everyday use including jewelry, buttons, belts, self-phones, tablets or even multivitamins and sensitization rate for nickel has been increasing over the years. Likewise, gold is one of the numerous metals which can induce contact sensitization, being designated as the allergen of the year 2001. Synthesized hydrophobic molecules like para-phenylenediamine (PPD) are common allergens. Indeed, this aromatic amine contained in many hair dyes and henna tattoos and characterized as a strong inducer of ACD, was designated as allergen of the year in 2006 (14). Likewise, another important allergen is 2,4-dinitroclorobenzene (DNCB), widely used in experimental models on ACD studies (15). DNCB is synthesized through chlorobenzene nitration and is used in the production of sulfur dyes (16). Interestingly, it has medical applications, as it is utilized in warts therapy (17), and its use has also been reported in the therapy for alopecia aerata (18). Another group of chemicals implied in ACD induction are parabens. These chemicals are widely used as anti- microbial preservatives and are common ingredients of food products as well as in cosmetics and medicinal applications (19). Interestingly, nail varnishes and preservatives as well as fragrances contained in shower gels and shampoos are responsible for the induction of patchy or diffuse ADC-associated lesions (1). Fragrances are either natural or chemical agents which enhance odor and although they are widely considered relatively harmless, they are the second most frequent contact allergen cause after nickel. Another well established and commonly implicated in ACD contact allergen is formaldehyde. This chemical is, under standard conditions, a colorless and odorous gas, derived from incomplete combustion of wood, tobacco, coal, and gasoline. Formaldehyde is, likewise, a common ingredient of numerous products including nail polish or personal hygiene products. Importantly the use of formaldehyde has been reduced over the years due to its negative “publicity” as carcinogenic factor and contact sensitizer (14). Propolis, a resinous substance found in poplars, and used by honeybees to seal their hive is considered a contact sensitizer responsible for ACD on

apiarists (1). This is an interesting finding due to the fact that propolis is also widely used as a component of natural medicine applications due to its anti-inflammatory, antiseptic and anesthetic properties (20). Importantly, sunscreens also hide “danger” due to some chemical ingredients which are incorporated as filters against UV radiation. Noteworthy, some physical filters such as zinc oxide, titanium dioxide are not considered as contact sensitizers, while other chemical filters often contained in sunscreens are more likely to cause sensitization (21). The above mentioned are just a few examples of the thousands of identified contact allergens which upon exposure can potentially induce the presentation of ACD.

1.4 Treatment of ACD

Upon the identification of the contact allergen responsible for ACD, it is of utmost importance to avoid further exposure of the patient to it, although this is not always possible (1). In cases of moderate ACD, avoiding the responsible contact allergen is sufficient for cure (2). Here, it is paramount that upon identification of the cause, the patient should use alternative, “safer” products instead. However, in some cases a prescription of therapeutic treatment may be needed. Thus, the utilization of topical corticosteroids is a routine therapeutical approach of albeit, moderate effectiveness, in many patients. Upon presentation of strong ACD symptoms, oral administration of corticosteroids might be needed. An example is the inducement of ACD by poison ivy (6) or PPD (2). Nevertheless, this type of therapy, though useful and necessary in some cases, would be better to avoid in chronic disease, due to potential severe side effects, such as osteoporosis and type II diabetes (2). It is important to note that, long term use of topical corticosteroids can likewise cause side effects including, skin atrophy, rebound dermatitis, dyspigmentation, telangiectasia or striae. To avoid these side effects, topical calcineurin inhibitors have also been used as an alternative therapy (6). Nonetheless, in severe acute phase ACD systemic corticosteroids may be the treatment of choice, as if contact dermatitis is not treated, the danger of it developing to a chronic condition underlies. In chronic cases where the individual does not respond to other therapies, suggested treatments are:

psoralen and UVA treatment, narrow- band UVB treatment, systemic treatment with immunomodulators, such as methotrexate, azathioprine and cyclosporine, and also targeted biologic therapy (1, 2). In mild cases, barrier creams and emollients could also be helpful. Information and awareness on potential contact allergens and products containing them is recommended, in order to avoid, as far as possible, repeated exposure. Unfortunately, there is no typical and standard treatment against ACD due partly to the fact that the clinical symptoms of the disease vary among individuals. Increasing knowledge on the pathogenesis and molecular mechanism involved is the key to find successful treatments. Furthermore, speedy, sensitive and reliable biomarkers would be a useful tool for proper diagnosis (2).

1.5 Extracellular Matrix (ECM) structure and functions

The extracellular matrix (ECM) is a complex network of molecules secreted by the cells with an inherent self- assembly ability, which provide tissue specificity, structure and organization (19, 22). The ECM is present in all tissues and organs where it provides both mechanical and biological support to cells (23-25). The ECM consists mainly of water, proteins and polysaccharides and more specifically, of proteoglycans (PGs), glycosaminoglycans (GAGs), collagens, elastins, fibronectin and laminins, as well as growth factors and enzymes (26-28). The components of this complex network participate in a wide variety of biological functions including, proliferation, adhesion, angiogenesis, cell motility, homeostasis as well as inflammation (22, 24, 29-31). The ECM components regulate biological functions by affecting specific signaling pathways, which finally support homeostasis, but in a pathological milieu participate in various disease progression. Importantly, in all tissues the initialization and propagation of inflammation can be supported by ECM- derived signals (32).

A prominent family of the ECM macromolecules is proteoglycans (PGs), molecules consisting of a protein core, where one or more glycosaminoglycan (GAG) chains are covalently bound in. PGs' molecular weight range is 11-220kDa, but is modified depending on the binding of specific GAG chains (33,

34). GAGs are linear polysaccharides, negatively charged consisting of repeating disaccharide units of 70 to 200 residues and form long, non branched chains. The disaccharitic building “blocks” consist of uronic acid and a hexozamine (glucosaminoglycan or galactosamine) (35). The types of GAGs are Hyaluronan (HA), Dermatan sulfate (DS), Chondroitin sulfate Keratan sulfate (KS), Heparin and Heparin sulfate (HS). GAG classification depends on the uronic acid form and the type of hexosamine incorporated in their repeating disaccharide units (32). Among the different GAG types, HA differs, as it is the only one not bound to protein cores, it is not sulfated and it is physiologically found in the form of free chains (32). PG properties depend on both their protein core and GAG decoration. Moreover, according to classification based on their localization, three groups of PGs have been determined, namely, the extracellular PGs, the cell membrane PGs and the intracellular PGs. The extracellular ones are secreted to the ECM upon synthesis and participate in the organization of the complex ECM network. Indeed, the Small- Leucine Rich Proteoglycans (SLRPs) and modular PGs are characterized by their extracellular localization. Modular PGs are further, depending on specific binding properties, classified to Hyalectans, also called Lecticans (HA and lectin binding) and the non HA binding PGs of the basement membrane. Hyalectans family members are brevican, neurocan, and versican. A characteristic structural feature of the hyalectan family is that their protein core is constituted of three domains. HA binds to the specific binding module localized to the N-terminal domain, while lectins bind to C-terminal. The third central domain has specific binding sites for other GAGs (36). The second PG localization is at the cell membrane. Cell membrane PGs are either transmebrane molecules (e.g syndecans, CD 44) whose GAG modifications are bound in to their extracellular domain or anchored to the membrane (e.g glypicans) Finally, the only known intracellular PG is denominated serglycan and is expressed by endothelial and hematopoietic cells (34).

Under pathological conditions an abnormal remodeling of the ECM is evident. One of the results of this remodeling is the liberation of Damage Associated Molecular Patterns (DAMPs), likewise demominated as alarmins. These

liberated endogenous molecules have intrinsic ability to trigger and sustain inflammation (37). The resulting inflammatory response induced by DAMPs is characterized as “sterile”, as it is not pathogen derived (38). Keratinocytes, as well as other cells with immune responsive functions, can in addition to cytokine secretion for immune modulation, also generate alarmins e.g. molecules with the ability to trigger immune responses (9). Under physiological conditions DAMPs, are intracellular endogenous molecules not in contact with the immune system and thus not recognized. However, under pathological conditions, such as stress or injury, apoptotic or activated cells release DAMPs to the ECM, causing innate immune system activation, which ultimately results in sterile inflammation (37, 39). Apart from released DAMPs of intracellular origin, DAMPs can also be products of the ECM, degraded, due to ECM protease action or through the action of enzymes secreted by apoptotic or necrotic cells (37). Some of the molecules shown to act as DAMPs are uric acid, ATP (40), heparan sulfate, biglycan (37), Low molecular weight hyaluronan (LMWHA) as well as many other components originating from the ECM and released upon degradation (38). It is well established that the ability of DAMPs to trigger sterile inflammation is directly correlated to several disease pathogenesis, including atherosclerosis, cancer and auto-immune diseases (39).

Noteworthy, another category of DAMPs liberated to the ECM, are Reactive Oxygen Species (ROS) (38). Specifically, ROS are chemically active small molecules resulting from incomplete reduction of oxygen (41). ROS are involved in programmed cell death or necrosis, mediate the expression of several genes by inducing or suppressing their transcription, and activate key cell signaling cascades (42). These highly active molecules are not only responsible for signal transduction, but also act as mediators of inflammation (43). Some examples of ROS are oxygen radicals including, superoxide anion ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), peroxyl ($RO2^{\bullet}$), peroxynitrite and alkoxyl ($RO^{\bullet}$) and also some non- radicals, that are either oxidizing agents and/ or are easily converted into radicals, namely hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), as well as hydrogen peroxide (H_2O_2). ROS can have both endogenous and exogenous origin and when their levels are increased, the

occurring state is characterized as “oxidative stress”. Importantly, even though there are many anti- oxidative self mechanisms in all tissues, under conditions of high ROS levels the anti- oxidative capacity of tissues is overridden and the condition of oxidative stress prevails (32). Indeed, in vertebrate organisms, cells have an evolutionary developed mechanism of antioxidant defense system with main function the removal and neutralization of ROS. Thus, antioxidant enzymes known as ROS scavengers are: superoxide dismutase (SOD) which coverts $O_2^{\bullet-}$ into H_2O_2, catalase, glutathione peroxidase (GPx) which converts H_2O_2 to H_2O, peroxidredoxins, and thioredoxins (Trxs). The condition of oxidative stress occurs, when ROS are so increased that the capacity of antioxidant enzymes is inadequate to neutralize them (43). Importantly, ROS have the ability to chemically degrade HA into smaller molecular weight fragments (42).

1.6 Hyaluronan (HA)

HA is one of the most important ECM components. This linear non- sulfated GAG is composed of repeating units of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) combined by β-1.3 and β-1.4 linkages (→ 3- β- D-GlcNAc (1 →4) β- D GlcUA (1→) (34, 44). Noteworthy, HA is the only non-sulfated GAG (45) and also, the only GAG not attached to a protein core, but found free to the ECM (46). Moreover, while all the GAGs are synthesized to the Golgi apparatus, HA is synthesized by the three hyaluronan synthases (HAS1, 2 and 3) at the plasma membrane (44) and secreted to the ECM during its synthesis (47). HA was first expressed about 500 million years ago in lower chordates, as revealed by philogenetic studies (48-50). This GAG was initially isolated from cat eye tissue in 1934 (51). The importance of HA for organism homeostasis is evident as it is expressed by and localized in all vertebrate tissues. Interestingly, nearly the half of the total HA in the human body is localized to the skin (52). More accurately, dermal fibroblasts produce and deposit most of skin HA to the dermis (53). Importantly, epidermal keratinocytes likewise, produce HA, and deposit it to the ECM of spinous and granular epidermal layers, while at the basal layer HA is mostly localized intracellularly (53).

HA is well established to participate in a wide variety of biological functions including cell adhesion, migration, proliferation, embryogenesis (54, 55), wound healing progression (56-58). Importantly, HA has been implicated in the pathogenesis of several types of cancer (59, 60) as well as in inflammation processes (61). This GAG exhibits a high turnover rate (46), enabling it to play an important role in the regulation of tissue homeostasis. Thus, HA mediates the formation of intercellular gels, by interacting with other ECM components, for instance, proteoglycans facilitating physiological tissue organization (62). Interestingly, every 24 hours 30% of the total HA is replaced by newly synthesized chains of this ubiquitous GAG (Misra et al., 2015). Noteworthy, HA affects body immune response and consequently the evolution of the inflammatory process (61). For instance, tumor necrosis factor- stimulated gene-6 (TSG-6) catalyses the formation of covalent bonds between HA and the heavy chains of inter-a-inhibitor (IaI), a procedure that promotes the resolution of inflammation (63). In fact, in keloid lesions, decreased TSG-6 levels were demonstrated to be part of the abnormal formation of scar tissue (63).

Importantly, HA functions are often opposing and directly dependent on its molecular weight. Hence, it is well established that high molecular weight HA (HMWHA) promotes tissue stability during homeostasis (64), as interactions between HMWHA and other ECM components, facilitate correct ECM structural organization. Additionally, by utilizing specific out-in signaling pathways HMWHA has immunosuppressive, anti- angiogenic and anti-inflammatory properties (47, 65) as well as to participate in cellular differentiation (47) and in wound healing processes (19). While HMWHA is normally deposited in tissues (64), LMWHA fragments are produced under stress conditions or as a result of injury either enzymatically by hyaluronidases, enzymes that degrade HA or chemically by action of ROS (40, 42). The truncated HA fragments are biologically active in a discrete manner as compared to its HMWHA counterparts (64). Thus, LMWHA fragments, in their function as DAMPs, promote the opposite effects to those of HMWHA, namely enhance inflammation, angiogenesis and tumorigenesis processes (47) as well as facilitate scar formation (46). The even smaller HA

fragments, tetrasaccharides have been shown to both ameliorate these effects (47) or to facilitate them in a manner dependent on the model system utilized (66).

In vertebrate tissues HA is detected either as a free molecule or in a complex with specific cell surface receptors or co- localized with other ECM components, including PGs in its role of a scaffold component in various forms. HA binding proteins are denominated hyaldherins and are capable of binding HA with covalent or electrostatic bonds. Many of HA biological roles are executed through its binding to the hyaladherin receptors (53). Indeed, through the conjunction of HA, these HA- hyaladherin complexes mediate cell- cell, cell- ECM and ECM components adherence (67).

1.7 Hyaluronan Synthases (HAS)

HA is synthesized by a three Hyaluronan Synthases (HAS1, 2 and 3), previously called hyaluronan synthetases, enzymes classified as glycosyltransferases with the ability to polymerase HA chains. These enzymes act in the obligatory presence of Mn^{2+} and Mg^{2+} and the use UDP sugars (GlcNAc and GlcA) as substrate. HA synthesis occurs *de novo*, as no primer is required, only the presence of UDP sugars and the activator Mg^{2+}. Notably, HAS enzymes have more than one functions, as they not only initiate and polymerase HA by connecting two sugars with two distinct linkages, but also, facilitate the transfer of the produced HA chains across the cell membrane and to the pericellular space (50). Specifically, the active site of HASs where HA polymerization occurs, is localized at the inner side of the plasma membrane and in continuation the synthesized HA chain is extruded to the outer side of the plasma membrane (68).

Interestingly, all species expressing *HAS* genes were found to express more than one. For instance, chordates were found to express three or four HASs and *Xenopous* has 3 isoforms and one non functional. Indeed, in addition to a common gene- ancestor, the existence of two gene duplications is hypothesized (50). In mammals three HAS isoforms have been identified with a molecular weight of approximately 63k, HAS1 HAS2 and HAS3 (44). The

three *HAS* genes are homologous and located on different chromosomes, specifically: *HAS1* on chromosome 19q13.4, *HAS2* on chromosome 8q24.12 and *HAS3* on chromosome 16q22.1 (69). Thus, the localization on different chromosomes implies ancient gene duplication origin (64). HAS expression is affected by the specific cell microenviroment and differs among different tissues (47). HAS1 and HAS2 produce HA of 200-2000kDa, even though HAS2 exhibits a tendency to synthesize chains at the higher range of the synthesis spectrum whereas HAS3 polymerases smaller chains with molecular weight 100-1000kDa (56, 70). Indeed, it is due to its size, that HA is secreted to the ECM not by exocytosis, like other GAGs, but during its synthesis by HASs (44, 62, 70).

HAS expression is affected by a plethora of factors and often differentially for each gene. Thus, growth factors and cytokines including, keratinocyte growth factor (KGF), epidermal growth factor (EGF), transforming growth factor- beta (TGF-b), bone morphogenenetic protein (BMP) superfamilies, tumor necrosis factor-alpha (TNF-α) and interleukin1- β (IL-1β), were demonstrated to increase HAS2 expression (71). Specifically, in keratinocytes it was determined that the EGF family members and especially heparin binding epidermal growth factor (HB-EGF), enhance HAS2 activity (36). Moreover, in keratinocytes HA synthesis is executed equally by HAS2 and HAS3, while fibroblasts mainly exhibit HAS2 activity (19). KGF, also stimulates the expression of the main HAS of the epidermis, the HAS3 enzyme (72, 73). Additionally, growth factors TGF-β1, FGF-2, EGF, and PDGF-BB were demonstrated to upregulate HAS expression in skin fibroblasts, resulting in increased HA production. In fact, TGF-β1 upregulated HAS1 and HAS2, while FGF-2, EGF, and PDGF-BB increased only HAS2 expression (74). In aged fibroblasts, HAS2 is downregulated, hence HA production is decreased (75). Additionally, glucocorticoids downregulate HAS2 expression at the transcriptional level and also, by the regulation of the mRNA turnover (71). Importantly, HAS1 was found to be upregulated during keratinocyte differentiation, highlighting this enzyme's importance for the process. HAS3 on the other hand, was correlated to the presentation of skin inflammatory conditions (76), including atopic dermatitis (77).

It is noteworthy that, HAS activity is regulated by a plethora of factors, but differentially for the 3 enzymes. In contrast to HAS2 and HAS3, HAS1 presents high dependency on availability of the respective substrates, as their low concentration abrogates HAS1 activity (78). On the other hand, HAS2 activity is enhanced under high GlcNAc levels (78). In fact, HAS3 is the most active glycosyltrasferase among the three HAS enzymes, as it exhibits high activity even at low UDP sugars levels (78). HAS3 activity however, may also be affected by substrate concentration (71).

1.8 Hyaluronidases (HYALs)

HYALs are enzymes that degrade HA with high affinity. These enzymes, however, also have the ability to degrade chondroitin and chondroitin sulfates albeit with low affinity (79). This HYAL property explains why several metazoans express HYALs, though they lack the ability to synthesize HA (71). In humans HYALs were determined to be expressed at very low levels, but they exhibit very specific and high activity for the HA substrate (79). In the human genome six mammalian *HYAL* genes have been detected so far (79), with the most active being HYAL1, HYAL2 and PH-20 (80). *HYAL1*, *HYAL2*, and *HYAL3* genes are located on chromosome 3p21.3, and *HYAL4*, *PHYAL1*, and *SPAM1*, are located on chromosome 7q31.3 (81). The first HYAL detected was denominated HYAL1 and is located to the lysosomes (79, 81). It is expressed as an isoform of 57kDA and as a processed isoform of 45kDa, but both present similar specificities (82). HYAL2 is located to the plasma membrane *via* a glycophosphatidyloinositol anchor located at the enzyme's C terminus, but it has also been detected to lysomes (36, 83). Specifically, HYAL2 degrades HMWHA into small size fragments of approximately 20kDa (81, 83). These fragments are endocytosed by cells and transported in endosome vesicles to lysosomes, where HYAL1 will further degrade these small fragments, to very small oligosaccharides mostly, tetrasaccharides (81). HYAL2 is expressed in two isoforms (82), with PH-20 isoform, encoded by the *SPAM1* gene, being anchored similarly to HYAL2 to the plasma membrane *via* a glycophosphatidyloinositol anchor (81).

HA degradation takes place by cleavage of β(1→4) glycosidic bond at C1 of the GlcNAc. In humans the most abundant HYALs are HYAL1 and HYAL2. Specifically, HYAL2 degrades HA with a slower rate and presents lower affinity for HA fragments smaller than 20kDa. HYAL1 is expressed in human somatic tissues and detected in plasma, while HYAL2 is present in most tissues with the exception of adult brain. Though PH-20 is the most active among the HYALs, it is expressed only on the surface of human sperm and the inner acrosomal membrane where it cleaves the digestion products of other HYALs into tetrasaccharides. As regarding, HYAL3, this hyaluronidase was demonstrated not to be significant for HA metabolism in mammals. HYAL3 has been detected in testes, brain and bone marrow tissues. HYAL4 is not an active hyaluronidase, but it presents affinity for chondroitin and chondroitin sulfate. HYALP1, was found to be transcribed in human tissues but not translated, though it was observed to be expressed and to replace PH-20 in Spam^{-}/$^{-}$ mice (36). Noteworthy, an important factor affecting HYAL activity is the microenvironment pH, as the PH-20 is most effective in neutral pH, while HYAL1 and 2 exhibit the highest activities in acidic milieu (51).

1.9 HA Receptors

1.9.1 CD44

The main HA receptor is denominated cluster of differentiation 44 (CD44) which has been characterized as a type I transmembrane glycoprotein (67, 84). Though, CD44 is encoded by a single gene with 10 constant and ten variant exons (85), it presents a variety of isoforms, due to alternative splicing and post- translational modifications (84). For instance, this transmebrane protein can be glycosylated at the N- and O- glycosylation sites (36) and GAG chains can be bound in (53). Interestingly, these modifications play a role in modulating receptor affinity for HA (86). Indeed, in epidermal keratinocytes interleukin-1β (IL-1β) was demonstrated to reduce CD44 Ser-325 phosphorylation, leading to increased affinity to HA (87). The molecular weight of the isoforms varies between 80-20kDA, with a main isoform of 85kDa (88). CD44 molecule consists of four domains, an HA binding, a

transmembrane, a cytoplasmic and a stalk domain. In order to acquire the ability to bind H, the receptor must be activated by post translational modifications including glycosylation at the extracellular domain or phosphorylation of serine amino acids in the cytosolic domain (36).

CD44 binds HA of all molecular weights, and complex formation triggers different cellular reactions depending on the molecular weight of the bound HA chain. Thus, HMWHA binding to CD44 suppresses angiogenesis, inflammation, migration, whereas LMWHA binding induces the opposite effects (36). The CD44 receptor plays an important role in skin homeostasis and maintenance of keratinocyte pericellular matrix and is expressed by cells residing in both dermal and epidermal sections of the human skin (89-91). Importantly, CD44- HA binding affects keratinocyte differentiation (92) and adhesion (93). Epidermal barrier function is another biological function with CD44 participation. Indeed, it has been suggested that CD44 contributes to the performance of the skin barrier through the facilitation of proper cell organization (94). The main isoform of the epidermis is epican, a heparan/ chondroitin sulfate proteoglycan form of CD44 (95). Importantly, the HS glycosylation of epican bestows to this CD44 isoform the ability of growth factor binding (96).

1.9.2 TLR4

Toll-like receptor 4 (TLR4) is a transmembrane protein intimately involved in the processes of innate immunity. Likewise, this receptor has the ability to bind HA (97). TLR4 belongs to the family TLR proteins, eleven of which (TLR1, TLR2, TLR3 TLR4, TLR5, TLR6, TLR7 TLR8, TLR9, TLR10 and TLR11) have been detected in human tissues (98). TLR family members are type I proteins that were firstly detected in drosophila. They have a leucine-rich repeat (LRP) domain at the extracellular domain and a carboxyl- terminal Toll-interleukin I receptor (TIR) domain intracellularly (99). In vertebrates they are highly conserved and recognize numerous ligands such as nucleic acids, lipids, lipoproteins and proteins. TLRs have the ability to recognize Pathogen Associated Molecular patterns (PAMPs), namely, specific molecules expressed in bacteria, fungi and viruses which upon binding to the respective

receptors, activate innate immunity *via* nuclear factor kappa-beta (NF-κB) (13, 19). The *TLR4* gene is located on chromosome 9q32-33 and contains four exons (100). The gene presents several single nucleotide polymorphisms (SNPs) (101). The receptor is widely known for its specific lipopolysaccharide (LPS) binding ability. Specifically, LPS produced by numerous gram negative bacteria, consists of a long and branched carbohydrate chain with a hydrophobic lipid A region attached on it. Immune system activation caused by this macromolecular glycolipid, occurs *via* the lipid A region whose specific structure differs among bacterial species (13, 102). It is noteworthy that, the binding between LPS and TLR4 is not direct, but non covalently mediated *via* an MD-2 adaptor protein (103).

The majority of TLRs with the exception of TLR3 and TLR4 members, act exclusively *via* the Myeloid differentiation factor 88 (MyD88) adaptor protein, leading to NF-κB activation and mitogen-activated protein kinases (MAPKs) activation and consequent transcription of pro- inflammatory cytokines (13). Thus, TLR3 acts *via* a separate adaptor protein, TIR- domain- containing adapter inducing interferon-β (TRIF), which functions in an autocrine manner and leads to late NF-κB activation (13, 104). Interestingly, TLR4 is able to induce downstream signaling through both MyD88 dependent and independent pathways. Generally, upon ligand binding to TLR4, the receptor is homodimerized, due to specific interactions inaugurated through the TIR domains. Subsequently, upon conformational changes in the receptor's structure, adaptor proteins are recruited *via* their TIR domains. The adaptor proteins with such domains, needed for TLR4 dependant immune response, are MyD88, MyD88-adapter-like (Mal) protein, known as TIR- domain-containing adapter protein (TIRAP) as well as, TIR- domain- containing adapter inducing interferon-β (TRIF) also known as TIR- domain- containing adapter molecule-1 (TICAM-1) and TRIF-related adapter molecule (TRAM), denominated likewise, as TIR-containing protein (TIRP), or TIR- containing adapter molecule-2 (TICAM-2) (101). As regarding the MyD88 dependent pathway, MyD88 signaling adaptor interacts with TLR4 *via* Mal bridging adaptor, while respectively to MyD88 independent pathway, TRIF signaling adaptor interacts with TLR4 *via* TRAM bridging adaptor. Noteworthy, it was

demonstrated that the bridging adaptor MyD88 and TLR4 have positively charged TIR domain, hence, these two dominions are mutually repelled. On the other hand, the Mal adaptor has negatively charged TIR domains, thus a link between TLR4 and the signaling adaptor is possible (104). Noteworthy, the LPS induced signaling is mediated mainly through the MyD88 dependant pathway. It has been determined that the MyD88 independent pathway participates in maturation of dendritic cells (101).

As regarding, receptor restricted downstream signaling in MyD88 dependant pathway IL-1- receptor- associated kinases (IRAKs) and tumor- necrosis-factor receptor- associated factor 6 (TRAF6) are activated, and as a result, inhibitor of nuclear factor-κB (IκB)- kinase complex (IKK) is triggered. Specifically, in the formation of the IKK complex, IKK-α, IKK-β IKK-γ participate, (known likewise as IKK1 and IKK2 and NF-κB essential modulator, NEMO, respectively). Consecutively, IKK phosphorylates IκB (family of inhibitors that keep NF-κB in inactivated form), which is ubiquitinilyzed and degraded whereupon NF-κB is released and translocates to the nucleus, which ultimately results in inflammatory cytokine production (105). Importantly, TRAF6 activation will also lead to Mitogen activated protein kinases (MAPK) triggering (104), with consequent activation of AP-1 transcription factor, also implicated in pro- inflammatory cytokine expression (106). As regarding the TRIF- dependant pathway, the interferon regulatory factor-3 (IRF3) transcription factor is activated, with consequent transcription of type I interferons (IFNs) and other co- stimulatory molecules (101). TRIF induces TNF-α production, whereupon this inflammatory mediator exhibits autocrine activity which results in late NF-κB activation (105).

Importantly, the TLR receptors exhibit the ability to recognize DAMPs, which results likewise in innate immune system activation (19, 103). TLR4 does not bind haptens directly, but it complexes with endogenous macromolecules generated by the breakdown of high molecular weight parent molecules, such as HMWHA. In *in vivo* experiments performed in mouse models a critical role of TLR4 was revealed, in the induction of skin sensitization, upon HMWHA degradation (103). Indeed, DAMPs are the cause of TLR4 (and also TLR2)-mediated pro- inflammatory cytokine production, such as interleukine-1 (IL-1),

IL-6, IL-12, IL-18, IL-13, TNF-α and interferon-α (INF-α), and consequent ACD presentation (107). Importantly, TLR4 was demonstrated to be a crucial part of the mechanism inducing ACD, as its binding to DAMPs, including LMWHA will trigger overexpression of IL-1β and TNF-α cytokines, which are required for mobilization and migration of Langerhan's and potentially dendritic cells that contribute to the transport of the antigens to skin- draining regional lymph nodes (107). Indeed, alarmins like DAMPs participate in several skin disorders including ACD. Upon exposure to allergens, alarmins such as ROS are liberated by keratinocytes, hence, a condition of stress occurs and the secreted alarmins bind with TLR4 and other TLRs, resulting in pro-inflammatory cytokine secretion, such as IL-1α, IL-1β, IL-18, IL-33 and IL-36. Indeed, the secretion of the respective cytokines constitutes an important step in ACD pathogenesis (9). Hence, blocking DAMP production is considered a promising therapeutic target for treating ACD (103).

Interestingly, nickel, the most frequent cause of ACD, has the ability to bind to human TLR4 due to the existence of two histidine residues, H456 and H458. Indeed, to the absence of the respective residues in the mouse TLR4 protein, is attributed the innate protection of these rodents for the presentation of contact hypersensitivity against nickel (13). Furthermore, TLR4, as well as TLR2, do not recognize haptens directly, but requires the binding of the hapten to host proteins for efficient recognition. As TLRs are responsible for the recognition of various pathogen derived factors it has been hypothesized that the skin bacterial flora is responsible for hapten recognition. However, germ free mice can be induced to develop contact hypersensitivity against TNCB, hence, the initial hypothesis of skin bacterial flora participation is probably not valid (13, 108).

1.9.3 RHAMM

Another HA receptor to whom significant biological roles have been attributed is receptor for hyaluronan mediated motility (RHAMM). Similarly to CD44, several isoforms of the receptor, due to alternative splicing, can occur. Furthermore, it has been determined that the isoforms are expressed in a cell specific manner (109). The molecular weight of this HA receptor is

approximately 85kDa (110). RHAMM was firstly detected as a soluble protein of 60kDa in the cell culture of heart fibroblasts (111). This receptor has been localized to the cytoplasm, the nucleus or located on the cell surface of fibrosarcoma cells (88). Importantly, in healthy, non-dividing adult cells RHAMM is not expressed or it presents low expression levels (88, 110). Upregulation of RHAMM has been reported in some cancer types (88), including, oral squamous cell carcinoma, breast and prostate cancer (110). Its upregulation in neoplasms has been suggested to be utilized as a prognostic marker (112, 113). RHAMM has been implicated, in the regulation of basic biological functions including cell growth (114), motility and transformation (115-117). Furthermore, this receptor has been correlated to pathological and physiological processes such as inflammation, differentiation, tissue remodeling and morphogenesis, matrix organization, angiogenesis (110).

Cytoplasmic RHAMM contains several specific kinase binding sites, the existence of a binding site for ERK1 being an important example (36). Moreover, to RHAMM has been assigned the ability to interact, in the role of an adaptor protein, with the cytoskeleton and specifically with actin, podosomes, mitotic spindle microtubules, the centrosome and other proteins of the cytoskeleton, mediating tumorigenesis- related processes (36, 118-120). Interestingly, cell- membrane RHAMM does not possess a transmembrane domain and is anchored to the plasma membrane *via* a glycosylphosphatidylinositol anchor (111, 121). It is well established that cell motility and invasion are affected by cell- surface RHAMM with the contribution of MAPK (ERK1,2) downstream signaling (122, 123). Moreover, RHAMM interacts with other receptors, including CD44, platelet- derived growth factor receptor (PDGFR), Ron- receptor tyrosine kinase e (119, 123), epidermal growth factor receptor (EGFR) as well as several hepatocyte growth factor receptors (HGFR) (110). Furthermore, RHAMM signaling has been directly correlated to the regulation of biological functions of cells originating from fibroblasts. Thus, transforming growth factor b (TGF-b) was demonstrated to affect RHAMM expression in fibroblasts and consequently these cells' cell motility (124). Moreover, RHAMM is directly involved in the

regulation of malignantly transformed fibroblasts migration, adhesion and growth as demonstrated in an *in vitro* fibrosarcoma model (116, 117).

1.9.4 ICAM

The intercellular adhesion molecule-1 (ICAM-1) is another receptor with the ability to bind HA (125). This protein is located to the plasma membrane, has a 55kDa protein core and its extracellular domain can be variously glycosylated. Indeed, the molecular weight of glycosylated forms of ICAM-1 ranges between 80- 114kDa (126). The ICAM-1 gene is located to chromosome 19 and has been shown to contain 7 exons and 6 introns (127). ICAM-1 activities have been correlated to HA-dependent signaling as is the case in the regulation of matrix metalloproteinase (MMP) expression in fibroblasts (128), and the mediation of LPS- dependent cytokine production in macrophages (125).

Interestingly, upon treatment of keratinocytes with the extracellular toxin streptolysin O, which is produced by certain streptococci and is characterized by ability to damage cell membranes, a strong upregulation of both CD44 and ICAM-1 was established (129).

1.9.5 LYVE-1

LYVE-1 is a HMWHA receptor expressed by lymphatic endothelial cells, whose structure was determined to be similar to that of the CD44 receptor. This transmembrane glycoprotein binds both soluble and immobilized HA and was shown to mediate the transport of HA to lymph from various tissues (36).

1.10 ROS and HA

ROS generation can be induced through the activities of various mediators including contact sensitizers. Importantly, ROS generation can in turn contribute to the release of other DAMPs (107). As mentioned above, it is well established that ROS engage in a chemical reaction with HA which results in HA chemical cleavage. Under, inflammatory conditions ROS generation is increased and in this milieu HMWHA acts as a protective factor against the degradation of lipids, proteins as well as other cellular components. Indeed,

HA serves as an extracellular target of ROS, which “neutralizes” ROS activities and thus prevents these dangerous species from entering the cell and incurring injury, which can ultimately lead to cell transformation or death. The resulting LMWHA fragments act as “danger signals” for immune system activation (130). Moreover, ROS not only degrade HA directly, but also affect HYAL action, as demonstrated in lung inflammatory conditions, through the upregulation of HYALs in a p38 MAPK dependant manner (131). It is important to point out, however that ROS have the ability to degrade all GAGs. Indeed, upon peroxinitrate ($ONOO^-$) decomposition, the resulting $^{\bullet}OH$ with the assistance of $^{\bullet}NO$, are responsible for GAG degradation through two distinct pathways. The first requires conversion of nitric oxide to nitrous acid and the other is mediated *via* peroxinitrate $ONOO^-$. The targeting of different GAG types and their subsequent degradation depends on the balance between nitric monoxide radical ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$) concentrations (132). Thus, in inflammatory conditions, $O2^{\bullet-}$ and $^{\bullet}NO$ levels are increased. When the latter two recombine, peroxynitrite is produced. Interestingly, peroxynitrite has the ability to degrade HMWHA, but not heparin/ heparan sulfate (42).

ROS are well established to participate in the progression of various pathological conditions (133). For instance, the degradation of HA localized to joint synovial fluid detected in patients with rheumatoid arthritis, has been attributed to topical, low expression of the protective antioxidant enzymes e.g. superoxide dismutase and catalase (42). Moreover, in the skin increased ROS levels result in decrease of epidermal antioxidants. Thus, ROS can directly or indirectly, for instance by proteolytic activation, degrade epidermal ECM components, such as collagens, hyaluronan and proteoglycans. Furthermore, ROS can induce dysfunctioning of basic cellular functions including fibroblasts and keratinocytes migration and proliferation as well as modifications of ECM components synthesis and remodeling (134).

The action of ROS has also been described during the resolution of the skin wounds. The participation of ROS is especially important in the resolution of skin chronic lesions which are described as condition of skin injury, where the lesion is unable to heal. Importantly, approximately 1% of global population

suffers from chronic wounds, which is determined as a both somatically and psychologically debilitating condition. Chronic wounds exhibit long term inflammation, impaired ECM response and an unsuccessful re-epithelialization. Noteworthy, the generation of ROS within the wounds is one of the factors responsible for this prolonged pathological condition (134).

1.11 NF-κB

The NF-κB is a family of transcription factors which are composed of homo or heterodimers. Up to date, five monomers located to the cytoplasm in inactive form have been identified: p65 (REL-A), p50 and p52, REL-B as well as cytoplasmic (c) REL. The, IκB molecules are responsible for keeping NF-κB in an inactive state (105). The NF-κB monomers have a ReI homology domain (RHD) of approximately 300 amino acids long, with 35-61% identity among different members. The RHD domain is responsible for the DNA binding of NF-κB, homo or hetero- dimerization and also, binding to IκB factors. This domain, however, is not accountable for the regulation of respective dimer transcriptional activity. The p50 and p52, NF-κB family members, arise from the longer p105 and p100 precursors, respectively. The p50/p65 is the most frequent dimer of NF-κB and was demonstrated to have the fastest traslocation rate upon activation from an ECM-dependent signal transduction (135). Thus, upon phosphorylation and proteolysis of IκB, NF-κB translocates to the nucleus, as stated above, where is mediates the transcription of various target genes (105).

1.12 HA metabolism in inflammatory conditions

HA metabolism has been correlated to various skin disorders. To begin with, in acute eczema, an inflammatory condition which is characterized by epidermal spongiosis, a significant HAS3 upregulation and consequent increased HA deposition were observed *in vitro*. Under, the same skin conditions, cadherins, important factors for cellular adherence were found to be downregulated and hence, keratinocyte cohesiveness was decreased and simultaneously water inflow was favored. Indeed, clinically spongiosis is occasionally detected in ACD. This finding can be partly explained by

production of IFN-γ by T cells, established to trigger keratinocyte apoptosis. In turn, apoptotic keratinocytes present downregulation of E-cadherin expression (136). Moreover, HAS3 expression levels were found to be elevated in skin biopsies of acute eczema patients, presumably because of pro- inflammatory factor action (76) and similar findings were observed in *in viv*o experiments (137).

Atopic dermatitis (AD), also known as atopic eczema, is the most frequent skin inflammation with genetic, immunological and autoimmune effects (138). AD patients present overexpression of HAS3 as shown by skin biopsies, while HAS1 expression was shown to be decreased (77). Interestingly, HAS synthase expression is regulated through multiple mechanisms in milieu of AD. Thus, HAS2 and HAS3 expression were demonstrated to be increased by pro- inflammatory cytokines, such as IL-4, IL-13, INF-γ (139), growth factors EGF and KGF (73, 90), as well by retinoic acid downstream signaling (140). Moreover, EGF expression was also found to be increased in AD patient biopsies, where HAS3 expression was upregulated as well. In agreement to these data, treatment of Reconstructed Human Epidermis (RHE) with EGF, resulted in HAS3 upregulation (77). HAS1 was suggested to be the main synthase responsible for HA production by keratinocytes, under physiological conditions (139, 141). However, other studies suggest that HAS3 is the main synthase in keratinocytes, whereas the synthesizing activity of HAS1 is mostly evident in fibroblasts (139, 141). Indeed, an interesting hypothesis has been proposed stating that the main difference between normal and pathological conditions, may be the balance between HAS isoforms expression (77).

The CD44 receptor participates in skin inflammatory mechanisms, albeit, sometimes in a conflicting manner. Thus, CD44 was demonstrated to regulate HA production in the skin, through a feedback mechanism. Indeed, in a CD44-deficient mouse model HA was found to be hyper- accumulated in both dermis and epidermis and keratinocyte proliferation was determined to be decreased. These findings were correlated to a disabled skin inflammatory response and defective tissue repair (142). In an early study, the existence of a positive correlation between CD44 expression and HA excess uptake was suggested (143). Moreover, this mechanism was indicated to be the key in the

resolution of inflammation in a mouse pneumonia model (144). In a separate CD44 deficient mouse model, the LPS induced NF-κB activation, as well as TNF-α, IL-1β and IL-6 cytokine production was found to be promoted. Furthermore, the same authors had demonstrated that the expression of TLR4 signaling inhibitors was decreased (145). Additionally, the same knock out mice presented defective epidermal permeability and homeostasis. On the other hand, in a delayed type hypersensitive dermatitis model, cell infiltration and cutaneous edema were decreased upon administration of an anti- CD44 antibody (146). Similarly, in CD44 knock out mouse model, the chemically induced ACD symptoms were found to be decreased (146). In a mouse model of colitis, deficiency in CD44 expression was, likewise, suggested to affect favorably the progression of the disease (146). On the other hand, the downregulation of CD44 expression in murine macrophages, induced increased NF-κB action, well correlated to an pro- inflammatory status (147). Likewise, some studies suggest that deficiency in CD44 expression does not affect ACD progression *in vivo.* A possible explanation of this finding could be the substitution of CD44 activities with other adhesion molecules, for instance E-selectin, I-selectin and ICAM-1 with the ability to mediate progression of inflammation. Hence, though CD44 plays key roles in skin inflammatory processes as well as in the inflammatory status of other tissues, other HA binding proteins could at least partly substitute the receptor's action (146).

Increased expression of the HYAL2 enzyme has been determined in pathological conditions (130). Thus, in an ACD keratinocyte model system HYAL1 and HYAL2 were found to mediate, at least partly, the degradation of hyaluronan (60). These authors had shown in NCTC2544 keratinocytes that the treatment with contact allergens results in HYAL upregulation, and increased HA degradation. Furthermore, this, process was found to be partly ROS- dependant, as pretreatment with anti oxidant DPI, decreased the degradation of HA (60). Moreover, *in vivo* overexpression of HYAL1, and consequently, increased LMWHA levels, resulted in enhanced dendritic cell migration from the skin. Indeed, in this study it was determined that the antigen presentation was executed more rapidly followed by an enhanced immune response (66). Interestingly, increased HYAL activity in allergy

models was established in early reports (148), whereas the inhibition of HYAL activity was demonstrated to result in diminished skin inflammation (108, 149).

The showed alterations of HYALs activities under inflammatory conditions are important, when taking into account the HMWHA well established anti-inflammatory effects. Thus, in inflammatory bowel disorders, Crohn's disease and ulcerative colitis, "stress cables" e.g. cross- linked HA chains forming cables and fibrils of HA have been detected at inflammation sites. These HA cables were shown to isolate pro- inflammatory mediators and hence prevent their action, while simultaneously they enhance tissue stability (47).

1.13 Wound healing

The important role of HA in the wound healing process has been widely studied. Thus, it has been demonstrated that HMWHA exhibits positive effects in wound healing. Indeed, gels containing HMWHA are being widely used on patients after surgery, to reduce scar formation. Specifically, during wound repair HA exhibits important roles in both structural and physiological functions (134). The process of wound healing includes four temporal phases often overlapping: homeostasis, inflammation; proliferation and migration and finally remodeling (150, 151).

During homeostasis a fibril clot is synthesized by growth factors and other molecules, with the scope to stop the bleeding and provide a suitable matrix for migrating cells, recruited to the site of injury (150). At the initiation of the process HMWHA is increased and high influx of inflammatory cells, neutrophils and leukocytes is observed (53). During the inflammatory phase keratinocytes, migrating to the wound region commence to synthesize HA (152), as well as the proliferating and migrating fibroblasts (150). The increased HA deposition modulates the functions of both inflammatory cells and dermal fibroblasts such as migration, synthesis of pro- inflammatory cytokines as well as phagocytosis of invading microbes. Furthermore, HA participates in the scavenging of ROS within the wounds (134).

However, the elevated available HMWHA supports LMWHA generation either through ROS or HYALs action (150). Importantly, the released LMWHA has been demonstrated to promote angiogenesis in the injured area (134). Subsequently, LMWHA binds the TLR2 and TLR4 receptors and the concomitant production of cytokines such as IL-6, TNF-α and IL-1β is triggered. Cytokines, in turn, enhance HAS upregulation, resulting in increased HA synthesis, which will again be degraded into LMWHA. Indeed, the resulting feedback mechanism will promote the sustaining of inflammation (150). Furthermore, HB-EGF is increased at the injured site, triggering HA production by the neighboring cells. An interesting crosstalk between keratinocytes and fibroblasts which migrate to the area, is the production of keratinocyte growth factor (KGF or FGF-7) by fibroblasts, and its binding on the respective FGFRIII2b receptor of epidermal keratinocytes, inducing enhanced keratinocyte proliferation (153).

As wound healing proceeds, and the HA levels reach a maximum, an instant decrease in the levels occurs, simultaneous to the appearance of proliferating fibroblasts at the injured site. This alteration in HA levels mediates chondroitin sulphate and collagen deposition. In fact, it seems that the decrease of HA levels, is the reason for keratan and dermatan sulfate synthesis in the Golgi apparatus (150). Subsequently, the, KS and DS containing proteoglycans support collagen polymerization, a procedure critical for wound closure and a part of the remodeling phase (150), a procedure, escorted by scar formation (53).

Interestingly, in fetus wound healing can be terminated without scar formation (during the first six months of pregnancy) (154). Hence, the differences between adult and fetal wound healing seem to be critical to scar formation. A basic difference between fetus and adult wound healing is the maintenance of increased HA levels during the whole wound healing process in fetus, as the decrease of HA levels does not occur (155). Another, significant difference is the increased expression of HYAL enzymes in adults, which results in enhanced HMWHA degradation (46). Moreover, addition of exogenous HYALs and the subsequent increased LMWHA production has been demonstrated to result in scar formation (46). Therefore, while HMWHA has

beneficial effects to the whole process *per se*, decrease of HMWHA and increase of LMWHA, seems to be one of the many reasons for scar formation. Moreover, it has been demonstrated that the molecular weight of HA modulates the type of the produced collagen that will, in turn, assist or inhibit scar formation. Large HA fragments modulate only type-III collagen production, abundant in fetus, while smaller fragments seem also to modulate the type-I collagen production, present in adult skin and promoter of scar formation (156). Importantly, a mixture of HA chains of various molecular weights HA seems to be more effective in the resolution of the wound healing process (157). Thus, it can be concluded that LMWHA has both beneficial and negative roles in the wound healing process. However, it must be noted that the amniotic fluid plays a vital role in the healing process of fetus and not only provides sterile conditions, but also contains numerous nutrients and growth factors (158). Additionally, the amniotic fluid plays a role in the promotion of the wound healing process by activating specific aspects of the immuno-response, production of cytokines, or by enhancing fibroblasts' migration to the wound spot (159).

2. Aims of the study

ACD is a significant health hazard globally and almost all individuals could at some point in their lives exhibit the symptoms of the disease. Therefore, it is deemed important to determine the mechanisms involved in this disease presentation and progression. The large number of patients suffering from ACD worldwide and the often debilitating effects the pathology induces justify alternative approaches in the facilitation of disease resolution. Contact allergens are present in numerous products of everyday life, hence, deeper knowledge of mechanisms of sensitization is critical for affective protection, diagnosis and treatment of ACD. The scope of the present study was to investigate the putative involvement of HA metabolism and TLR4 signaling in the process of contact allergen mediated human NCTC2544 keratinocyte sensitization. Defining the mechanisms involved, could facilitate the protection against the disease as well as the development of alternative therapeutical approaches.

3. Materials and Methods

3.1 Cell culture

All the experiments were conducted on NCTC2544 human keratinocyte cell line (Istituto Zooprofilattico di Bresia, Italy). Culture media was RPMI 1640 supplemented with Fetal Bovine Serum (FBS) 10% v/v. As antimicrobial agents 100IU/ml penicillin and 100μg/ml streptomycin were used. Cells were cultured as monolayers at temperature of 37^{o}C and 5% CO_2. All cell culture reagents were purchased from Biosera.

3.2 Chemicals and Reagents

As skin sensitizers, the allergens 2,4-dinitrochlorobenzene (DNCB) and para-phenylendiamine (PPD) were used, supplied by Sigma (USA). The utilized concentrations of PPD (30μg/ml) and DNCB (2μg/ml) were earlier established to strongly induce keratinocyte sensitization (160) and had likewise been utilized in a previous study (60). Allergens were diluted in DMSO, also used as vehicle control at 0.2% final concentration. LMWHA (15-40kDa) was provided by R&D Diagnostics and diluted in ddH_2O and utilized at 250μg/ml; the concentration of LMWHA with strongest effect on Il-18 production as previously determined (60).

Keratinocytes were treated with the neutralizing antibody against TLR4 (Sigma) at 2μg/ml and 4μg/ml for one hour, before the addition of allergens. Lipopolysaccharide from Escherichia coli (LPS) was purchased from Sigma Chemical Co. (St. Louis, Mo., USA) and was utilized at 500ng/μl as a positive control for TLR4 activation (103, 161).

Aristolochic acid (a-acid) (Sigma) was utilized, in accordance to a previous study (Nikitovic et al., 2015), at the concentration of 40μM. The cells were treated with a-acid for one hour before the addition of the allergens.

3.3 Transfection with short interfering RNA (siRNA)

Silencing of TLR4 gene was performed with short interfering RNA method. Specifically, NCTC2544 were seeded in T25 culture flasks (1.000.000 cells per flask). The sequence was taken from Zhang and Li (162) (sense sequence: 5' GCUGAUGCCGCUGAUGCCA 3', anti-sense sequence: 5' UGGCAUCAGCGGCAUCAGC 3'). Three different concentrations of siRNA sequence were assessed (25nM, 50nM, 100nM) in order to establish the most efficient downregulation conditions. This approach demonstrated that 100nm of siTLR4 induced the strongest effect and this concentration was further utilized in all TLR4-silencing experiments. As a negative control, scrambled siRNA (siNegative) was used. The transfection was performed with Lipofectamine^{TM}2000 (Invitrogen) protocol. Lipofectamin and RNA sequences were diluted in Opti-MEM © (Invitrogen) culture media, left for five minutes in room temperature and then they were mixed and left for 20minutes in room temperature, before they were added to the cultured cells, according to manufacturer's instructions.

3.4 Western Blot

NCTC 2544 were seeded in T25 culture flasks, 1.000.000 cells per flask. The harvested NCTC2544 were lysed with RIPA solution. Electrophoresis of the samples was performed in 10% polyacrylamide gel, followed by transfer to nitrocellulose membrane in 10mM CAPS buffer. Thereinafter, membranes were blocked with PBS containing 0.1% Tween and 5% v/v milk powder, overnight at 4^{o}C. Primary antibodies were diluted in PBS containing 0.1% Tween and 1% v/v milk powder. Primary antibodies supplied from Santa Cruz Biotechnology are: TLR4, HAS1, HAS2, HAS3, NF-κB-p65, p-NF-κB-p65 (Ser536), all used in 1:200 dilution. Actin was purchased from Millipore and used in 1:5000 dilution. Secondary peroxidase-conjugated antibodies provided by Millipore, were used to visualize the binding of the primary to the targeted proteins upon incubation, and the final image was obtained by Luminata^{TM}Crescendo western HRP Substrate, Millipore. Anti-mouse, anti-rabbit and anti-goat were diluted in PBS containing 0.1% Tween and 1% v/v

milk powder in 1:5000 dilution. The Blots were analyzed using ImageJ software.

3.5 RNA-isolation and Real-Time PCR

Total RNA was isolated with Trizol (Invitrogen) according to manufacturer's instructions. 1000ng of the total RNA of each sample were used for the production of complementary DNA (cDNA) with TAKARA PrimeScript reagent kit (TAKARA, Japan) according to manufacturer's instructions. Real-Time PCR was performed in Mx3005P cycler (Stratagene) with the KAPA SYBR® FAST Universal qPCR kit (KAPA Biosystems) in a total volume of 20 µl. Specific gene primers were used for the detection of the investigated genes (HAS1, HAS2, HAS3, TLR4, HYAL1, HYAL2, CD44). As a house keeping gene for the quantification of the results, GAPDH was used. For each optimized assay, a standard curve was run. This curve provided a linear plot of threshold cycle (Ct), against log (dilution). The quantification of each gene was according to the concentrations of a standard curve and was presented as arbitrary units.

Table1 Sequences for primers used in Real-time PCR.

Gene	**Forward**	**Reverse**
HAS1	5' GGT GGG GAC GTG GGA TC 3'	5' ATG CAG GAT ACA CAG TGG AAG TAG 3'
HAS2	5' GTG TTA TAC ATG TCG AGT TTA CTT CC 3'	5' GTC ATA TTG TTG TCC CTT CTT CCG C 3'
HAS3	5' GGT ACC ATC AGA AGT TCC TAG GCA GC 3'	5' GAG GAG AAT GTT CCA GAT GCG 3'
HYAL1	5' CCG GTG CTG CCC TAT GTC 3'	5' AGG CTG TGC TCC AGC TCA TC 3'
HYAL2	5' GGC GCA GCT GGT GTC ATC 3'	5' CCG TGT CAG GTA ATC TTT GAG GTA CT 3'

TLR4	5’ CAG AAC TGC AGG TCC TGG 3’	5’ GTT CTC TAG AGA TGC TAG 3’
GAPDH	5’ GGA AGG TGA AGG TCG GAG TCA 3’	5’ GTC ATT GAT GGC AAC AAT ATC CAC T 3’
CD44	5’ GGT CCT ATA AGG ACA CCC CAA AT 3’	5’ AAT CAA AGC CAA GGC CAA GA 3’

3.6 Immunofluorescence

NCTC2544 cells were seeded onto round coverslips in 24-well plates (120.000 cells per well) and incubated for 24h. Upon treatment completion and culture media was removed, the cells were fixed with a solution of 5% formaldehyde and 2% sucrose diluted in PBS for 10minutes in room temperature. After two washes with PBS, the cells were permeated with Triton-X diluted in PBS for 10minutes at room temperature. After permeability dilution was removed and coverslips were washed with PBS, primary antibody (anti-NF-κB by Santa Cruz) diluted in 1% FBS, PBS in a dilution of 1:50, was added and incubated for 1h. For HA localization Hyaluronic acid Binding Protein (HABP) was used, supplied from Millipore at 1:100 dilution. After three washes, secondary antibodies were added (anti-rabbit Alexa Fluor in 1:250 dilution and streptavidin from Invitrogen for HABP in 1:200 dilution) and incubated in the dark for 1h. Nuclei were stained with TOPRO-3, by Invitrogen, in 1:500 dilution for 20minutes in the dark. Finally, coverslips were placed on glass slides using glycerol and observed with confocal microscopy. The images were captured by Leica Confocal Software.

3.7 Proliferation assay

NCTC2544 cells were seeded into 96-well plates. Four different concentrations of NCTC2544 were tested (1500, 3000, 6000 and 9000 cells per well), in order to find the appropriate one for the assay, which was found to be 3000 cells per well (Figure 19A). For the experiment, after 24h incubation in culture media, 125μg/ml and 250μg/ml LMWHA were added for

48h. Then, cells were lysed and their number was measured by fluometric CyQUANT cell Proliferation Assay kit. This method measures DNA content upon binding of a fluorescent dye on it. On every experiment a standard curve was used to quantify the result. Fluorescence was measured by fluorimeter (Biotek) using 485/528nm at emission filters. All samples were used in triplicates.

3.8 Statistical analysis

Each experiment was performed at least three times with representative results reported. For all experiments, statistical significance was evaluated by Student's t test or one-way ANOVA analysis with Tukey's post-test, using GraphPad prism (version 4.0) software.

3.9 Optimizations

As RNA interference methodology specific for the TLR4 had not been previously performed in the NCTC2544 keratinocytes, the concentration of the TLR4 specific sequence, inducing the maximum downregulation of the receptor had to be determined. Hence, three concentrations of the TLR4 specific sequence were used (25nM, 50nM, 100nM), with 100nM achieving 63% downregulation at the transcriptional level and 55% downregulation at the protein level. Therefore, the 100nM concentration was utilized in the experiments with siRNA against TLR4. However, as the studied allergens increase TLR4 levels, the siRNA effect was annulled by the newly synthesized TLR4. In order to investigate the effects of contact allergens on TLR4 deficient cells, TLR4 neutralizing antibody was utilized. The first concentration used was 2μg/mL as in Galbiati, Papale (160), which was sufficient to decrease IL-18 production caused by PPD and DNCB. In the present study, for maximum blocking of the receptor two concentrations were utilized: 2μg/mL which was determined by Galbiati, Papale (160) to decrease IL-18 production caused by PPD and DNCB and a higher concentration of 4μg/mL. The utilized concentrations of PPD and DNCB have been used in

numerous previous studies on the related topic, as well as the utilized control with DMSO (60, 160, 163).

In continuation with previous studies (60, 160, 163), the treatment duration for PPD and DNCB was 24h. However, when NF-κB activation was studied, this time point was not effective. Hence, 1h, 2h and 3h treatments were tested with the maximum activation of NF-κB being at 2h, as determined by Western blot (*data not shown*). Likewise, the same time point was used in immune fluorescence for the examination of NF-κB.

Likewise, in Western blot experiments, the antibodies for all the investigated proteins were optimized, utilizing different concentrations and the one with the clearest image was used in the experiments that followed. Similarly, antibodies used in confocal microscopy, were firstly tested in order to obtain a strong fluorescent signal without background fluorescence (*data not shown*). The concentrations are mentioned in the respective materials and methods sections.

Finally, before performing proliferation assay, the appropriate cell number seeded in every well had to be determined. Four different seeding numbers were tested, 1500, 3000, 6000 and 9000 cells per well, with 3000 being ascertained as the most suitable. This seeding number revealed maxim proliferative ability during the incubation time. LMWHA was tested not only in the concentration used in previous experiments (60), but also at a lower one (125μg/ml).

4. Results

4.1 Contact allergens upregulate TLR4 in a concentration dependent manner

As TLR4 was demonstrated to participate in allergen-mediated keratinocyte activation (160), the present thesis aimed to further examine its mechanisms of action. The implication of TLR4 in inflammatory mechanisms was previously shown by numerous studies (108, 164-167). Additionally, it seems to be part of contact allergen-induced keratinocyte activation, as a neutralizing antibody against TLR4, decreases the contact allergen mediated IL-18 production (160). In this study, after treating keratinocytes for 24h with increasing concentrations of the two allergens, PPD (30μg/mL, 45μg/mL and 60μg/mL) and DNCD (1μg/mL, 1.5μg/mL and 2μg/mL), a notable concentration dependent upregulation of TLR4 expression (Figure 1) was observed at both protein ($p \leq 0.01$ for 30μg/mL, 45μg/mL PPD and 2μg/mL DNCB, $p \leq 0.001$ for 60μg/mL PPD) and mRNA level ($p \leq 0.01$ for 30μg/mL, 45μg/mL PPD, $p \leq 0.001$ for 60μg/mL PPD and $p \leq 0.05$ for 2μg/mL DNCB). These data, well in agreement with the previously shown contribution of TLR4 to contact allergen-induced IL-18 production (160), suggest that TLR4 is able to affect the contact allergen mediated activation of keratinocytes.

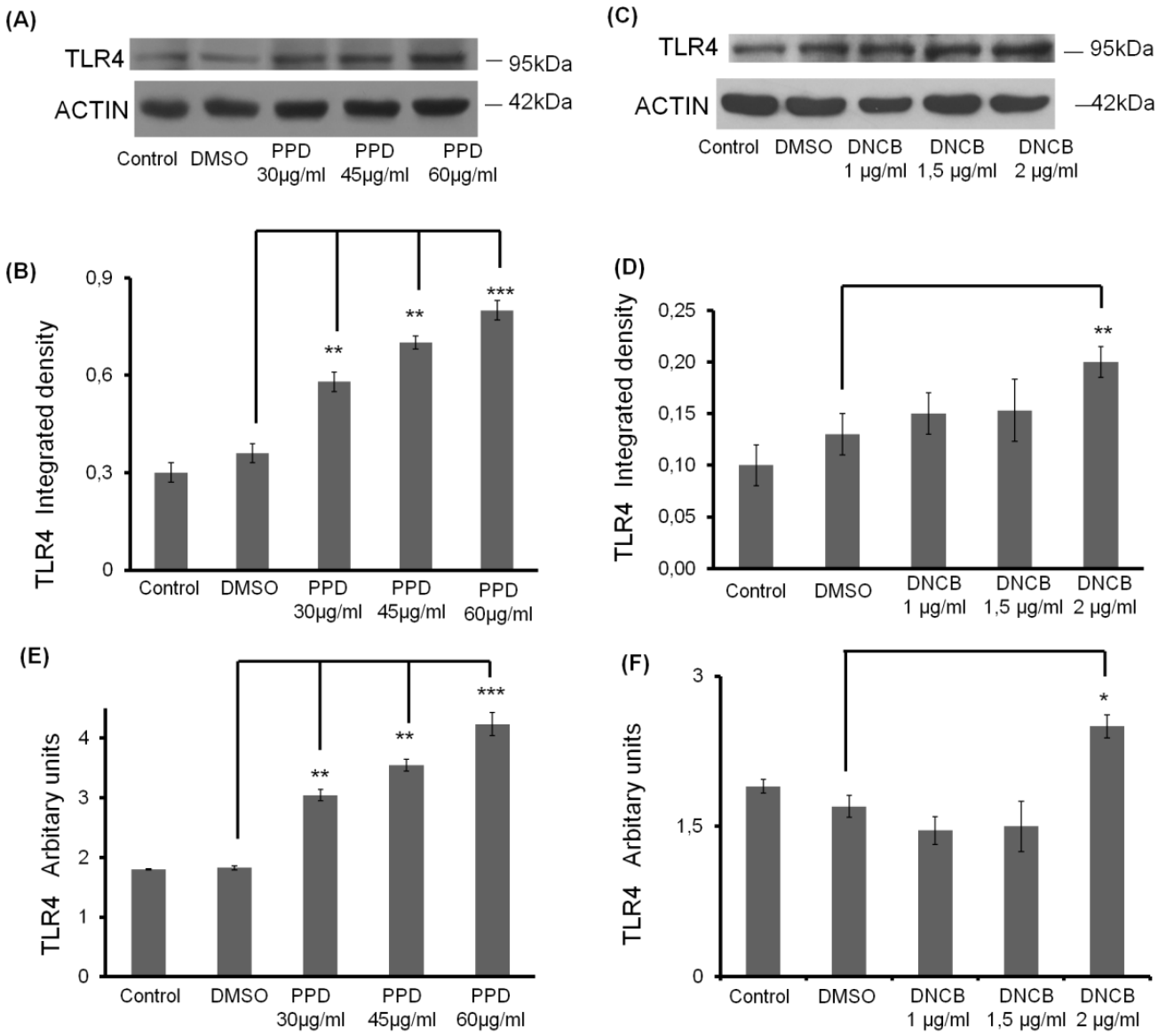


Figure 1 Effect of PPD and DNCB treatment on TLR4 expression. NCTC2544 cells were treated for 24h with increasing concentrations of PPD (30µg/ml, 45µg/ml, 60µg/ml), and DNCB (1µg/ml, 1.5µg/ml, 2µg/ml) (DMSO - negative control) before harvesting. **(A)**, **(C)** Representative blots of TLR4 protein (95kDa) and actin (42kDa) are presented. **(B)**, **(D)** TLR4 protein bands were densitometrically analyzed and adjusted against actin. **(E)**, **(F)** TLR4 mRNA expression was evaluated by Real- time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0,05, ** p≤0,01, ***p≤0,001.

4.2 The effect of contact allergens on HAS1 expression

Taking into account that inflammatory mediators were shown to affect HA synthesis (150), the present study aimed to examine the putative effects of

contact allergens on HAS expression in the following step of the present study. Therefore, NCTC2544 were treated for 24h with PPD (30μg/mL) and DNCB (2μg/mL) and HAS1 expression was initially estimated. The use of Western blot and Real- time PCR, demonstrated an increase in HAS1 expression at both the protein and mRNA levels (p≤0.05) (Figure 2).

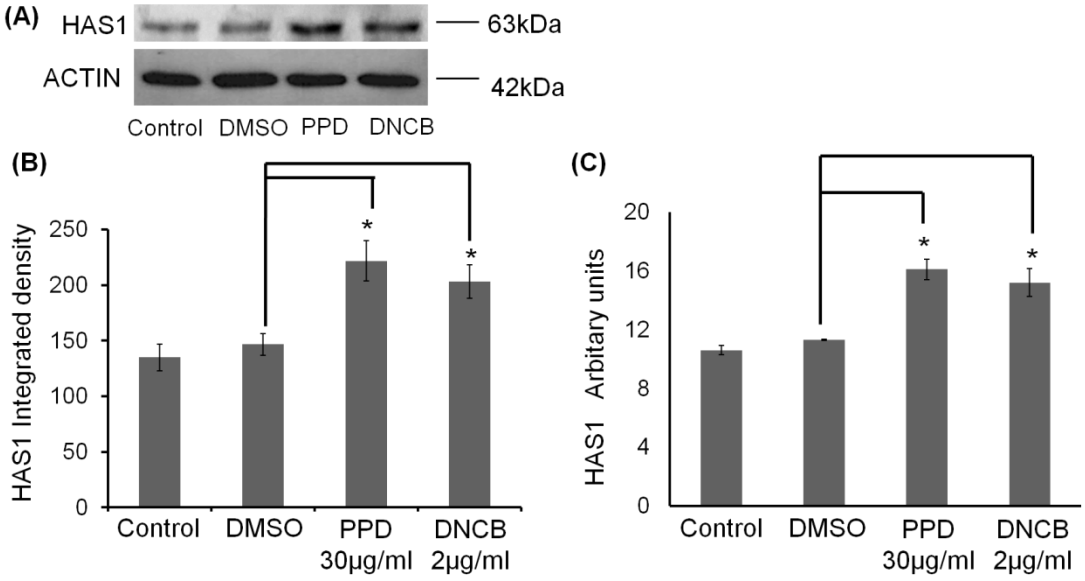


Figure 2. Effect of allergen treatment on HAS1 expression. NCTC2544 cells were treated for 24h with PPD 30μg/ml and DNCB 2μg/ml (DMSO - negative control) before harvesting. **(A)** Representative blots of HAS1 protein (63kDa) and actin (42kDa) are presented. **(B)** HAS1 protein bands were densitometrically analyzed and adjusted against actin. **(C)** HAS1 mRNA expression was evaluated by Real- time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0.05, ** p≤0.01, ***p≤0.001.

4.3 Contact allergens induce an upregulation of HAS2 expression

The putative effects of contact allergens on HAS2, the main HA synthase of fibroblasts, but likewise expressed in keratinocytes (70, 90), were also examined. Previously, it had been shown that pro- inflammatory cytokines increase HAS2 expression in keratinocytes (77). The utilization of western blot and Real- time PCR in the present study demonstrated that contact allergens

significantly upregulated HAS2 expression (p≤0.05), at both the transcriptional and translational levels (Figure 3).

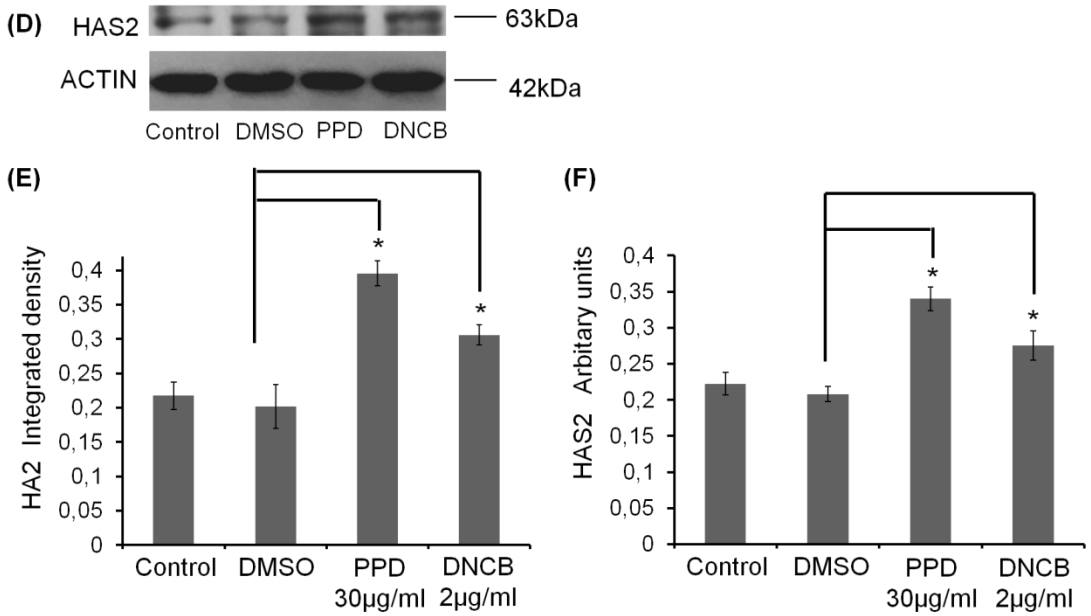


Figure 3. Effect of contact allergens on HAS2 expression. NCTC2544 cells were treated for 24h with PPD 30µg/ml and DNCB 2µg/ml (DMSO - negative control) before harvesting. **(A)** Representative blots of HAS2 protein (63kDa) and actin (42kDa) are presented. **(B)** HAS2 protein bands were densitometrically analyzed and adjusted against actin. **(C)** HAS2 mRNA expression was evaluated by Real- time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0.05, ** p≤0.01, ***p≤0.001.

4.4 Contact allergens cause upregulation of HAS3 expression

HAS3, mainly expressed in keratinocytes (72), has previously been demonstrated to play a role in skin inflammation (76) and atopic dermatitis (AD) (77). The treated with PPD (30µg/mL) and DNCB (2µg/mL) keratinocytes were shown to have an upregulated HAS3 expression (p≤0.05) (Figure 4) at both protein and mRNA level. These data therefore suggest that contact allergen treatment enhances HA synthesis of human keratinocytes.

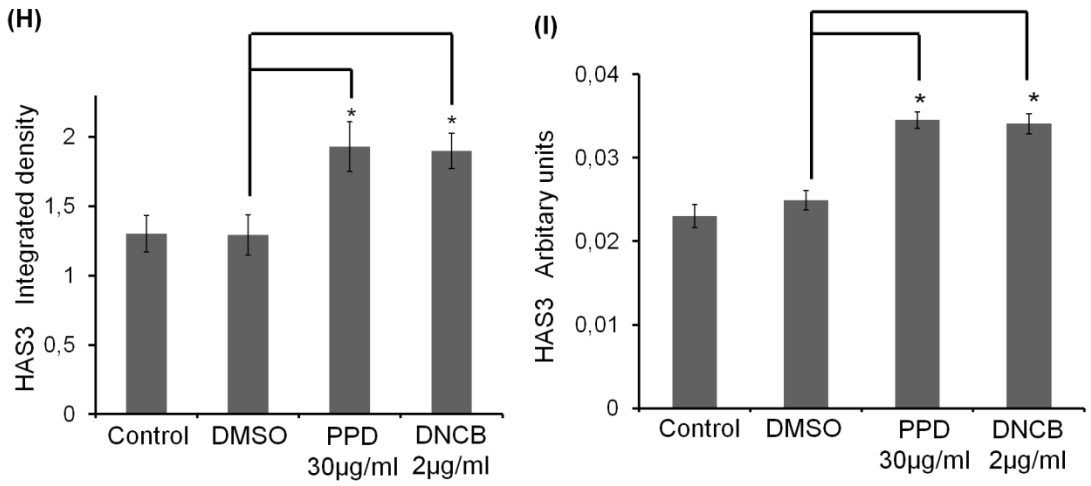


Figure 4. Effect of allergen treatment on HAS3 expression. NCTC2544 cells were treated for 24h with PPD 30µg/ml and DNCB 2µg/ml (DMSO - negative control) before harvesting. **(A)** Representative blots of HAS3 protein (63kDa) and actin (42kDa) are presented. **(B)** HAS3 protein bands were densitometrically analyzed and adjusted against actin. **(C)** HAS3 mRNA expression was verified by Real- time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0.05, ** p≤0.01, ***p≤0.001.

4.5 LMWHA upregulates TLR4 expression by keratinocytes

LMWHA has been characterized as DAMP (168), an endogenous factor able to trigger and sustain inflammation. Previously, it had been shown that LMWHA affects keratinocyte activation, whereas in the present study the putative contribution of TLR4 signaling to this mechanism was examined. NCTC2544 keratinocytes were treated with LMWHA (250µg/mL) (Figure 5) and its possible effect on TLR4 expression was examined. This strategy revealed a significant upregulation of TLR4 expression at both the protein (p≤0.001) and mRNA (p≤0.001) levels, in treated with LMWHA keratinocytes. These data suggest that LMWHA could modulate keratinocyte activation through a TLR4- dependent mechanism.

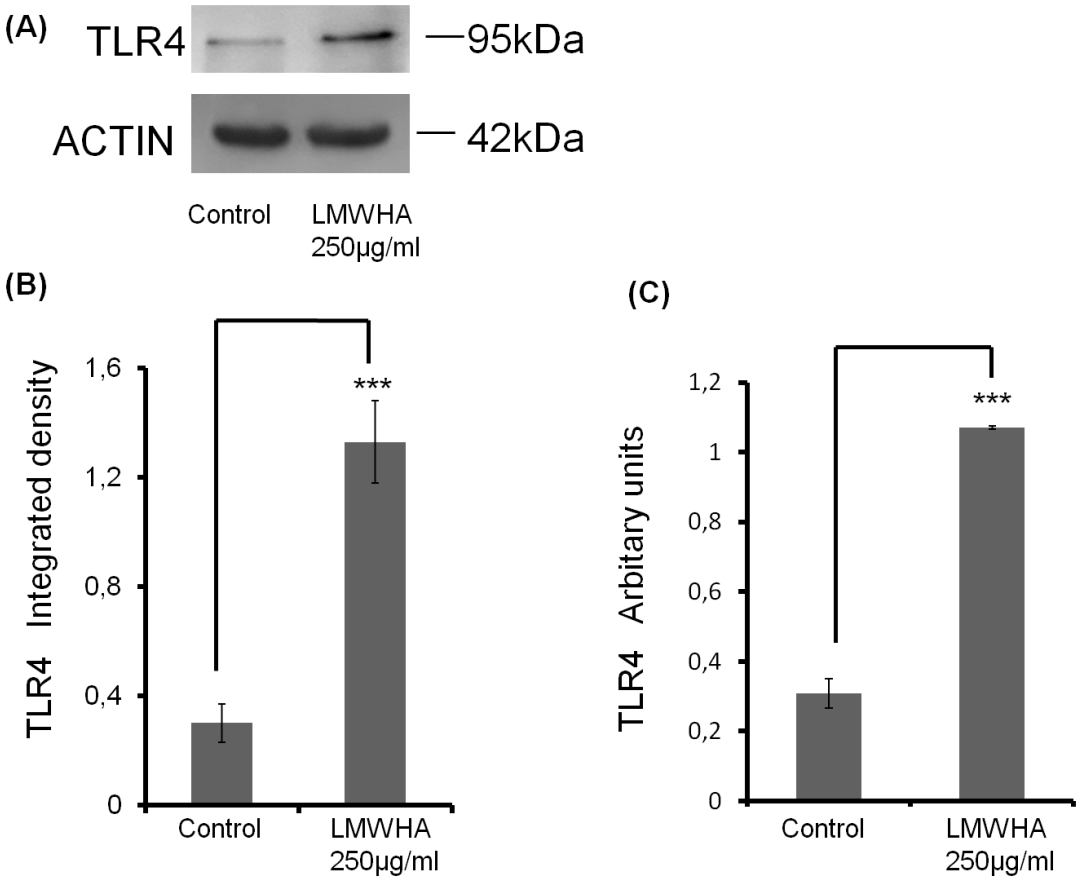


Figure 5. Effect of LMWHA treatment on TLR4 expression. NCTC2544 cells were treated for 24h with LMWHA 250µg/ml before harvesting. **(A)**, Representative blots of TLR4 protein (95kDa) and actin (42kDa) are presented. **(B)** TLR4 protein bands were densitometrically analyzed and adjusted against actin **(C)** TLR4 mRNA expression was evaluated by real time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0,05, **p≤0,01, ***p≤0,001.

4.6 LMWHA upregulates HAS1 and HAS3

Taking into account the shown upregulation of HAS1 expression upon treatment with contact allergens, this study aimed to examine the putative effect of LMWHA. Thus, the expression of HAS1 upon treatment with LMWHA (250µg/mL) was also determined (Figure 6). Western blot and Real-time PCR demonstrated a significant upregulation of HAS1 expression (p≤0.01 and

p≤0.001 respectively). These data indicate the existence of a possible feedback loop involving LMWHA signaling.

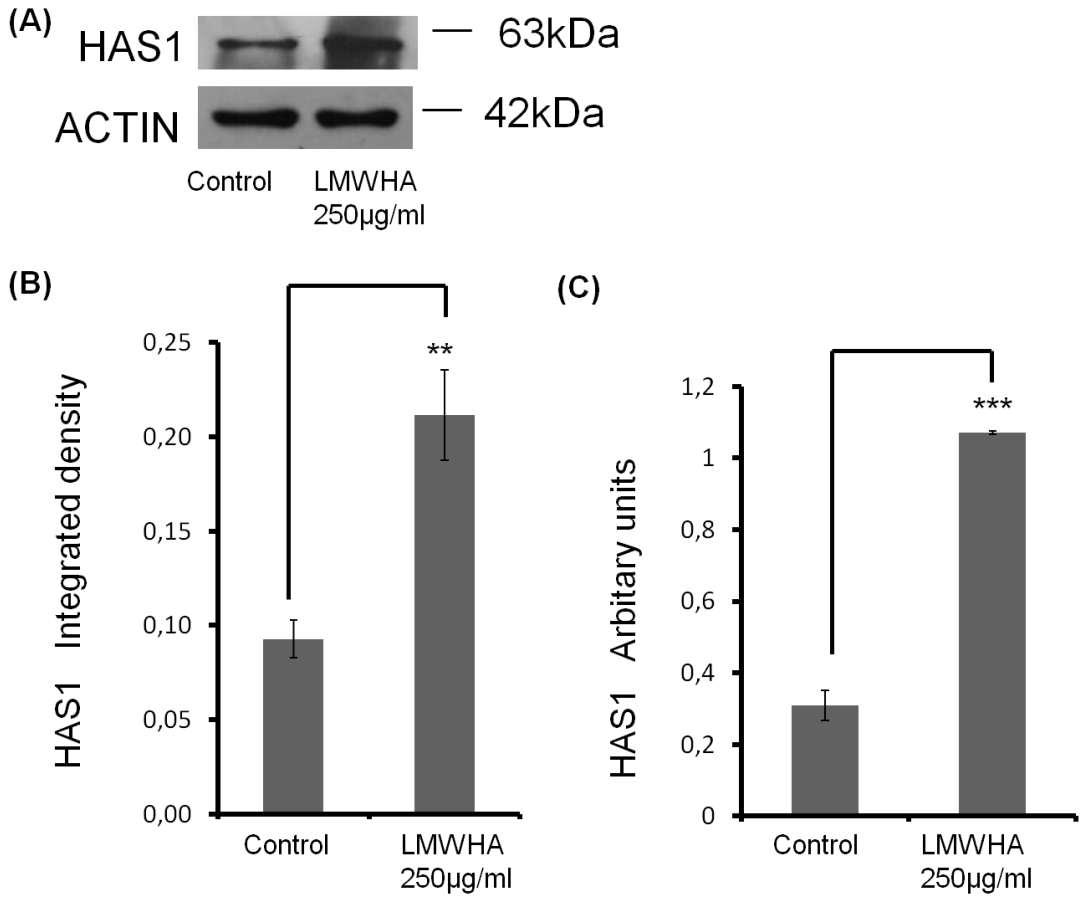


Figure 6. Effect of LMWHA treatment on HAS1 expression. NCTC2544 cells were treated for 24h with LMWHA 250μg/ml before harvesting. **(A)** Representative blots of HAS1 protein (63kDa) and actin (42kDa) are presented. **(B)** TLR4 protein bands were densitometrically analyzed and adjusted against actin. **(C)** HAS1 mRNA expression was verified by Real- time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0.05, ** p≤0.01, ***p≤0.001.

Similarly, putative effects of LMWHA on HAS3 expression were examined (Figure 7). Likewise to the demonstrated effect on HAS1, LMWHA in a concentration of 250μg/mL, increased HAS3 protein (p≤0.05) and mRNA (p≤0.05) expression. This finding, in combination to demonstrated HAS1 upregulation, indicates that during keratinocyte activation HA production of

various molecular weights is upregulated, enhancing thus, the simultaneous degradation to LMWHA, and finally the sustaining of inflammation.

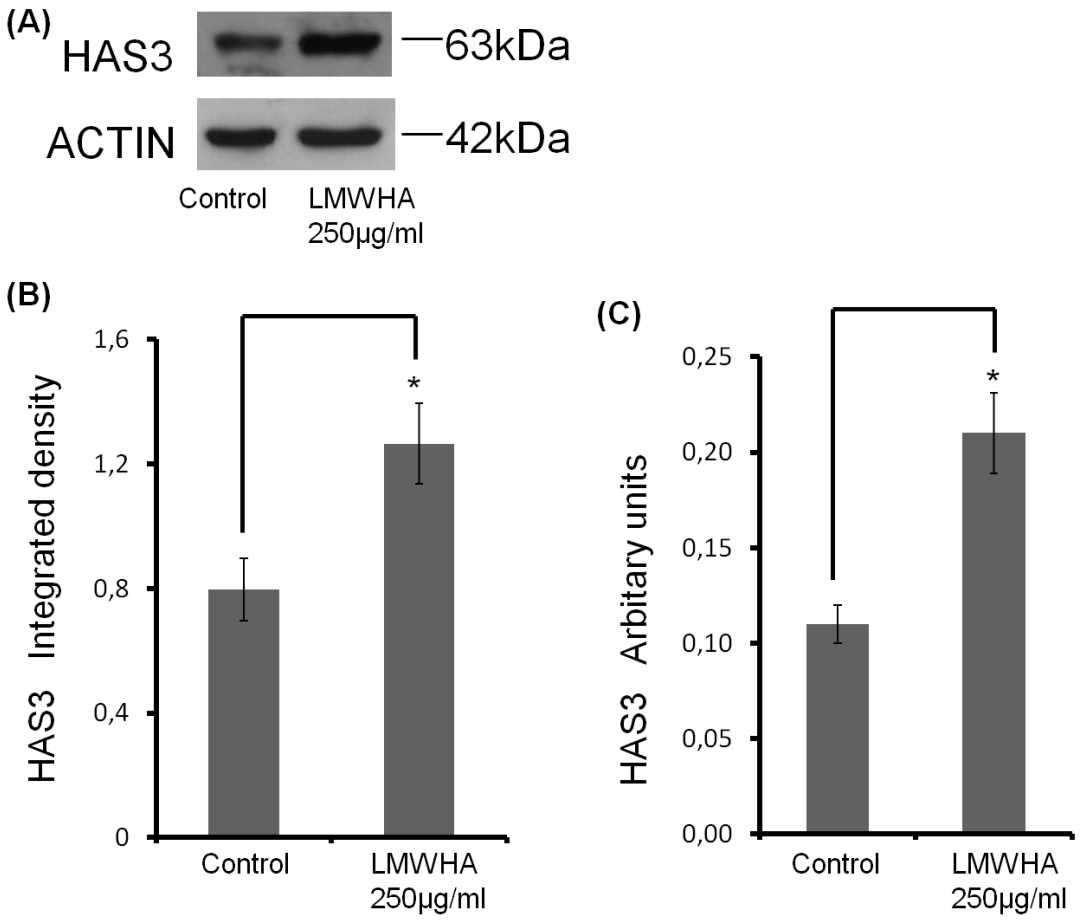
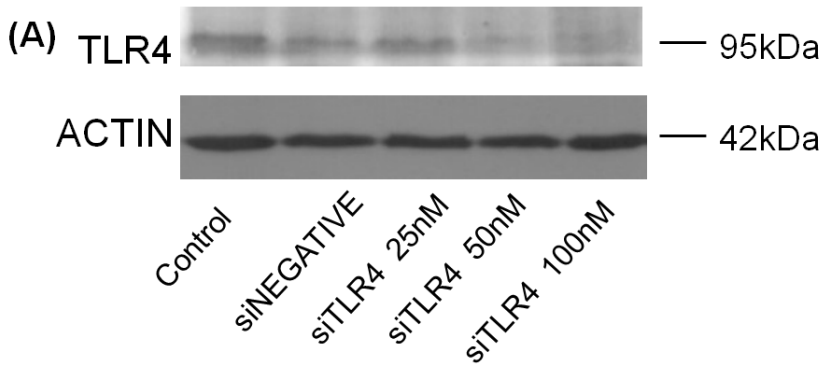


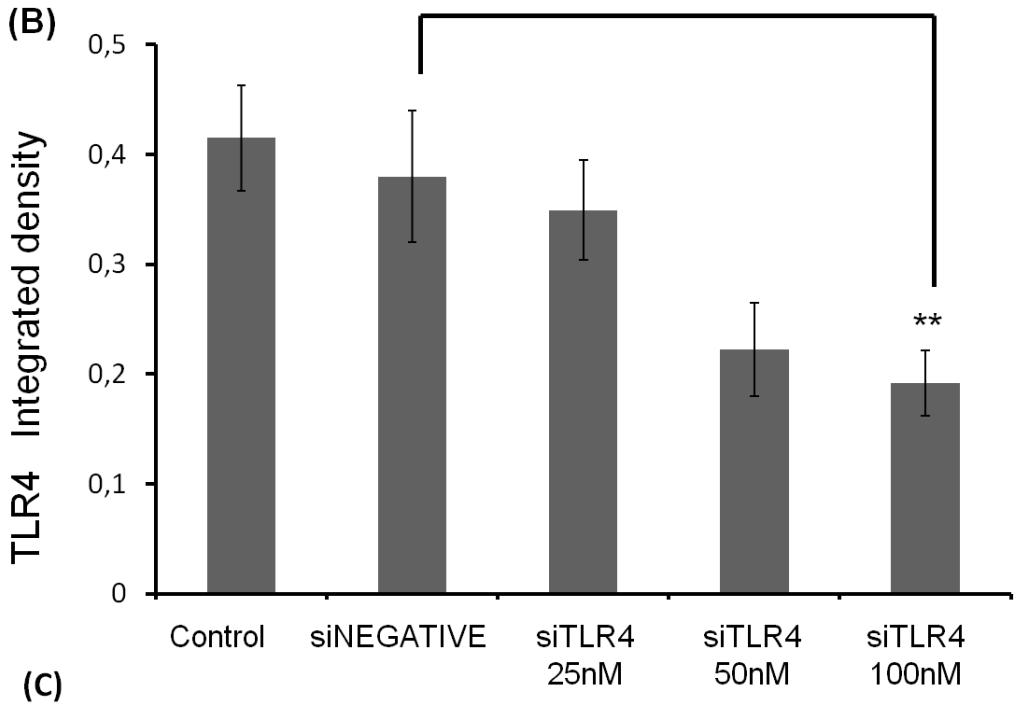
Figure 7. Effect of LMWHA treatment on HAS3 expression. NCTC2544 cells were treated for 24h with LMWHA 250μg/ml before harvesting. **(A)** Representative blots of HAS3 protein (63kDa) and actin (42kDa) are presented. **(B)** TLR4 protein bands were densitometrically analyzed and adjusted against actin. **(C)** HAS3 mRNA expression was evaluated by Real- time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0.05, ** p≤0.01, ***p≤0.001.

4.7 The role of TLR4 in the process of keratinocyte sensitization

4.7.1 Optimization of siTLR4

In continuation, the study focused on the potential involvement of TLR4 in the mechanisms of keratinocyte activation. Therefore, to further investigate the role of TLR4, the silencing of the TLR4 gene utilizing the siRNA method was performed. To achieve a satisfying downregulation of the targeted gene, three different concentrations of the specific siRNA were tested (25nM, 50nM and 100nM), as shown in Figure 8. The most efficient downregulation of 55% at the protein level ($p \leq 0.01$) and a 63% downregulation at the mRNA level ($p \leq 0.01$) was achieved with the utilization of the 100nM concentration of siTLR4 (Figure 8) which was further used in all siRNA experiments.


(A)
TLR4
95kDa
ACTIN
42kDa
Control
siNEGATIVE
siTLR4 25nM
siTLR4 50nM
siTLR4 100nM


(B)
TLR4 Integrated density
0,5
0,4
0,3
0,2
0,1
0
**
Control
siNEGATIVE
siTLR4 25nM
siTLR4 50nM
siTLR4 100nM

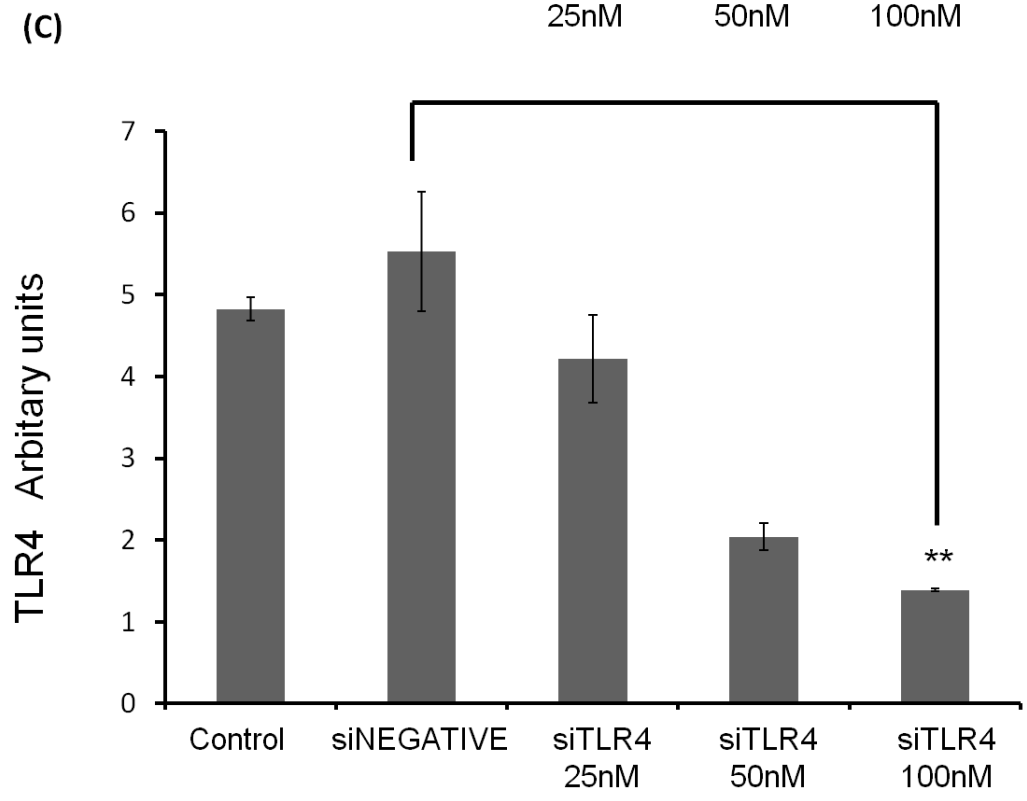

(C)
TLR4 Arbitary units
7
6
5
4
3
2
1
0
**
Control
siNEGATIVE
siTLR4 25nM
siTLR4 50nM
siTLR4 100nM

Figure 8. Transfection with siRNA specific for TLR4. NCTC2544 cells were transfected with three different concentrations (25nM, 50nM and 100nM) of siRNA sequence specific for TLR4 gene for 24h before harvesting. As control a non specific RNA sequence was used (siNegative). **(A)** Representative blots of TLR4 protein (95kDa) and actin (42kDa) are presented. **(B)** TLR4 protein bands were densitometrically analyzed and adjusted against actin. **(C)** TLR4 mRNA expression was determined by real time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0.05, ** p≤0.01, ***p≤0.001.

4.7.2 HAS1 is downregulated in TLR4 deficient cells

The next step of the present study, was to examine whether the demonstrated modulations of HAS enzymes' expression were perpetrated through TLR4 downstream activities. Therefore, after downregulation of the TLR4 gene, HAS1 expression was measured. Both HAS1 protein (p≤0.001) and mRNA (p≤0.01) levels were downregulated, as demonstrated in Figure 9. This finding shows a correlation between the synthesis of the studied GAG and TLR4 downstream signaling.

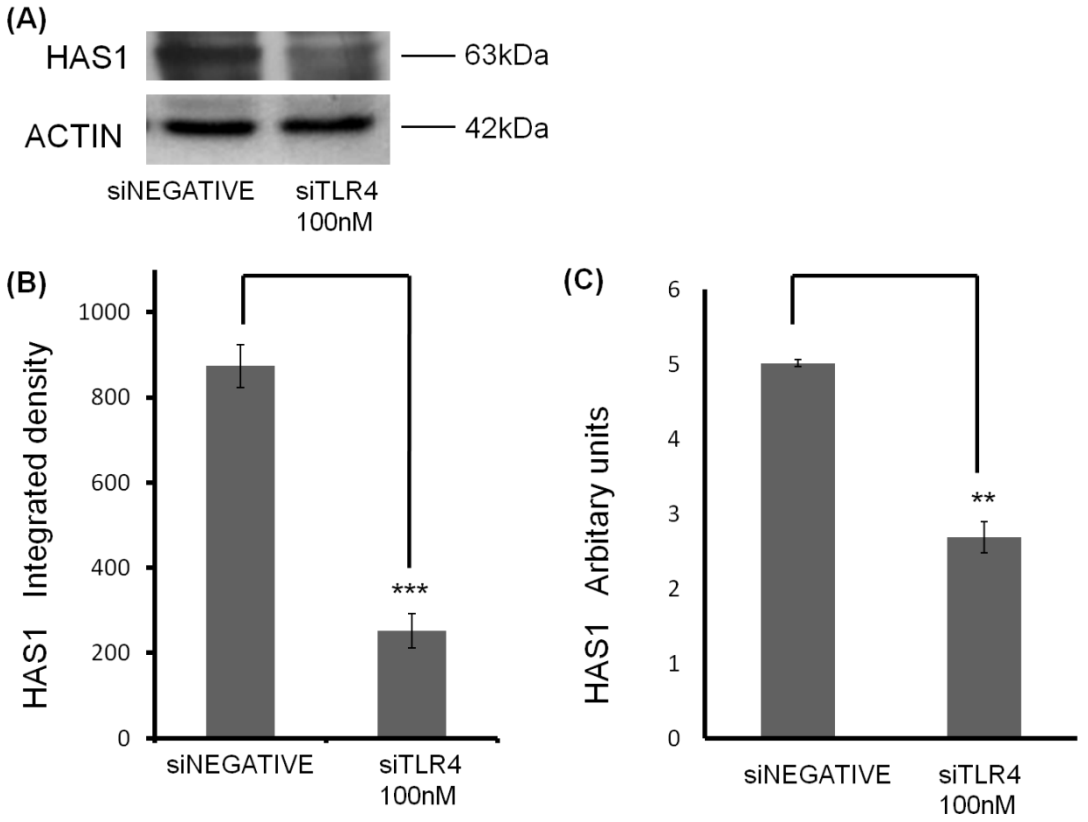


Figure 9. Effect of TLR4 downregulation on HAS1 expression. NCTC2544 cells were transfected with 100nM of siRNA sequence specific for TLR4 gene for 24h before harvesting. As control a non specific RNA sequence was used (siNegative). **(A)** Representative blots of HAS1 protein (63kDa) and actin (42kDa) are presented. **(B)** HAS1 protein bands were densitometrically analyzed and adjusted against actin. **(C)** HAS1 mRNA expression was evaluated by Real- time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0.05, ** p≤0.01, ***p≤0.001.

4.8 Effect of TLR4 downregulation on HAS3 expression

The expression of HAS3 in TLR4 deficient keratinocytes, was likewise estimated. Similarly to HAS1, HAS3 was also found to be decreased in keratinocytes whose TLR4 expression was attenuated as shown by Western blot (p≤0.05) and Real- time PCR (p≤0.05) (Figure 10). In combination, to the shown decrease of HAS1, these data suggest that TLR4 downstream signaling affects HAS isoforms expression in keratinocytes.

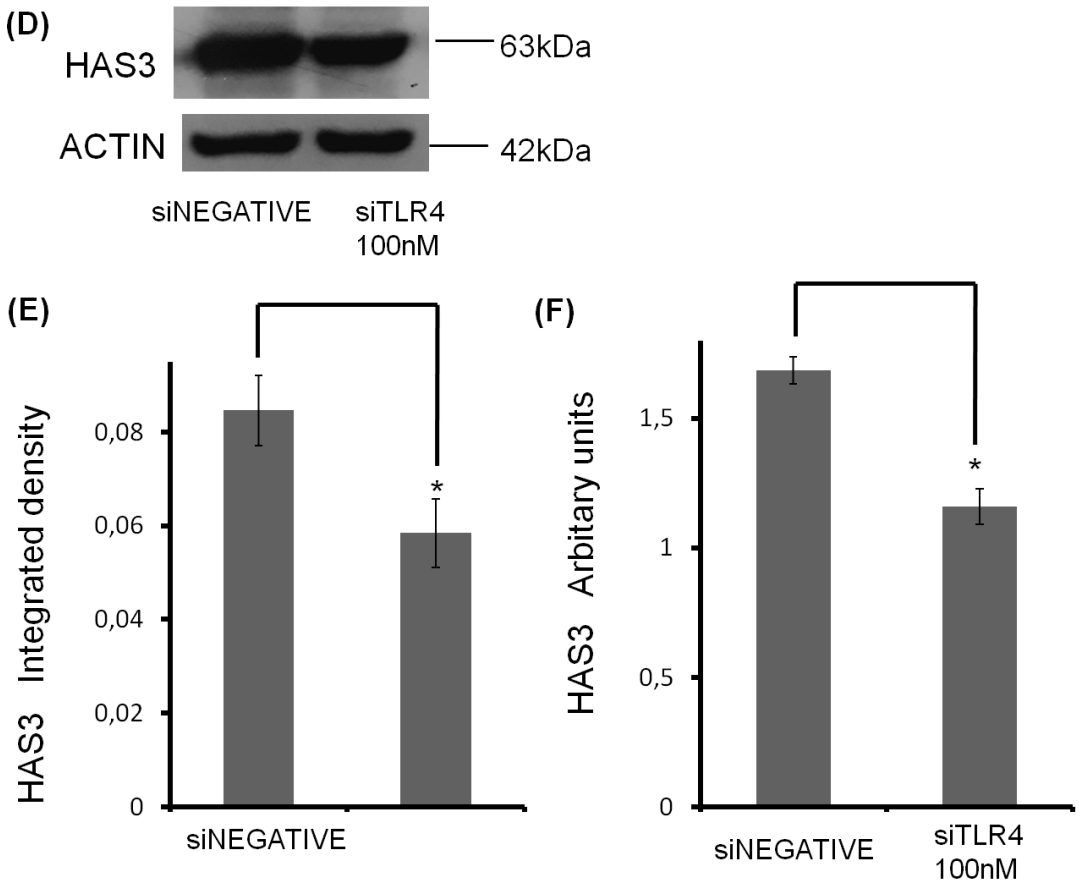


Figure 10. Effect of TLR4 downregulation on HAS3 expression. NCTC2544 cells were transfected with 100nM of siRNA sequence specific for TLR4 gene for 24h before harvesting. As control a non specific RNA sequence was used (siNegative). **(A)** Representative blots of HAS3 protein (63kDa) and actin (42kDa) are presented. **(B)** HAS3 protein bands were densitometrically analyzed and adjusted against actin. **(C)** HAS3 mRNA expression was determined by Real- time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0.05, ** p≤0.01, ***p≤0.001.

4.9 HA is decreased in TLR4 deficient cells

Additionally, the present thesis aimed to assess the effect of TLR4 on HA localization. The utilization of immunofluorescence demonstrated that HA was deposited to the pericellular matrix by NCTC4544 cells, in accordance to a

previous study (60). Upon downregulation of TLR4 expression, a decrease in HA signal was established (Figure 4G), demonstrating that HA pericellular deposition depends on TLR4 expression. These data are well correlated to the dependence of HAS1 and HAS3 expressions on TLR4 showed in this study.

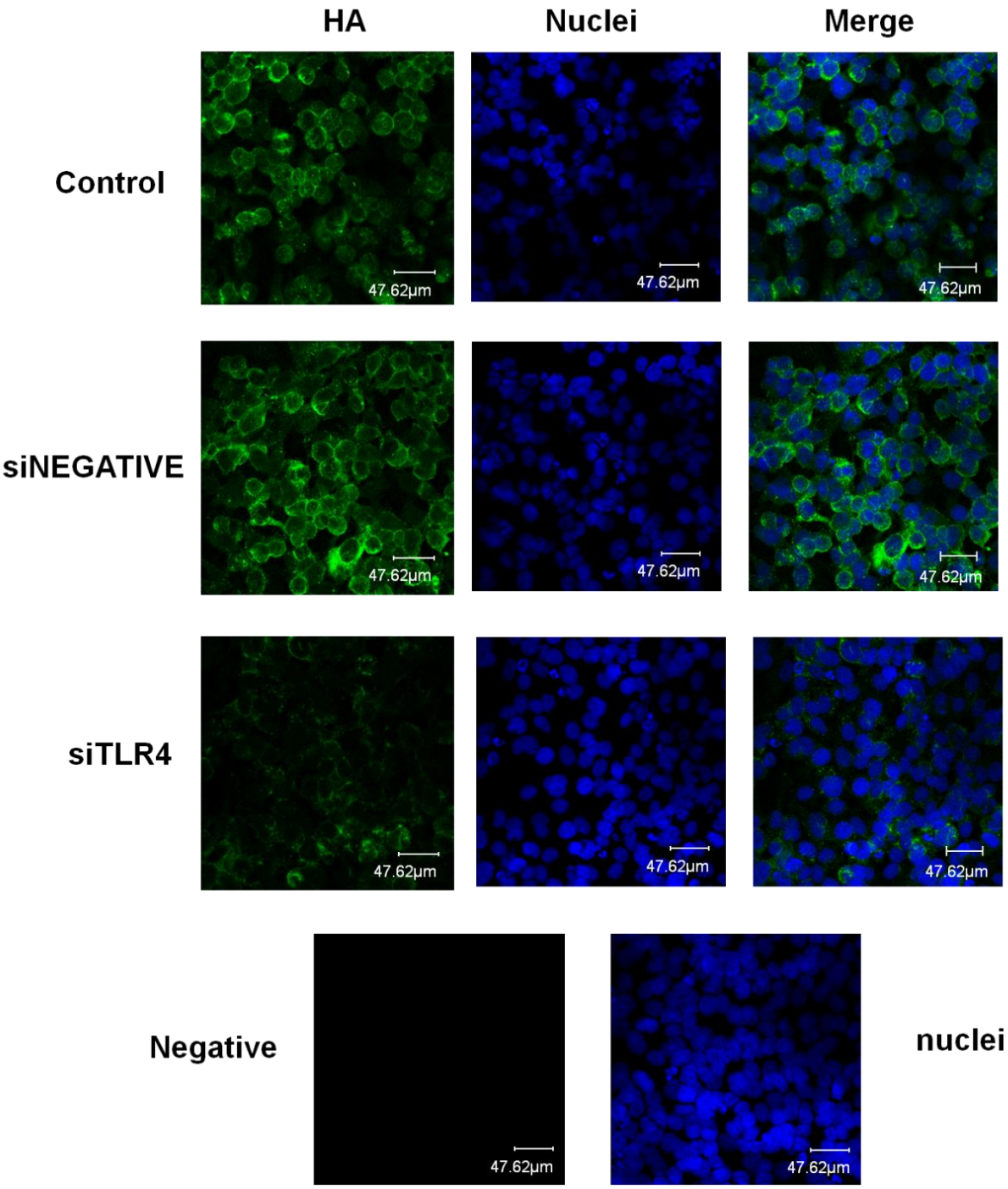


Figure 11. Effect of transfection with siRNA against TLR4 on HA localization NCTC2544 cells were transfected with 100nM of siRNA sequence specific for TLR4 gene for 24h before harvesting. As control a non specific RNA sequence was used (siNegative). HA was stained with green fluorescent antibody and the nuclei are presented in blue, after TOPRO staining.

4.10 Role of TLR4 on HA metabolism in keratinocytes exposed to PPD and DNCB

4.10.1 HAS1 and HAS3 allergen mediated upregulation, is decreased when TLR4 is blocked

Downregulating TLR4, with the siRNA method could not be used in combination with allergens, due to the shown upregulation of TLR4 expression by both PPD and DNCB. Both allergens were demonstrated to override the silencing of the TLR4 gene (*data not shown*). Thus, for the combination of allergen treatment and blocking of TLR4 action, a neutralizing antibody against TLR4 was utilized. This strategy aimed to block the allergen mediated TLR4 downstream signaling. In a previous study (160), the same approach was demonstrated to decrease keratinocyte IL-18 production upon exposure to contact allergens.

NCTC2544 were treated for 1h with anti-TLR4 2μg/ml and then, PPD (30μg/ml) (Figure 12A, C) and DNCB (2μg/ml) (Figure 12B, D) were added for 24h. As shown in Figure 12 neutralizing TLR4 antibody decreases PPD and DNCB mediated HAS1 and HAS3 upregulation ($p \leq 0.05$).

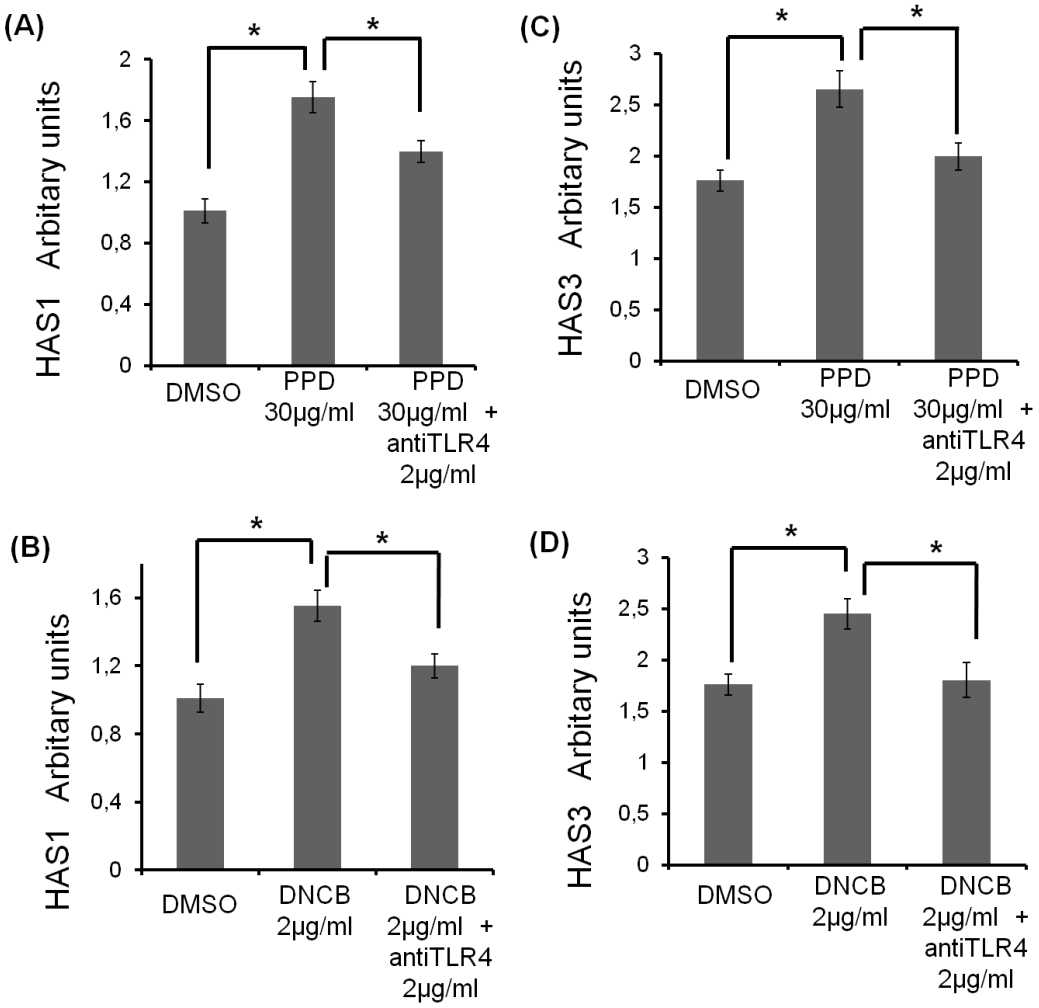


Figure 12. Effect of blocking TLR4 on PPD and DNCB mediated HAS1 and HAS3 upregulation. NCTC2544 cells were treated for 1h with a neutralizing antibody against TLR4 (2µg/mL) and then with PPD 30µg/ml and DNCB 2µg/ml for 24h, or just with PPD 30µg/ml and DNCB 2µg/ml for 24h (DMSO – negative control), before harvesting. **(A)**, **(C)** PPD 30µg/ml and **(B)**, **(D)** DNCB 2 µg/ml before harvesting. HAS1 and HAS3 mRNA expression was determined by Real- time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance * p≤0.05, ** p≤0.01, ***p≤0.001.

4.10.2 The effects of TLR4 on contact sensitizers mediated HYALs expression

Previously, it was demonstrated that exposure of keratinocytes to contact allergens causes an upregulation of HYAL isoforms. Specifically, HYAL1 was

increased upon both PPD (30μg/ml) and DNCB (2μg/ml) treatment, while HYAL2 was increased only by DNCB (60). In this study, whether this upregulation is mediated, among others, by TLR4 was investigated. Utilization of the anti- TLR4 antibody did not affect the basal levels of HYAL1 and HYAL2 expression (Figure 13 A, D). In continuation, NCTC2544 cells were pre- incubated for 1h with two concentrations of anti-TLR4 2μg/ml and 4μg/ml and then, exposed to PPD (30μg/ml) and DNCB (2μg/ml) for 24h. Real- time PCR verified the increase in HYAL1 levels PPD (Figure 13B, $p \leq 0.05$) and DNCB (Figure 13C, $p \leq 0.01$) treatments and increased HYAL2 expression after PPD treatment (Figure 13 E, $p \leq 0.05$), as previously shown (60). Interestingly, the upregulation of HYAL isoforms' expression due to the allergens' action was annulled in the presence of anti- TLR4 in at a concentration of 4μg/ml ($p \leq 0.05$). The above data suggest that TLR4, at least partially, contributes to the contact sensitizer effect.

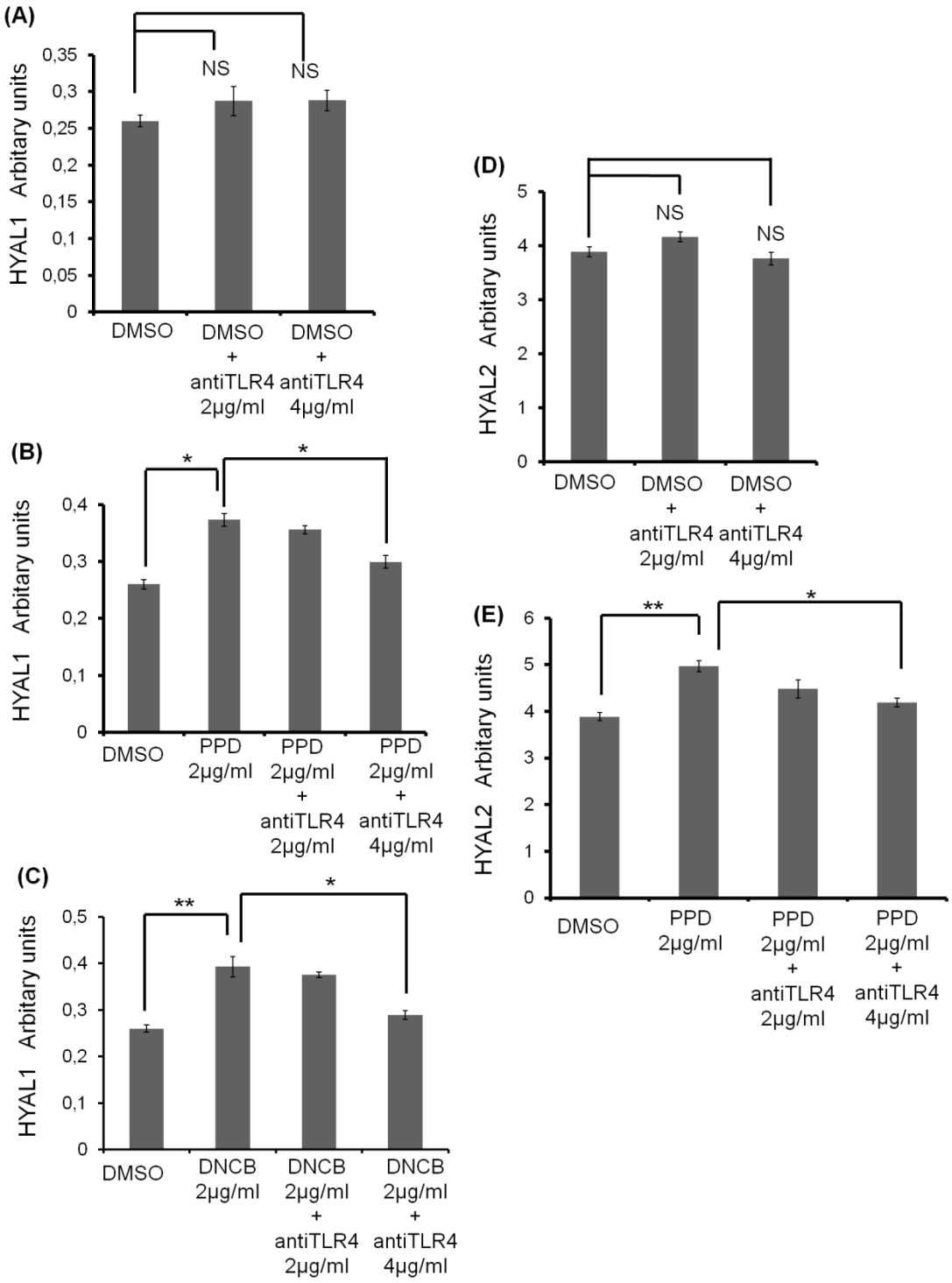


Figure 13. Effect of a neutralizing antibody against TLR4 on PPD and DNCB mediated HYAL1 and HYAL2 upregulation. NCTC2544 cells were treated for 1h with a neutralizing antibody against TLR4 2µg/mL and 4µg/mL and then with PPD 30µg/ml and DNCB 2µg/ml for 24h, or just with PPD 30µg/ml and DNCB 2µg/ml for 24h (DMSO- negative control), before harvesting. **(A), (B), (C)** HYAL1 and **(D), (E)** HYAL2 mRNA expression was determined by Real- time PCR. The results represent

the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0,05, ** p≤0,01, ***p≤0,001.

4.11 Effect of PPD and DNCB on NF-κB activation

Under inflammatory conditions, NF-κB is activated, which causes a multitude of cellular responses including TNF-α, IL-1 and IL-8 cytokine release (169). In the present study, NCTC2544 cells were treated with PPD (30μg/ml) and DNCB (2μg/ml) for two hours. Indeed, as NF-κB activation is prior to cellular responses, the appropriate treatment time had to be optimized. First, a 24h treatment, analogous to all other experiments, was performed, but it revealed no significant changes in NF-κB activation. Then 1h, 2h and 3h treatments were tested (*data not shown*). In these experiments maximum activation of NF-κB, upon contact allergen treatment, was determined to be at the 2h point (*data not shown*). LPS (500ng/ml) was used as positive control as it is a well established activator of TLR4 and NF-κB (103, 161). Western blot analysis revealed a contact sensitizer- mediated NF-κB activation by both allergens as shown in Figure 14 (p≤0.05 for PPD and p≤0.01 for DNCB). Furthermore, NF-κB translocation to the nucleus upon 2h allergen treatment was detected by immunoflurorence (Figure15).

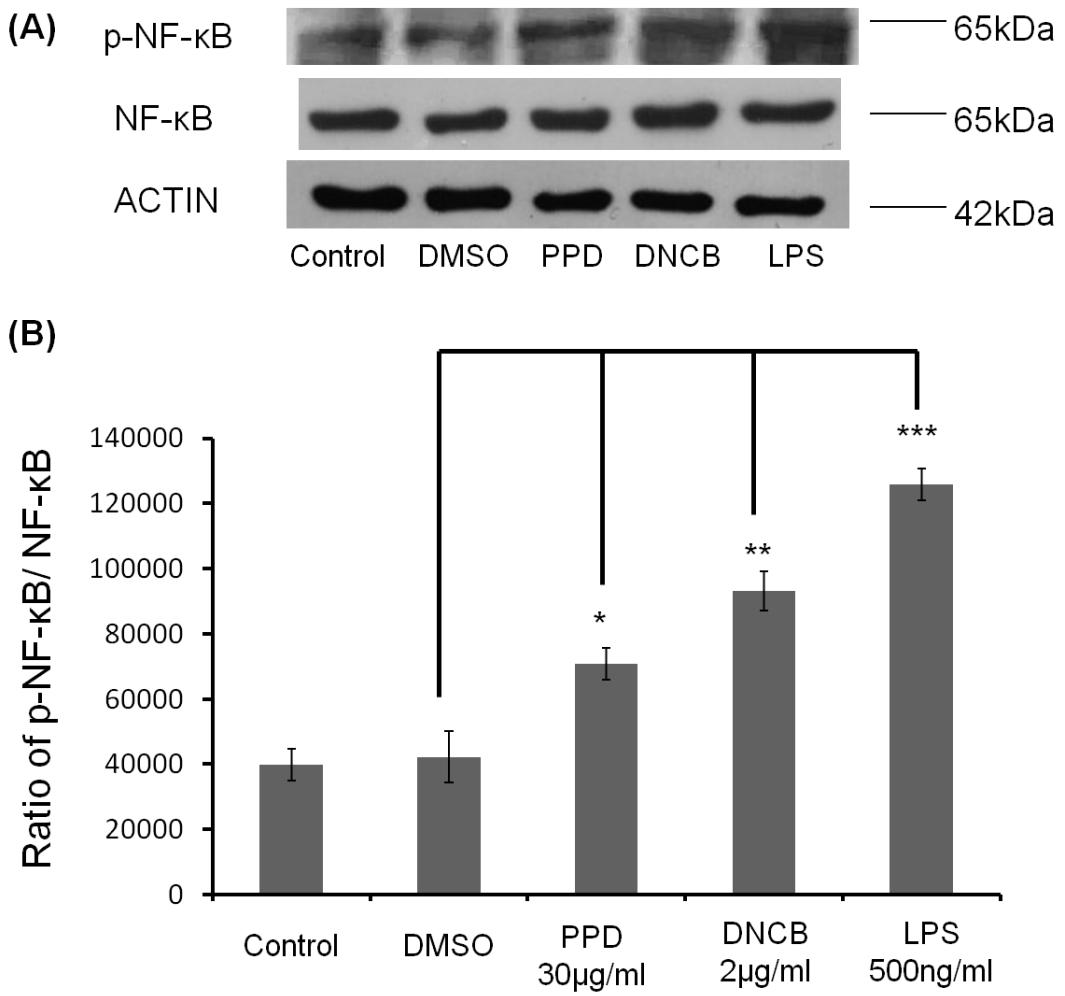


Figure 14. Effect of PPD and DNCB on NF-κB activation. NCTC2544 cells were treated with PPD 30µg/ml, DNCB 2µg/ml and LPS 500ng/ml as positive control, for 2h (DMSO - negative control) before harvesting. **(A)** Representative blots of NF-κB protein (65kDa), p- NF-κB (65kDa) and actin (42kDa) are presented. **(B)** NF-κB, p-NF-κB protein bands were densitometrically analyzed and adjusted against actin and the ratio of p-NF-κB/ NF-κB is presented. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0.05, ** p≤0.01, ***p≤0.001.

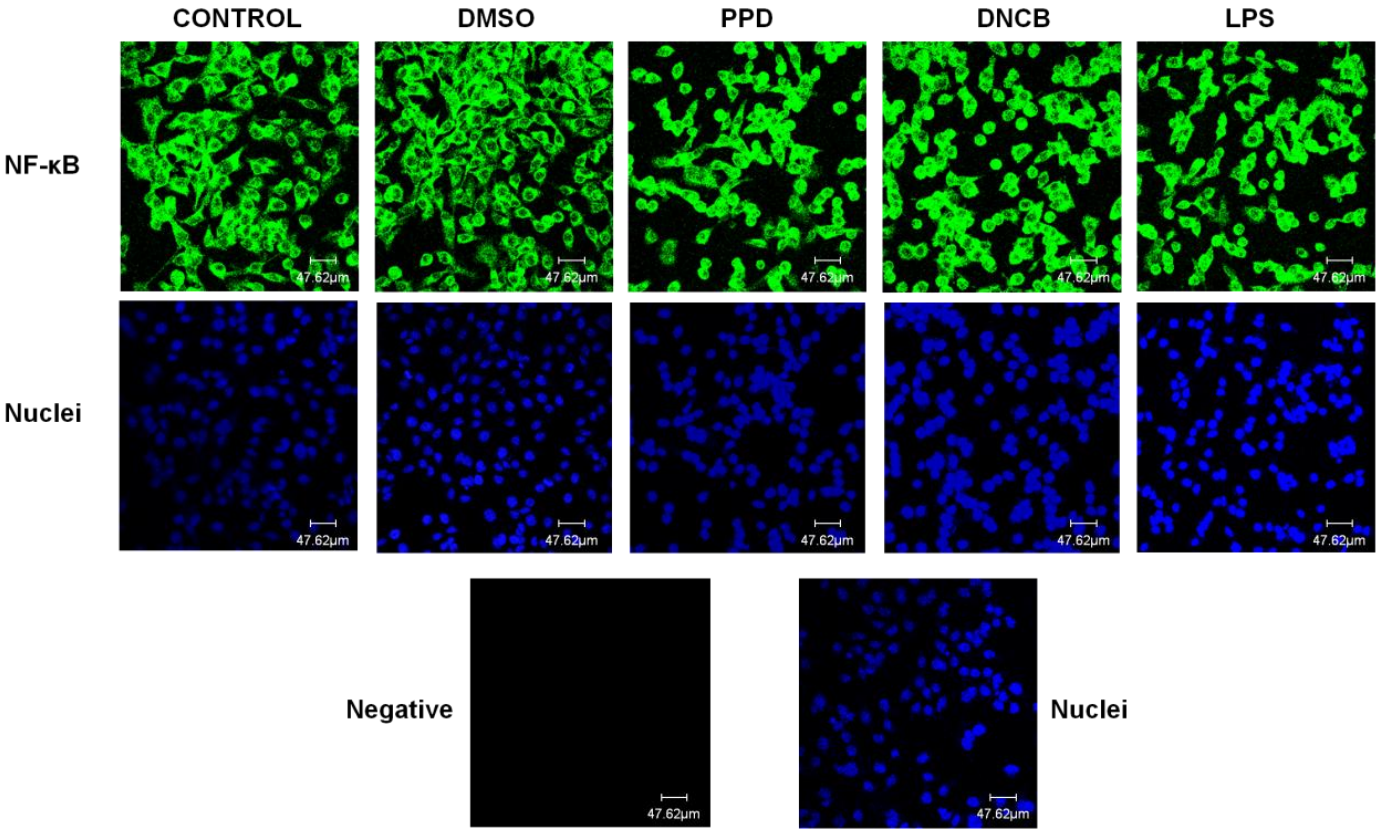


Figure 15. Effect of PPD and DNCB on NF-κB localization. NCTC2544 cells were treated with PPD 30µg/ml, DNCB 2µg/ml and LPS 500ng/ml as positive control (DMSO – negative control), for 2h before harvesting. NF-κB was stained with green fluorescent antibody and the nuclei are presented in blue after TOPRO staining.

4.12 Effect of TLR4 on contact allergen mediated NF-κB activation

Western blot analysis revealed that PPD- and DNCB-dependent NF-κB activation was attenuated upon blocking TLR4 with a specific neutralizing antibody (Figure 16). These data suggest that PPD and DNCB- dependent NF-κB activation is partly mediated through TLR4 downstream signaling.

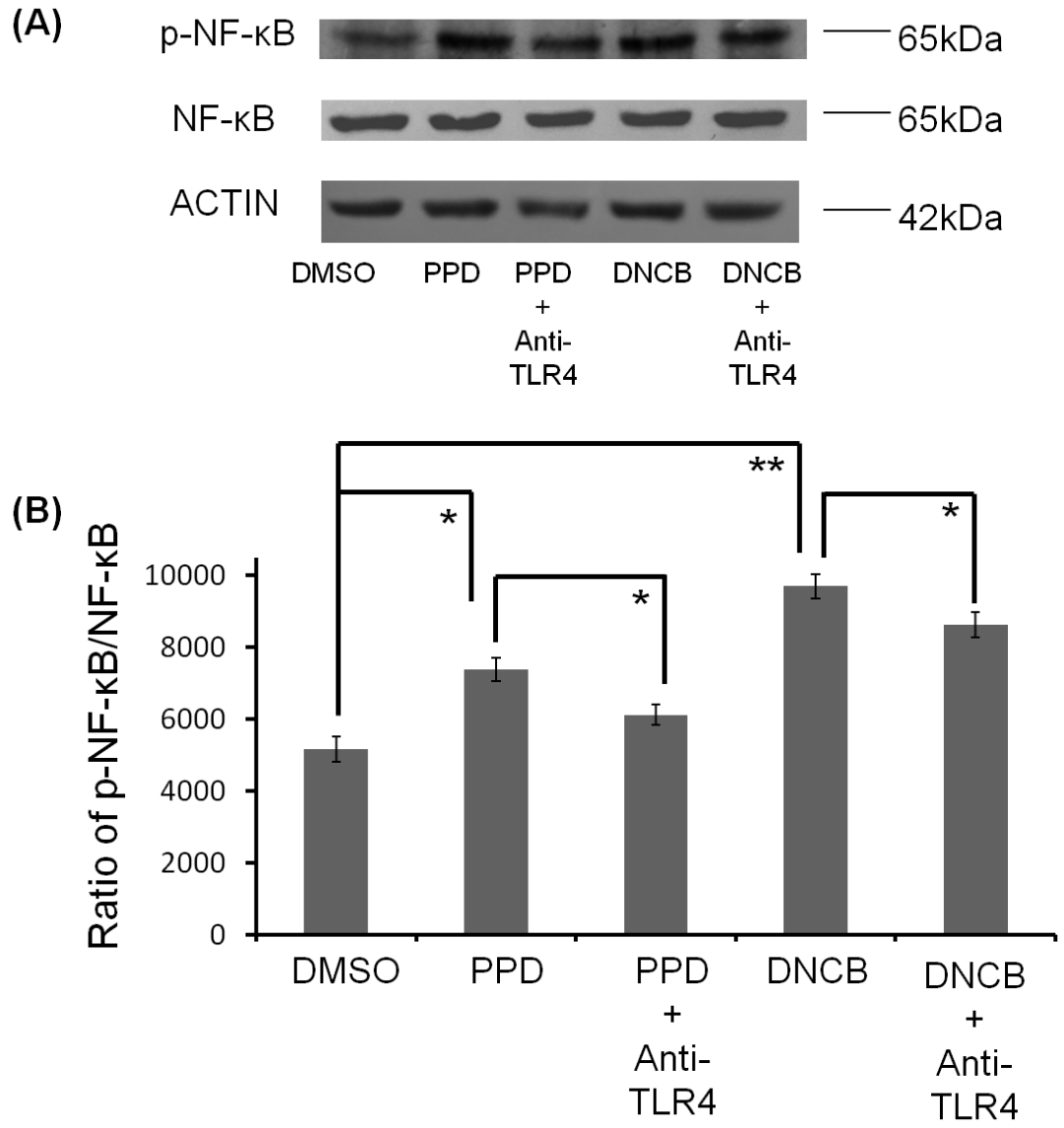


Figure 16. Effect of blocking TLR4 on NF-κB activation. NCTC2544 cells were treated either with a neutralizing antibody against TLR4 (4 μg/mL) for 1h and in continuation with PPD 30μg/ml and DNCB 2μg/ml during 2h or just with PPD 30μg/ml and DNCB 2μg/ml for 2h (DMSO - negative control) before harvesting. **(A)** Representative blots of NF-κB protein (65kDa), p- NF-κB (65kDa) and actin (42kDa) are presented. **(B)** p-NF-κB, NF-κB protein bands were densitometrically analyzed and adjusted against actin and the ratio of p-NF-κB/ NF-κB is presented. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0,05, ** p≤0,01, ***p≤0,001.

4.13 The effect of LMWHA on NF-κB activation

In order to examine the direct effect of LMWHA on NF-κB activation, keratinocytes were exposed to 250μg/ml of LMWHA. This approach resulted in enhanced NF-κB activation (p≤0.05), as shown in Figure 17A, B. On the other hand, a-acid, an inhibitor of HYALs activity and hence HA degradation, attenuated PPD and DNCB- mediated NF-κB activation (p≤0,05) (Figure 17C, D). In control experiments, a-acid was found not to affect the basal levels of keratinocyte NF-κB activation. These data correlate well with the supposition that LMWHA directly participates in NF-κB activation in this cell-type.

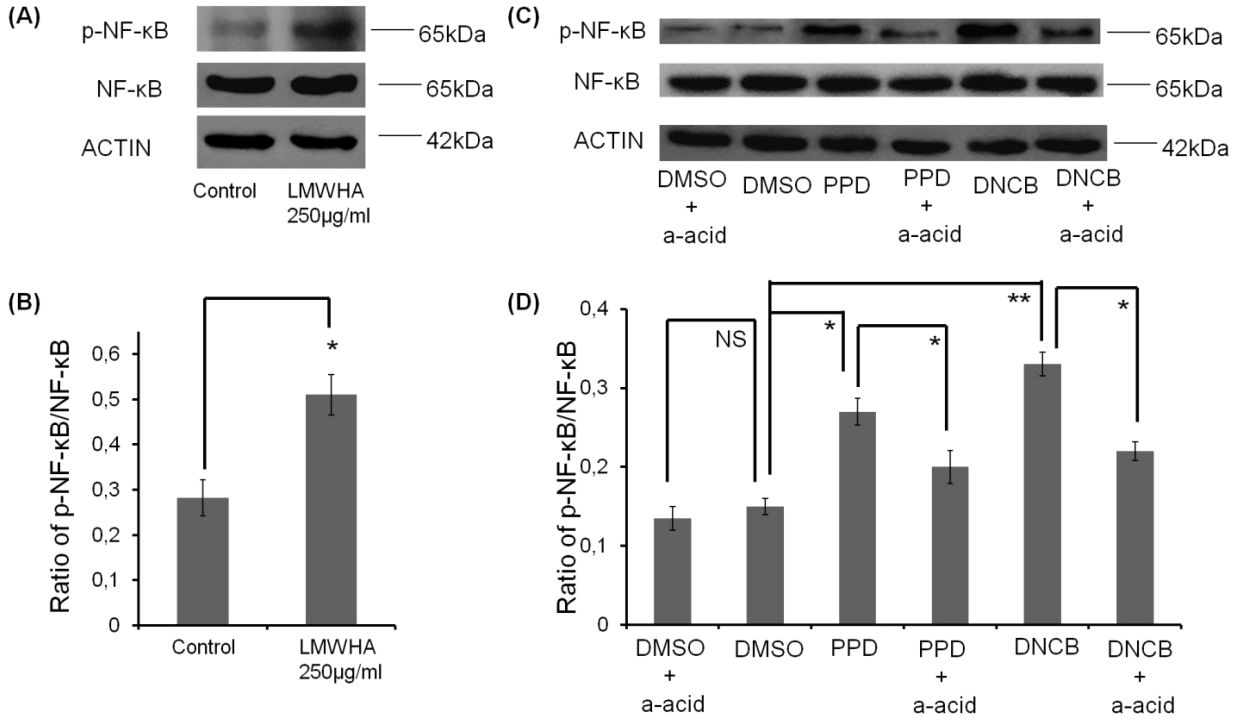


Figure 17. LMWHA effect on NF-κB activation (A), (B) NCTC2544 cells were treated for 2h with LMWHA 250μg/ml before harvesting. **(A)** Representative blots of NF-κB (65kDa), p- NF-κB (65kDa) and actin (42kDa) are presented. **(B)** p-NF-κB, NF-κB protein bands were densitometrically analyzed, adjusted against actin and the ratio of p-NF-κB/ NF-κB is presented. **(C), (D)** NCTC2544 cells were treated either with a-acid (40μM) for one hour and in continuation with PPD 30μg/ml, DNCB 2μg/ml for 2h, or just with PPD 30μg/ml and DNCB 2μg/ml for 2h (DMSO - negative control), before harvesting. **(C)** Representative blots of NF-κB (65kDa), p- NF-κB (65kDa) and actin (42kDa) are presented. **(D)** NF-κB, p-NF-κB protein bands were densitometrically analyzed and adjusted against actin and the ratio of p-NF-κB/ NF-κB is presented. The results represent the average of three separate experiments in

triplicate. Means ± SEM plotted; Statistical significance *p≤0,05, **p≤0,01, ***p≤0,001.

4.14 Effect of contact sensitizers on CD44 expression

In a previous study it was demonstrated that the effects of PPD, DNCB and LMWHA on keratinocyte sensitization were partly mediated through CD44 downstream signaling (60). In this study it was examined whether PPD and DNCB affect CD44 expression. The utilization of Real- time PCR demonstrated that PPD and DNCD do not affect CD44 expression (P=NS) (Figure 18).

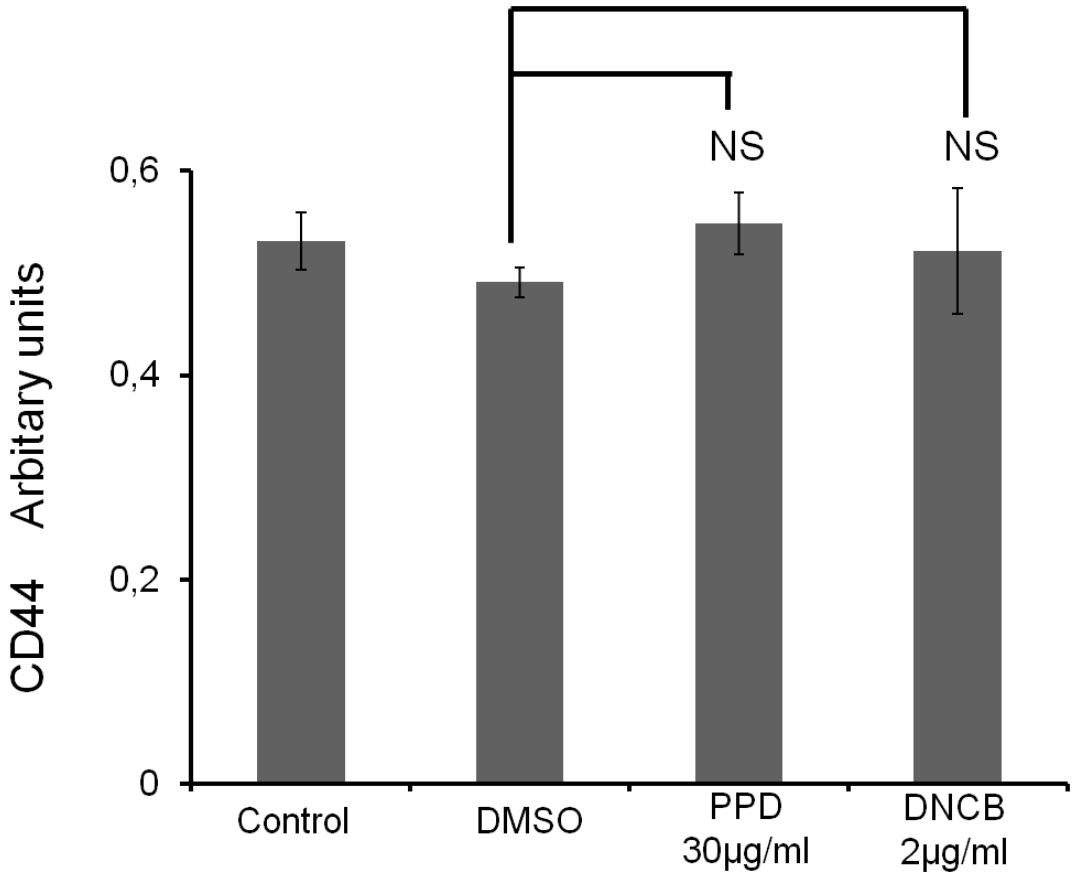


Figure 18. Effect of PPD and DNCB on CD44 expression. NCTC2544 cells were treated for 24h PPD 30µg/ml and DNCB 2µg/ml (DMSO – negative control) before harvesting. CD44 mRNA expression was verified by Real- time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0.05, ** p≤0.01, ***p≤0.001.

4.15 LMWHA inhibits keratinocyte proliferation

In addition to its contribution to the mechanisms of contact allergen mediated keratinocyte sensitization, HA is well established to be important for the wound healing process (19). During the processes of wound healing, which presents aspects of sterile inflammation, HA was shown to affect keratinocyte proliferation in a manner dependent on size and model utilized (46, 170, 171). Therefore, in the model system used in this study, the proliferation rate of keratinocytes, after LMWHA treatment was tested (Figure 19). First, an optimization of the appropriate cell number was performed (Figure 19A) and consecutively the effect of LMWHA on keratinocyte growth was assessed. The highest utilized concentration (250µg/ml) of LMWHA induced a significant inhibition of the keratinocyte proliferation rate (Figure 19B).

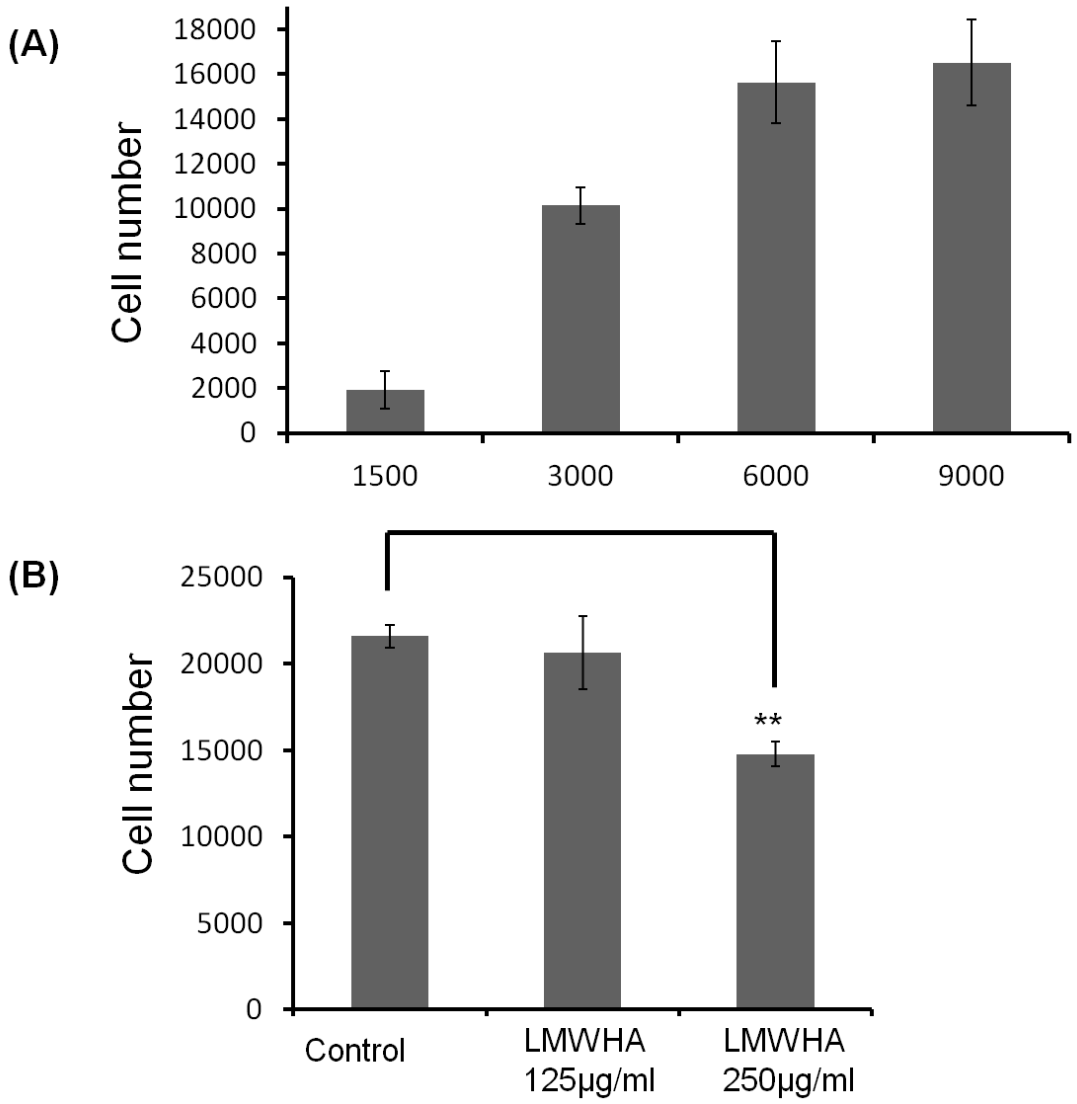


Figure 19. Effect of LMWHA on NCTC2544 cell proliferation. **(A)** Four different cell numbers were seeded in 96-well plates (1500, 3000, 6000, 9000) grown in culture media. **(B)** NCTC2544 cells were treated for 48h with LMWHA 125µg/ml and 250µg/ml before harvesting. Control cells were grown in cell culture media. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p≤0.05, ** p≤0.01, ***p≤0.001.

5. Discussion

TLRs were firstly established to mediate pathogen associated inflammatory responses, but over the years increasing data demonstrated important roles of the receptors in the processes of sterile inflammation (172). Importantly, TLR4 was shown to be implicated in several keratinocyte functions, such as proliferation. Thus, in HaCat cell line TLR4 acted as a negative regulator of proliferation (173). Moreover, *in vivo* experiments suggested that the activation of TLR4 by ligands released upon injury will result in the induction of IL-23 release by keratinocytes and the presentation of the atopic dermatitis phenotype (174). Furthermore, TLR4 expression has been correlated positively with inflammatory conditions, for instance, upon LPS treatment the receptor was increased in a synovial sarcoma cell line (175).

Keratinocytes express TLR4 and also secrete pro- inflammatory cytokines including, IL-1β and IL-18 as a response to exposure of various haptens (13, 176). Importantly, TLR4 expression was found to be increased in the skin of atopic dermatitis patients (177). Moreover, TLR4 downstream signaling has previously been correlated to ACD (9, 13, 160, 178, 179). Indeed, TLR4-deficient macrophages were shown to exhibit reduced cytokine expression upon activation (172). Interestingly, mice lacking TLR4 were not sensitized by IL-12, whereas mice deficient in both TLR2 and TLR4 did not respond to contact allergens 2,4,6 trinitroclorobnzene (TNCB), oxazolone and fluorescein isothiocynate (FITC) insult (13). Nickel, a well known contact sensitizer, was shown to induce in endothelial cells a pro- inflammatory cytokine IL-8 production, whereas the inhibition of MyD88, a TLR downstream mediator, abrogated the nickel-dependent IL-8 production (180). Likewise, the inhibition of TLR4 activity decreases chemical allergen mediated release of IL-18, which is a well established marker of keratinocyte sensitization (160, 163, 181). Hence, a correlation between TLR4 availability and cytokine release is arguable. In the present study, the contact sensitizers, PPD and DNCB, were demonstrated to increase keratinocyte TLR4 expression, in a concentration dependent manner. These data are similar to previous reports where the existence of feedback mechanisms between cytokine release and TLR4 expression, has been suggested. For instance, IL-33 released by murine

macrophages causes an upregulation of their TLR4 levels (182). Similarly, macrophages infected by lactate dehydrogenase- elevating virus (LDV) exhibited increased TLR4 expression (183). Indeed, LDV virus activates innate immune cells causing cytokine, including TNF-α, secretion to which these authors attributed TLR4 upregulation (183). Moreover, in mouse articular chondrocytes, IL-1β treatment was shown to stimulate TLR4 expression (184). Furthermore, in *in vivo* models, where mice were treated with DNCB, an increase in TLR4 levels was determined in the skin (185). Hence, it could be hypothesized that in NCTC2544 keratinocyte model, cytokine release triggered by allergens is responsible for the shown TLR4 upregulation.

HMWHA (200-2000kDa) is produced by HAS 1 and 2 whereas HAS3 produces HA of 100-1000kDa as recently discussed (19). Importantly, HAS enzymes are differentially expressed in pathological conditions (60, 77, 186-188). In the present study it was demonstrated that the three HA synthases were upregulated upon exposure of keratinocytes to PPD and DNCB contact allergens. Previously, HAS2 mRNA was shown to be increased upon inflammatory cytokine treatment in endothelial cells (49), suggesting that these mediators regulate HAS expression, as well as HA synthesis. As earlier demonstrated, blocking of TLR4 results in decreased allergen mediated IL-18 production (160). Moreover, in an *in vivo* model, TLR2 and TLR4 deficiency was accompanied by lower deposition of HA on the cell surface and decreased inflammatory response to lung injury (57). Likewise, in a mouse model, TLR4 deficiency was correlated to reduced oxidative stress and inflammation (189). In a separate murine TLR4 knock out model, a protective effect against brain injury was shown through decreased NF-κB activation (190). Additionally, in an *in vitro* study on a human synovial sarcoma cell line, LPS treatment and the resulting inflammatory conditions, initiated an increase in HAS2 and HAS3 (175). Indeed, in the present study TLR4- deficient keratinocytes exhibited significantly decreased HAS1 and HAS3 expression levels, implying a direct correlation between HA synthesis and TLR4-dependant signaling. The finding that allergens upregulate HA synthases, is supported by respective data in mouse synovial fibroblasts (191). More

accurately, mouse synovial fibroblasts inflammatory response was attenuated when HA synthesis was inhibited by the downregulation of the HAS genes. Therefore, these data argue that HA production is important for inflammatory conditions as produced HMWHA is a substrate for both HYALs and ROS activities, resulting in its degradation to LMWHA fragments. Importantly, in the present study TLR4- deficient keratinocytes exhibited significantly decreased HAS1 and HAS3 expression levels, implying a direct correlation between HA synthesis and TLR4- dependant signaling. Indeed, numerous endogenous molecules have been determined as TLR4 ligands including biglycan, CD138, α-crystallin A chain, β-defensin 2, endoplasmin, fibrinogen, fibronectin, heparan sulphate, HMGB1, HSP22, HSP60, HSP70, HSP72, hyaluronan, monosodium urate crystals, S100 proteins and tenascin-C, among others, as previously discussed by Yu, Wang (192). Some of these ligands e.g fibronectin, laminin or tenascin-C are important components of the epidermal ECM and of the epidermal- dermal junction and known to be involved in the regulation of skin homeostasis (193, 194). Therefore, it is conceivable that the binding of these molecules to TLR4, under physiological conditions, can mediate respective receptor downstream signaling, regulating the basal levels of HAS isoforms expression. Previously, HAS2 mRNA was shown to be increased upon treating endothelial cells with IL-1β and TNF-α inflammatory cytokines (49), suggesting that these mediators regulate HAS expression and HA synthesis. Moreover, as reviewed by Ghatak, Maytin (150), TNF-α and IL-1β, whose expression is mediated by TLR4, have been demonstrated to affect HA deposition in several cell types.

LMWHA is also an established DAMP, as well as a TLR receptor ligand. Indeed, LMWHA is known to affect innate immune system activation, resulting in the release of inflammatory mediators (57, 195). Indeed, LMWHA, whose structure with its repeating disaccharides resembles that of pathogens, is recognized by TLRs, and thus, bestows to this size HA the ability to induce inflammatory response (172). In the present study, well correlated to earlier findings, it is demonstrated that treating keratinocytes with LMWHA enhances NF-κB activation. In a previous study, on the human keratinocyte model NCTC2544, it was demonstrated that LMWHA activates keratinocytes, as

monitored by IL-18 production (60). Importantly, it is well established that LMWHA can bind the TLR4 receptor and thus, to participate in immunomodulation. This is well corroborated by a study, where cytokine and chemokine production triggered by HA fragments in peritoneal macrophages, was found to be annulled in $TLR2^{-/-}$ and $TLR4^{-/-}$ cells (172). Furthermore, activation of TLR4 by HA was demonstrated in both *in vivo* and *in vitro* models (167, 184, 196). Previously, in HaCat keratinocyte cell line, LMWHA of 6-50kDa caused an upregulation of TLR4 expression (157), which correlates well to the enhancement of TLR4 levels, demonstrated in the present study. Endogenous LMWHA is generated either by chemical degradation due to ROS or hyaluronidase enzymatic action (79, 160, 197). Noteworthy, in inflammatory conditions, ROS generation is increased whereas, it was demonstrated in murine skin that the inhibition of ROS action with antioxidants reduces skin sensitization by allergens (149, 198). The same authors had likewise demonstrated that, the inhibition of HYALs activity with aristolochic acid reduced contact sensitization. Moreover, it was previously shown, that allergens increase the expression of HYALs, decreasing HA deposition to keratinocyte pericellular matrix by increased degradation of HMWHA to small HA fragments (60).

Binding of LMWHA to the TLR4 receptor triggers downstream signaling which leads to NF-κB activation as shown in dendritic cells (199). Separate studies had likewise demonstrated that HA fragments induce NF-κB activation without specifying the mechanism(s) involved (166, 200). Moreover, LMWHA was shown to mediate cytokine and chemokine production *via* TLR4 (172). Indeed, TLR4 deficient macrophages, do not release inflammatory cytokines and chemokines in response to LMWHA stimulus (57). Furthermore, small HA fragments also induce maturation and migration of dendritic cells through TLR4 activation, a process important for the innate immune response (60, 166). Here, the effect of LMWHA on the expressions of the LMWHA receptor TLR4, as well as on HAS1 and HAS3 expressions was investigated, in a keratinocyte model system. This approach demonstrated an increase of the respective target expressions. The increase of LMWHA endogenous levels is a result of contact allergen treatment is hypothesized. As mentioned above,

both HA synthases (in this study) and HYALs (60) are increased in keratinocytes exposed to PPD and DNCB contact sensitizers. Hence, the enhanced synthesis of HMWHA, due to increased expression of HAS isoforms, will in combination to HYALs and ROS activities result in increased LMWHA generation. As LMWHA was shown to further increase IL-18 production (60), as well as HAS isoforms expression it could be argued that this GAG assists in sterile inflammation sustenance.

As HAS and HYAL expression are increased in keratinocytes upon PPD and DNCB treatments, it was considered significant to examine if this upregulation is TLR4 mediated. Moreover, in the present study, it was shown that the basal levels of HAS isoforms are diminished in TLR4 deficient keratinocytes. Hence, upon blocking TLR4 with a neutralizing antibody, the contact allergen-dependent increase in HAS1, HAS3, HYAL1 and HYAL2 expression was found to be attenuated. Therefore, blocking TLR4 downstream signaling will also result in lower HA synthesis, decreased release of LMWHA and attenuated inflammatory response. These data are in agreement with previous studies where TLR4- deficient mice have dampened ability to respond to an insult (57, 201). Importantly, it has been suggested that the inhibition of TLR4 downstream signaling is a promising therapeutic approach for inflammation- correlated pathologies (103, 202, 203). The importance of HYALs contribution to the process of TLR4 mediated inflammation was also shown in HYALs- deficient mouse synovial fibroblasts, where the cytokine-mediated overexpression of TLR4 was found to be attenuated (191). In the same study, the downregulation of HYAL isoforms in combination to treatment with anti- oxidants reduced the release of inflammatory mediators.

The triggering of TLR4 leads to downstream NF-κB activation and is directly correlated to inflammatory responses (169, 204). Increased NF-κB activation has been observed in several inflammatory conditions, such as inflammatory bowel diseases, rheumatoid arthritis and asthma (177). At this point, it is noteworthy to mention that lypopolysaccharide (LPS), detected to the outer membrane of Gram- negative bacteria, is a well established pathogen activator of TLR4 downstream signaling (102). Hence, in this study LPS was utilized as a reliable a positive control for the activation of TLR4 and

downstream mediators. In fact, LPS is well established to activate TLR4, with consequent downstream activation of NF-κB with consequent cytokine secretion, as demonstrated in several cell lines including mouse embryonic fibroblasts (205), as well as keratinocytes (176). Indeed, therapeutic approaches against inflammation target the NF-κB pathway (206, 207). For instance, the flavone apigenin, which exhibits anti- inflammatory activities, has been demonstrated to suppress LPS mediated cytokine secretion partly *via* suppressing NF-κB activation in placenta and fetal membranes (208). Similarly, in periodontal ligament cells apigenin-7-glycoside decreased LPS mediated inflammation by inhibiting NF-κB and MAPK intracellular pathways (209). In fact, apigenin downregulated, among others, the expression of several cytokines including IL-1β, IL-2, IL-6, IL-8, as well as TNF-α. Furthermore, the same substance inhibited production of cytokines, such as IL-1β, IL-2, IL-6 and TNF-α in human epithelial cells (210). In keratinocytes, apigenin not only reduced the production of inflammatory cytokines, but was also found to decrease the LPS mediated increase in TLR4 levels and NF-κB activation (161). Furthermore, in a different study, DNCB treatment *in vivo* induced increased TLR4 and NF-κB expression in rat skin, as well as cytokine, including IL-18, release. These effects were attenuated by a flavonoid preparation which specifically inhibits NF-κB *via* TLR4 neutralization (185). Additionally, the cytokine IL-1β is able to activate NF-κB in mouse chondrocytes, with resultant upregulation of the cytokines' transcription (184). Oral exposure to diisononyl phthalate (DINP) and sensitization by FITC in mice, resulted in increased NF-κB activation, IL-18 release and an ACD like phenotype, which was attenuated by the utilization of an NF-κB inhibitor (211). In the present thesis, PPD and DNCB treatments enhanced NF-κB activation, as demonstrated by increased p-NF-κB levels, and also, by the shown NF-κB translocation from the cytoplasm to the nuclei of the cells. Importantly this activation is mediated partly *via* TLR4 receptor, as blocking of TLR4 diminished the effect. Moreover, previously, it was shown that, inflammatory cytokines increased HAS genes expression, whereas the inhibition of NF-κB ameliorated the latter effect (49, 212), which well correlates to the data of the present study. Importantly, in atopic dermatitis patients, NF-κB levels nuclear levels are increased when compared to healthy skin (177). In order to confirm

whether the observed contact sensitizers' dependent NF-κB activation is mediated at least partly by TLR4, the receptor was blocked with a neutralizing antibody. Indeed, this approach resulted in attenuated NF-κB activation well in agreement with the above mentioned studies. Furthermore, in the present thesis, a specific inhibitor of HYAL activity, a-acid, was utilized in order to diminish HA degradation. As a result, the allergen- mediated NF-κB activation of keratinocytes was decreased. On the other hand, the exposure of keratinocytes to LMWHA resulted in enhanced NF-κB activation. These data in combination to the previously shown, attenuated keratinocyte activation by contact allergens upon a-acid treatment (60), support the suggestion that allergen- dependent NF-κB activation and downstream inflammatory signaling, are partly executed *via* LMWHA effects.

Moving on to a different topic, the important role of HA in the wound healing process, which is a separate model of sterile inflammation, has been widely discussed. At the initiation of the process HMWHA deposition was found to be increased (53) whereas, as wound healing proceeds, an instant decrease of HA levels occurs that will finally lead to an enhanced, important to wound closure, collagen deposition. This type of wound healing results in scar formation (53). Interestingly, in fetuses wound healing can be terminated without scar formation (154). A basic difference between fetus and adult wound healing are the sustained HA levels as the decrease of HA deposition does not occur in fetuses (155). Furthermore, adult tissues exhibit higher HYAL isoforms expression, as compared to fetal skin (46). Therefore, while HMWHA exhibits beneficial effects on the wound healing process, decrease of High and increase of Low Molecular weight HA, seems to contribute to scar formation. Indeed, in the present study, it was investigated whether LMWHA affects keratinocyte proliferation, as cell proliferation is part of the wound healing process and contributes to dermal pathologies. An inhibition of keratinocyte growth by LMWHA is demonstrated. Previously it was demonstrated that HA fragments have the ability to inhibit fibroblast proliferation in a molecular weight and concentration dependent manner (213). In another study, LMWHA resulted in inhibition of fibroblast proliferation *via* membrane hyperpolarization. In fact, the authors suggested that the

effects were not concentration dependant (214). Furthermore, chronic wounds are characterized by increased secretion of pro- inflammatory cytokines; whereas LMWHA has been demonstrated to promote angiogenesis at the injured area (134). ROS is also an important factor in injuries as it degrades HMWHA and thus LMWHA levels are increased (41). The ability of LMWHA and contact allergens to modulate keratinocyte growth may, likewise, contribute to the contact dermatitis disease phenotype. Further investigation is needed in order to clarify the complex roles of HA in wound healing process.

6. Conclusions

In conclusion, the present thesis suggests that during keratinocyte activation by contact allergens, TLR4 and HA interactions have an important role. Upon exposure of keratinocytes to contact allergens, HMWHA is cleaved to LMWHA, either enzymatically by HYALs or chemically by ROS action. This will result in increased LMWHA levels. Upon binding of LMWHA to TLR4 the receptor is activated with the resulting downstream triggering of NF-κB and its translocation to the nucleus. The translocated NF-κB will engage in transcriptional regulation of target genes with the consequent inflammatory cytokine production. This chain of events, in turn, triggers increased TLR4 expression. HASs upregulation will provide even more HMWHA as substrate for the degrading factors ROS and the also upregulated HYALs. Importantly, when TLR4 is blocked, the contact allergen mediated effects on HAS and HYAL isoforms expression and also NF-κB activation, are diminished, demonstrating the importance of the receptor to the immune response. Therefore, keratinocyte activation by contact allergens is partly mediated *via* a LMWHA/ TLR4/ NF-κB signaling axis.

The results of the thesis are summarized in Figure 20 both graphically (A, B, C) → used as graphical abstract in Kavasi, Berdiaki (215) and as a chart (D):

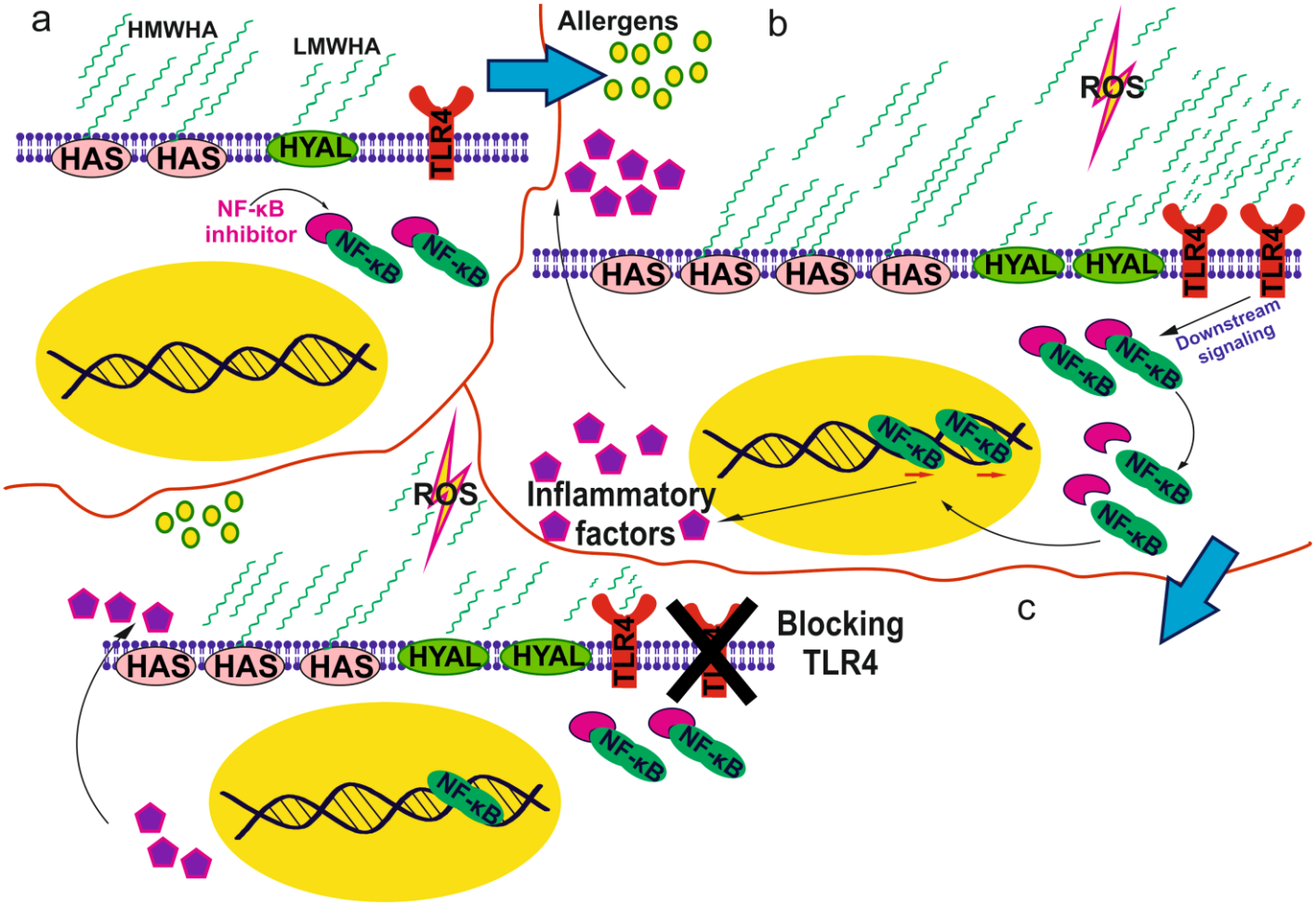


Figure 20 (a-c): The role of HA-TLR4 interaction in the process of contact allergen-induced keratinocyte activation. Keratinocyte cell membrane is presented graphically in **(a)** inactivated condition, **(b)** in activated by contact allergens condition and **(c)** upon blocking of TLR4 receptor. Allergens upregulate HASs, HYALs and also TLR4. As a result, HMWHA is increased and degraded to LMWHA by HYALs and ROS, hence, LMWHA levels are elevated. NF-κB is downstream activated, causing the transcription of inflammatory factors. When TLR4 is neutralized, the effects are all diminished.

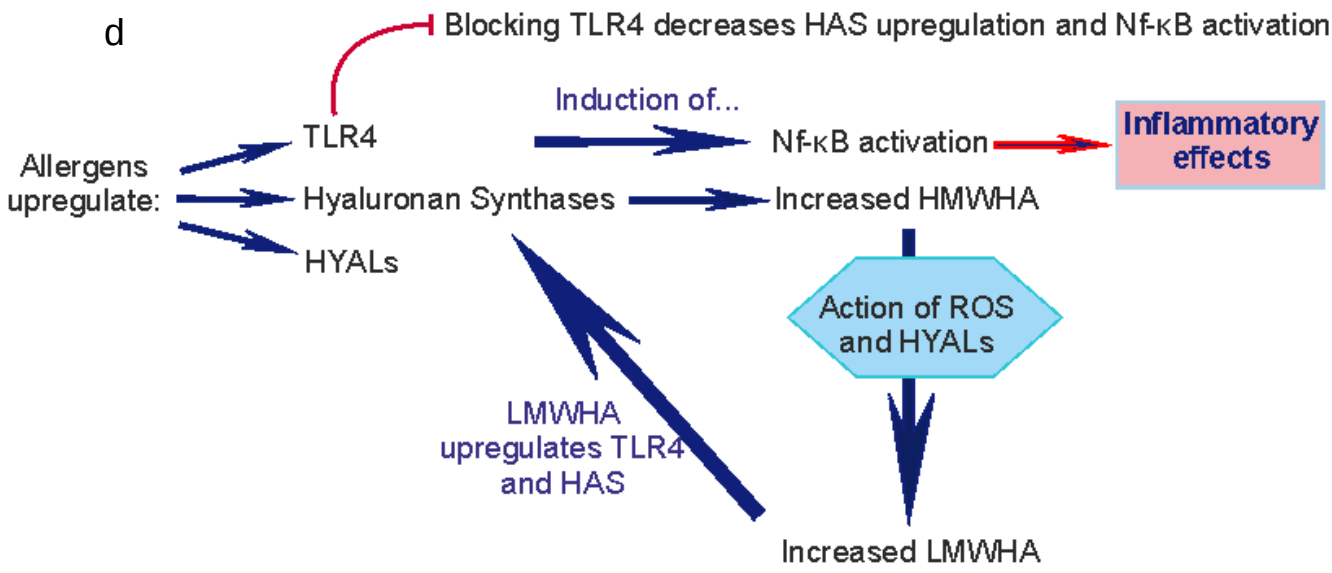


Figure 20 (d). Contact allergens, in a manner dependent on TLR4/ NF-κB downstream signaling, induce TLR4, HASs and HYALs overexpression resulting in increased synthesis and degradation of HA. Released pro- inflammatory LMWHA fragments facilitate TLR4, HAS and HYALS overexpression and sustain keratinocyte sensitization.

7. Future approaches

Further investigation is needed in order to clarify all the steps of HA/ TLR4/ NF-κB signaling pathway. An important factor that could be examined is the CD44, HA receptor. CD44 is a main HA receptor whose activities regulate many biologic functions including inflammation (49, 216). Previously it was demonstrated that inflammatory effects of PPD were diminished upon utilization of a neutralizing antibody specific for CD44 (60). To be more accurate, the term “inflammatory effects” was quantified by IL-18 assay, as it is well established that contact sensitizers induce the production of IL-18 in keratinocytes (160). Thus, it was shown that the blocking of the CD44 receptor, by a specific neutralizing antibody, ameliorated the PPD mediated IL-18 production, as well as that the allergen dependent IL-18 secretion was decreased after anti-TLR4 treatment (160). Furthermore, others have demonstrated in murine macrophages that TLR4 and CD44 form a complex correlated to enhanced cytokine release (217). Complex formation was determined to be dependent on HA binding (145, 217, 218). Indeed, LMWHA activates both CD44 and TLR4 leading to downstream NF-κB activation, which finally results in the upregulation of cytokine release (184). Several reports suggest a contribution of CD44 to ACD progression (160, 219). CD44 and HA binding was also demonstrated to be PPD modulated in keratinocytes (60). Hence, the role of CD44 could be important in the progression of sterile inflammation and is potentially an interesting factor for further investigation in keratinocyte sensitization model.

Deeper understanding of factors implicated in sterile inflammation state will be a useful tool to design new therapeutic approaches for ACD. In addition, the present therapies are focused on relief of the symptoms and not in attenuating the “root” of the problem.

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Appendix

In the appendix the two publications Kavasi et al., 2019 and Kavasi et al., 2017 are included:

- Kavasi, R.M., Berdiaki, A., Spyridaki, I., Papoutsidakis, A., Corsini, E., Tsatsakis, A., Tzanakakis, G., Dragana, N., 2019. Contact allergen (PPD and DNCB)-induced keratinocyte sensitization is partly mediated through a low molecular weight hyaluronan (LMWHA)/TLR4/NF-kappaB signaling axis. Toxicology and applied pharmacology, 114632.
- Kavasi, R.M., Berdiaki, A., Spyridaki, I., Corsini, E., Tsatsakis, A., Tzanakakis, G., Nikitovic, D., 2017. HA metabolism in skin homeostasis and inflammatory disease. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 101, 128-138.

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Contact allergen (PPD and DNCB)-induced keratinocyte sensitization is partly mediated through a low molecular weight hyaluronan (LMWHA)/ TLR4/NF-κB signaling axis

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ABSTRACT

Allergic contact dermatitis (ACD) is caused by topical exposure to chemical allergens. Keratinocytes play a key role in innate immunity, as well as in ACD progression. The transmembrane Toll-like receptor 4 (TLR4), strongly implicated in skin inflammation, has the ability to bind Damage Associated Molecular Patterns (DAMPs), like Low Molecular Weight Hyaluronan (LMWHA). Previously, we had determined that *p*-phenylenediamine (PPD) and 2,4-dinitrochlorobenzene (DNCB) modulate keratinocyte HA deposition in a manner correlated to their sensitization. In the present study, we aimed to investigate putative co-operation of HA and TLR4 in the process of PPD and DNCB-induced keratinocyte activation. Contact sensitizers were shown to significantly increase the expression of Hyaluronan Synthases (HAS) and TLR4 in NCTC2544 human keratinocytes, as demonstrated by western blot and Real-Time PCR. These data, in correlation to earlier shown enhanced HA degradation suggest that the contact sensitizers facilitate HA turnover of keratinocytes and increase the release of pro-inflammatory, LMWHA fragments. Treatment with exogenous LMWHA enhanced TLR4, HAS levels and Nuclear factor-kappa beta (NF-κB) activation. PPD, DNCB and LMWHA-effects were shown to be partly executed through TLR4 downstream signaling as shown by Real-Time, western blot, siRNA and confocal microscopy approaches. Specifically, PPD and DNCB stimulated the activation of the TLR4 downstream mediator NF-κB. Therefore, the shown upregulation of TLR4 expression is suggested to further facilitate the release of endogenous, bioactive HA fragments and sustain keratinocyte activation. In conclusion, keratinocyte contact allergen-dependent sensitization is partly mediated through a LMWHA/TLR4/ NF-κB signaling axis.

1. Introduction

Allergic contact dermatitis (ACD) is the most frequent among skin diseases and results upon skin contact with allergens. In Europe, almost 20% of general population presents ACD to one or more contact allergens. ACD is an undesired side effect observed with many products, including cosmetics, natural extracts, drugs, chemicals and medical devices (Peiser et al., 2012; McKim Jr. et al., 2012). This particular type of allergy occurs after the mistaken response of the immune system, resulting in sterile inflammation. The term "sterile" refers to the type of inflammation caused chemically or by injury and not by pathogens (Chen and Nunez, 2010; Kurbert et al., 2016). Over the last decades, a great deal of progress has been made in the understanding of the ACD underlying mechanisms activation (Corsini et al., 2018; Bechara et al., 2018; Helou et al., 2019), resulting in the publication of the first adverse outcome pathway (AOP) framework (OECD, 2014), The Adverse

Abbreviations: TNCB, 2, 4, 6- trinitrochlorobenzene; DNCB, 2,4-dinitrochlorobenzene; ACD, Allergic contact dermatitis; a-acid, Aristolochic acid; DAMPs, Damage Associated Molecular Patterns; DINP, diisononyl phthalate; (DMSO), dimethyl sulfoxide; ECM, extracellular matrix; FITC, fluorescein isothiocynate; GAGs, glucosaminoglycans; HMWHA, High Molecular Weight Hyaluronan; HAS, Hyaluronan Synthases; HA, Hyaluronan; HABP, Hyaluronic Acid Binding Protein; IL, Interleukine; LDV, lactate dehydrogenase-elevating virus; LLR, leucine rich repeat domain; LPS, Lipopolysaccharide; LMWHA, Low Molecular Weight Hyaluronan; NF-κB, Nuclear factor-kappa beta; PPD, *p*-phenylenediamine; PGs, proteoglycans; ROS, Reactive Oxygen Species; siRNA, short interfering RNA; TLR4, Toll-like receptor 4; (TNF-α), Tumor necrosis factor-alpha

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Outcome Pathway for Skin Sensitization Initiated by Covalent Binding to Proteins, OECD Series on Testing and Assessment, No. 168, OECD Publishing, Paris (https://doi.org/10.1787/9789264221444-en). Contact allergens are low molecular weight chemicals (< 1000 Da) (Martin, 2012) the so-called haptens, unable to be recognized by the immune system due to their size (Corsini and Kimber, 2007). Their absorption by the skin is followed by binding to skin macromolecules mainly proteins (haptenization), leading to the formation of a complete antigen (key event 1 or molecular initiating event in AOP), which leads to keratinocytes' activation, a key event at cellular level (key event 2). The other key event at cellular level is activation of dendritic cells (key event 3), which is caused by hapten-protein complexes as well as by signaling from activated keratinocytes. Dendritic cells subsequently mature and migrate out of the epidermis to the local lymph node where they display the hapten-protein complex to naive T-lymphocytes, inducing differentiation and proliferation of allergen chemical-specific T-cells (Key event 4). Keratinocytes, which are the main components of the body external barrier, are important mediators in all phases of ACD progression, representing the starting point of primary inflammation and the driving of chemical skin sensitization (Corsini et al., 2013, 2018).

The extracellular matrix (ECM) is a complex network of molecules secreted by the cells, consisting of proteoglycans (PGs), glucosaminoglycans (GAGs), collagens and laminins (Jarvelainen et al., 2009; Tzanakakis et al., 2018). ECM has an inherent ability to self-assemble that provides tissue specificity, structure and organization (Kavasi et al., 2017). The ECM components participate in a variety of biological functions such as proliferation, adhesion, angiogenesis, cell motility, inflammation, (Badylak, 2002; Nikitovic et al., 2014) by activating specific signaling pathways, and finally mediating disease progression. Consequently, in all tissues the initialization and propagation of inflammation can be supported by ECM-derived signals (Nikitovic et al., 2014).

Indeed, under pathological conditions, ECM is modified which results in release of Damage Associated Molecular Patterns (DAMPs) (Chen and Nunez, 2010). Thus, DAMPs or, endogenous molecules that trigger and sustain inflammation, are usually released to the ECM, or originate from this network under stress conditions (Hernandez et al., 2016). It is well established that the ability of DAMPs to trigger inflammation is related to several diseases including atherosclerosis, cancer and auto-immune diseases (Hernandez et al., 2016). Noteworthy, Reactive Oxygen Species (ROS) are also a category of DAMPs liberated to the ECM (Kurbert et al., 2016). Examples of secreted DAMPs are uric acid, ATP (Corsini et al., 2013), heparan sulfate, biglycan and low molecular weight hyaluronan (LMWHA) as well as other components originating from the ECM and released upon degradation (Chen and Nunez, 2010).

Hyaluronan (HA), an important component of the ECM, is a linear, non-sulfated GAG composed of repeating disaccharides of *N*-acetylglucosamine and glucuronic acid units (Weigel et al., 1997). In mammals High Molecular Weight Hyaluronan (HMWHA) is synthesized by three isoforms of Hyaluronan Synthases (HAS)-1, 2 and 3 (Stern et al., 2006) localized to the plasma membrane, in contrast to all other GAGs synthesized in the Golgi apparatus (Weigel et al., 1997; Itano et al., 1999). HAS gene expression is altered under several conditions, *e.g.*, HAS1 is upregulated during keratinocyte differentiation and HAS3 was shown to play a role in Atopic Dermatitis (AD) (Malaisse et al., 2014; Kavasi et al., 2017). HAS2 is responsible for the bulk of HA synthesis by fibroblasts, whereas, both HAS2 and 3 are equally important for keratinocyte HA synthesis (Itano et al., 1999).

The biologic actions of HA are mainly executed through its specific receptors cluster of differentiation 44 (CD44) and receptor for hyaluronan mediated motility (RHAMM). CD44 is established to be important for skin homeostasis (Brown et al., 1991) and plays a role in several cellular functions including metastasis, wound healing and inflammatory response (Bajorath, 2000). Degradation of HA is mediated by enzymes with high affinity to HA, hyaluronidases (HYAL). The main HYAL isoforms in human are HYAL2 located on the plasma membrane or in lysosomes and HYAL1 deposited specifically to the lysosomes (Stern and Jedrzejas, 2006). Importantly, approximately 50% of the total HA in the body is produced in the skin and specifically by dermal fibroblasts and epidermal keratinocytes (Stern and Maibach, 2008), with important role in skin homeostasis (Passi et al., 2004; Passi, 2018). In our previous study, we demonstrated a modification of HA deposition in keratinocytes mostly due to upregulation of HYAL isoforms, upon contact allergen treatment (Nikitovic et al., 2015).

Despite the simplicity of HA structure, it exhibits a wide range of biological functions, often opposing, depending on the molecular weight of HA chains. Indeed, HMWHA possesses anti-angiogenic, anti-inflammatory and immunosuppressive abilities (Stern et al., 2006). On the other hand, LMWHA fragments induce angiogenic, inflammatory and tumorigenic effects (Stern et al., 2006; Passi et al., 2019). These fragments are generated either enzymatically by HYAL isoforms, or chemically by ROS (Corsini et al., 2013). LMWHA fragments (10-100 kDa) liberated during tissue damage or inflammation, act as DAMPs (Scheibner et al., 2006). Toll-like receptors (TLRs) are a family of transmembrane receptors, important for the innate immune system, that specifically recognize and respond to DAMPs (Dusio et al., 2011). Moreover, LMWHA fragments were shown to trigger inflammatory responses *via* TLRs (Scheibner et al., 2006).

TLR4 is one of the 11 TLRs identified in humans, of which LMWHA is an established ligand (Termeer et al., 2002). It is a homodimer with a leucine rich repeat domain (LLR) usually anchored to the plasma membrane, although in keratinocytes it has also been identified to the cytoplasm (Begon et al., 2007). Several studies demonstrate a direct correlation between HA and TLR4 in immune system activation (Taylor et al., 2004; Watanabe et al., 2016). Furthermore, the contribution of TLR4 in inflammatory mechanisms was previously shown by numerous studies (Termeer et al., 2002; Watanabe et al., 2016; Dickinson and Wondrak, 2018). Additionally, it seems to be implicated in contact allergen-induced keratinocyte activation (Galbiati et al., 2014; van der Veen et al., 2016). In this study, we wanted to assess the putative contribution of TLR4 signaling to HA-dependent mechanisms of contact allergen-induced keratinocyte activation. We investigated in depth the connection among them by means of pharmacological inhibition and silencing, showing for the first time that contact allergens upregulated TLR4, HAS1-3 further facilitating the release of bioactive HA fragments and sustaining keratinocyte activation, central for acquisition of skin sensitization.

2. Materials and methods

2.1. Cell culture

All the experiments were conducted on NCTC2544 human keratinocyte cell line (Istituto Zooprofilattico di Bresia, Italy). Culture media was RPMI 1640 supplemented with Fetal Bovine Serum (FBS) 10% *v*/v. As antimicrobial agents 100 IU/ml penicillin and 100 μg/ml streptomycin were used. Cells were cultured as monolayers at 37 °C temperature and 5% CO_2. All cell culture reagents were purchased from Biosera.

2.2. Chemicals and reagents

As skin sensitizers, the allergens 2,4-dinitrochlorobenzene (DNCB) and *p*-phenylendiamine (PPD), supplied by Sigma (USA), were used. The utilized concentrations of PPD (30 μg/ml) and DNCB (2 μg/ml) were earlier established to strongly induce keratinocyte sensitization (Galbiati et al., 2014) and have likewise been utilized by us in a previous study (Nikitovic et al., 2015). Allergens were diluted in dimethyl sulfoxide (DMSO), also used as negative control at 0.2% final concentration. LMWHA (15-40 kDa) (R&D Diagnostics) was diluted in

ddH_2O and utilized at 250 μg/ml; the concentration of LMWHA with strongest effect on IL-18 production as previously determined (Nikitovic et al., 2015). Aristolochic acid (a-acid) (Sigma) was utilized, in accordance to our previous study (Nikitovic et al., 2015), at the concentration of 40 μM, with which the cells were treated for one hour before the addition of the allergens.

Keratinocytes were treated with the neutralizing antibody against TLR4 (Sigma) at 2 μg/ml and 4 μg/ml for 1 h, before the addition of allergens. Lipopolysaccharide from *Escherichia coli* (LPS) was purchased from Sigma Chemical Co. (St. Louis, Mo., USA) and was utilized at 500 ng/μl as a positive control for TLR4 activation (Kuzmich et al., 2017; Kim et al., 2018).

2.3. Transfection with siRNA

Silencing of the TLR4 gene was performed using short interfering RNA (siRNA) methodology. Specifically, NCTC2544 were seeded in T25 culture flasks (1×10^6 cells per flask). The siRNA sequence was adopted from Zhang and Li, 2015 (sense sequence: 5' GCUGAUGCCG CUGAUGCCA 3′, anti-sense sequence: 5' UGGCAUCAGCGGCAUCAGC 3′). Three different concentrations of siRNA were assessed (25 nM, 50 nM, 100 nM) in order to establish the most efficient downregulation conditions. This approach demonstrated that 100 nm of siTLR4 induced the strongest effect and this concentration was further utilized in all TLR4-silencing experiments (Supplementary Fig. 1). As a negative control, non-specific scrambled siRNA (siNegative) was used. The transfection was performed with Lipofectamine™2000 (Invitrogen), according to manufacturer's instructions.

2.4. Western blot

Initially, NCTC 2544 were seeded in T25 culture flasks, at the density of 1×10^6 cells per flask. The harvested NCTC2544 were lysed with RIPA solution. Electrophoresis of the samples was performed in 10% polyacrylamide gel, followed by transfer to nitrocellulose membrane in 10 mM CAPS buffer. Therein, membranes were blocked with PBS containing 0.1% Tween and 5% *v*/v milk powder, overnight at 4 °C. Primary antibodies were diluted in PBS containing 0.1% Tween and 1% v/v milk powder. Primary antibodies supplied by Santa Cruz Biotechnology were: TLR4, HAS1, HAS2, HAS3, NF-κB-p65, p-NF-κB-p65 (Ser536), all used in 1:200 dilution. Actin was purchased from Millipore and used at 1:5000 dilution. Secondary peroxidase-conjugated antibodies provided by Millipore, were used to visualize the binding of the primary to the targeted proteins upon incubation and the final image was obtained by Luminata™Crescendo western HRP Substrate (Millipore). Anti-mouse, anti-rabbit and anti-goat were diluted in PBS containing 0.1% Tween and 1% v/v milk powder in 1:5000 dilution. Blots were analyzed using ImageJ software.

2.5. RNA-isolation and Real-Time PCR

Total RNA was isolated with Trizol (Invitrogen), according to manufacturer's instructions. 1000 ng of total RNA of each sample were used for the production of complementary DNA (cDNA) with TAKARA PrimeScript reagent kit (TAKARA, Japan), according to manufacturer's instructions. Real-Time PCR was performed in an Mx3005P cycler (Stratagene) using the KAPA SYBR® FAST Universal qPCR kit (KAPA Biosystems) in a total volume of 20 μl. Specific gene primers were used for the detection of the investigated genes (HAS1, HAS2, HAS3, TLR4, HYAL1, HYAL2) as shown in Table 1. As a house keeping gene for the semiquantification of the results, GAPDH was used. For each optimized assay, a standard curve was run. This curve provided a linear plot of threshold cycle (Ct), against log (dilution). The semiquantification of each gene was calculated according to the concentrations of a standard curve and was presented as arbitrary units.

2.6. Immunofluorescence

NCTC2544 cells were seeded onto round coverslips in 24-well plates (120.000 cells per well) and incubated for 24 h. Upon treatment completion, culture media was removed, The cells were fixed with a solution of 5% formaldehyde and 2% sucrose diluted in PBS for 10 min at room temperature. After two washes with PBS, the cells were permeated with Triton-X diluted in PBS for 10 min at room temperature. After permeation, Triton-X dilution was removed and the coverslips were washed with PBS, when NF-κB specific antibody (Santa Cruz) diluted in 1% FBS-PBS (1:50) was added and incubated for 1 h. For the detection of HA localization Hyaluronic Acid Binding Protein (HABP) (Millipore) was used, at 1:100 dilution. After three washes, respective secondary antibodies were added (anti-rabbit Alexa Fluor at 1:250 or streptavidin for HABP at 1:200 dilution, Invitrogen) and incubated in the dark for 1 h. Nuclei were stained with TOPRO-3 (Invitrogen), at 1:500 dilution for 20 min in the dark. Finally, coverslips were placed on glass slides using glycerol and observed with confocal microscopy. The images were captured by Leica Confocal Software.

2.7. Statistical analysis

Each experiment was performed at least three times and representative data are presented. Statistical significance was evaluated by Student's *t*-test or one-way ANOVA analysis with Tukey's post-test, using GraphPad prism (version 4.0) software.

3. Results

3.1. PPD and DNCB upregulate TLR4 in a concentration dependent manner

As TLR4 was demonstrated to participate in allergen-mediated keratinocyte sensitization (Galbiati et al., 2014; van der Veen et al., 2016), we aimed to further examine its mechanisms of action. In this study, after treating keratinocytes for 24 h with increasing concentrations of the two allergens, PPD (30 μg/l, 45 μg/ml land 60 μg/ml) and DNCD (1 μg/ml, 1.5 μg/ml and 2 μg/ml), a notable concentration dependent upregulation of TLR4 expression (Fig. 1) was observed at both protein ($p \leq .01$ for 30 μg/ml, 45 μg/ml PPD and 2 μg/ml DNCB, $p \leq .001$ for 60 μg/ml PPD) and mRNA level ($p \leq .01$ for 30 μg/ml, 45 μg/ml PPD, $p \leq .001$ for 60 μg/ml PPD and $p \leq .05$ for 2 μg/ml DNCB).

3.2. Contact allergens increase HAS1, HAS2 and HAS3 expression

Considering the fact that inflammatory mediators increase HAS isoform expression in other models (Ghatak et al., 2015), we investigated the putative effect of PPD and DNCB. Therefore, NCTC2544 cells were treated for 24 h with PPD (30 μg/ml) and DNCB (2 μg/ml) and HAS1, HAS2 and HAS3 expressions were measured. Western blot and Real-Time PCR demonstrated an increase in HAS isoform expression at both protein and mRNA levels ($p \leq .05$) (Fig. 2).

3.3. LMWHA enhances TLR4, HAS1 and HAS3 expression

LMWHA has been described as a DAMP (Scheibner et al., 2006). Previously, we had shown that HA affects keratinocyte sensitization, whereas in the present study we examined putative contribution of TLR4 signaling in this mechanism. NCTC2544 keratinocytes were treated with LMWHA (250 μg/ml) in order to examine its possible effect on TLR4 expression. This strategy revealed a significant upregulation of TLR4 at both protein ($p \leq .001$) and mRNA ($p \leq .001$) levels, in treated cells (Fig. 3A, B, C). These data suggest that LMWHA partly modulates keratinocyte activation through a TLR4-dependent mechanism. Furthermore, we also analyzed HAS isoform expression upon treatment with LMWHA (250 μg/ml). Western blot and Real-Time PCR

Table 1
Sequences for primers used in Real-Time PCR.

Gene	Forward	Reverse
HAS1	5′ GGT GGG GAC GTG GGA TC 3′	5′ ATG CAG GAT ACA CAG TGG AAG TAG 3′
HAS2	5′ GTG TTA TAC ATG TCG AGT TTA CTT CC 3’	5’ GTC ATA TTG TTG TCC CTT CTT CCG C 3’
HAS3	5′ GGT ACC ATC AGA AGT TCC TAG GCA GC 3’	5′ GAG GAG AAT GTT CCA GAT GCG 3’
HYAL1	5’ CCG GTG CTG CCC TAT GTC 3’	5′ AGG CTG TGC TCC AGC TCA TC 3’
HYAL2	5’ GGC GCA GCT GGT GTC ATC 3’	5’ CCG TGT CAG GTA ATC TTT GAG GTA CT 3’
TLR4	5’ CAG AAC TGC AGG TCC TGG 3’	5′ GTT CTC TAG AGA TGC TAG 3’
GAPDH	5′ GGA AGG TGA AGG TCG GAG TCA 3’	5’ GTC ATT GAT GGC AAC AAT ATC CAC T 3’

demonstrated a significant upregulation of HAS1 ($p \leq .01$ and $p \leq .001$ respectively) and HAS3 expression ($p \leq .05$) as presented in Fig. 3D, E, F, G, H and I.

3.4. HAS1 and HAS3 expression and HA deposition of TLR4 deficient cells

Furthermore, we wanted to examine the potential involvement of TLR4 in HA downstream mechanisms contributing to keratinocyte activation. Therefore, in order to examine the role of TLR4, silencing of the TLR4 gene was performed utilizing short interfering RNA. Three different concentrations of the specific siRNA were tested (25 nM, 50 nM and 100 nM), as presented in Supplementary Fig. 1. The most efficient downregulation (55% at the protein level, $p \leq .01$, and 63% downregulation at the mRNA level, $p \leq .01$) was achieved utilizing the 100 nM concentration which was further used in all siRNA experiments.

Initially, after downregulation of the TLR4 gene, HAS1 and HAS3 expression were assessed. This approach demonstrated that HAS1 protein and mRNA levels were strongly downregulated ($p \leq .001$ and $p \leq .01$ respectively); whereas HAS3 protein and mRNA levels were moderately decreased ($p \leq .05$) (Fig. 4). These data suggest a correlation between HA synthesis and TLR4 downstream effects. Additionally, we aimed to assess the effect of TLR4 on HA localization. The utilization of immunofluorescence demonstrated that HA was deposited to the pericellular matrix by NCTC4544 cells, in accordance to our previous study (Nikitovic et al., 2015). Upon downregulation of TLR4 expression, a decrease in HA signal was established (Fig. 4G), demonstrating

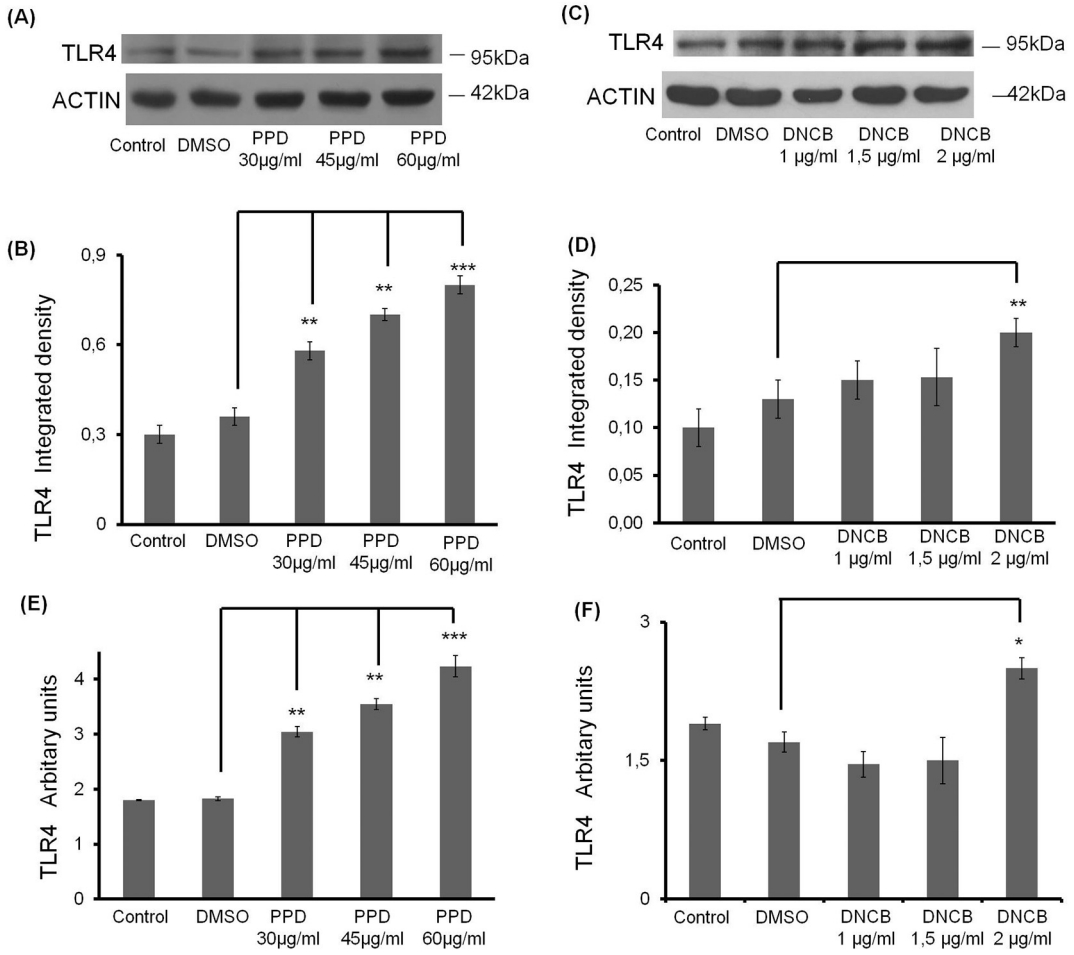

Fig. 1. Effect of PPD and DNCB treatment on TLR4 expression. NCTC2544 cells were treated for 24 h with increasing concentrations of PPD (30 μg/ml, 45 μg/ml, 60 μg/ml), and DNCB (1 μg/m, 1.5 μg/ml, 2 μg/ml) (DMSO - negative control) before harvesting. (A), (C) Representative blots of TLR4 protein (95 kDa) and actin (42 kDa) are presented. (B), (D) TLR4 protein bands were densitometrically analyzed and adjusted against actin. (E), (F) TLR4 mRNA expression was evaluated by Real-Time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *$p \leq .05$, ** $p \leq .01$, ***$p \leq .001$.

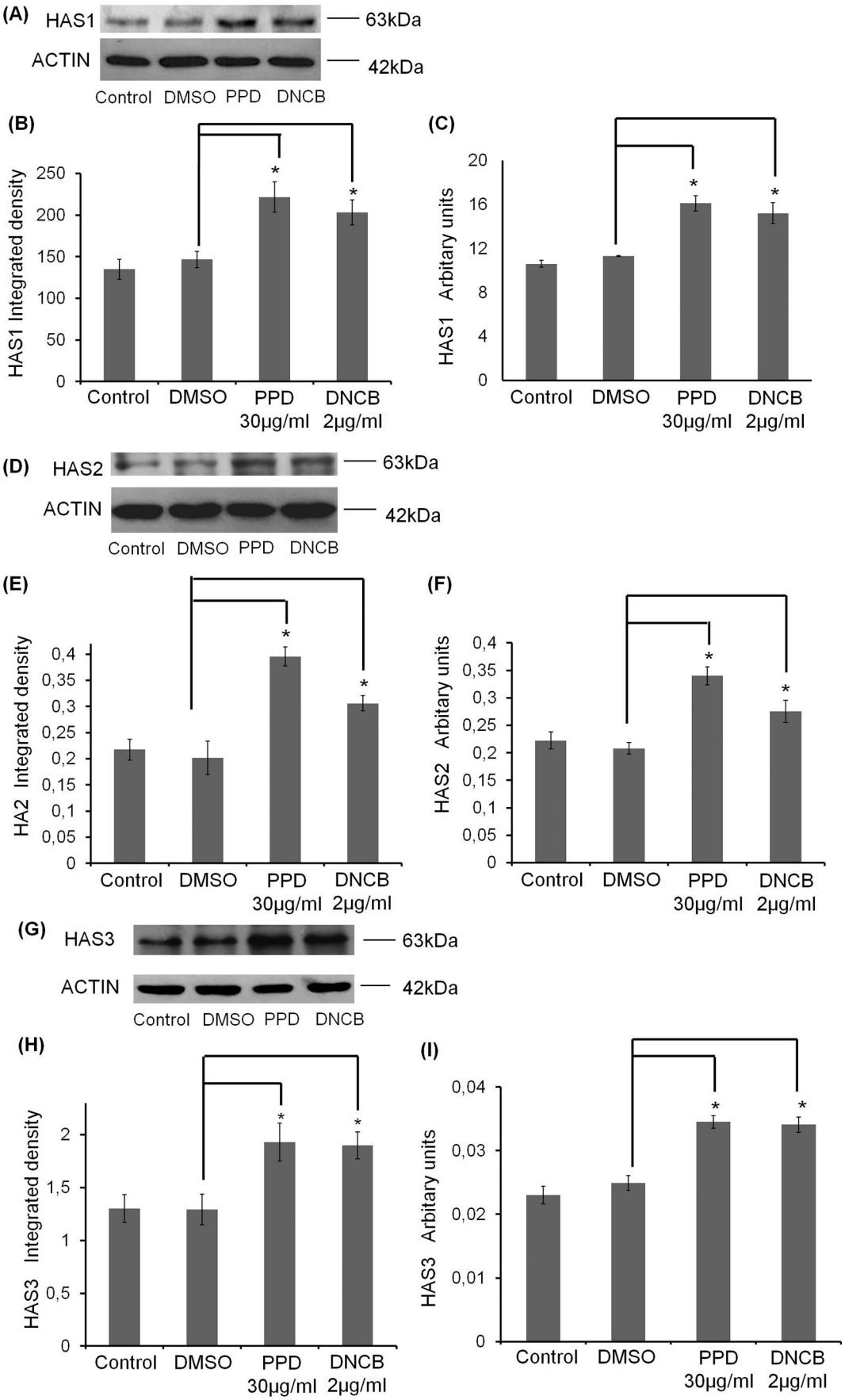

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Fig. 2. Effect of PPD and DNCB treatment on HAS1, HAS2 and HAS3 expression. NCTC2544 cells were treated for 24 h with PPD 30 μg/ml and DNCB 2 μg/ml (DMSO - negative control) before harvesting. (A), (D), (G) Representative blots of HAS1, HAS2 and HAS3 proteins (63 kDa) and actin (42 kDa) are presented. (B), (E), (H) HAS1, HAS2 and HAS3 protein bands were densitometrically analyzed and adjusted against actin. (C), (F), (I) HAS1, HAS2 and HAS3, mRNA expression was evaluated by Real-Time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *$p \leq .05$.

that HA pericellular deposition depends on TLR4 expression. These data are well correlated to the dependence of HAS1 and HAS3 expressions on TLR4 shown in this study.

3.5. Role of TLR4 on HA metabolism in keratinocytes exposed to PPD and DNCB

Downregulating TLR4, with the siRNA method could not be used in combination with allergens, due to the shown upregulation of TLR4 expression by both PPD and DNCB. Indeed, both allergens were demonstrated to override the silencing of the TLR4 gene, (data not shown). Thus, for the combination of allergen treatment and blocking of TLR4 action, a neutralizing antibody against TLR4 was utilized. This strategy aimed to block the allergen mediated TLR4 downstream signaling. In a previous study (Galbiati et al., 2014) the same approach demonstrated a decrease in NCTC2544 keratinocyte IL-18 production upon exposure to PPD and DNCB.

NCTC2544 cells were pretreated for 1 h with anti-TLR4 2 μg/ml and then, PPD (30 μg/ml) (Fig. 6A, C) and DNCB (2 μg/ml) (Fig. 5B, D) were added for 24 h. As shown in Fig. 5 blocking TLR4 decreases PPD and DNCB mediated HAS1 and HAS3 upregulation ($p \leq .05$).

Previously, we demonstrated that exposure of keratinocytes to contact allergens causes an upregulation of HYAL isoforms. Specifically, HYAL1 was increased upon both PPD (30 μg/ml) and DNCB (2 μg/ml) treatment, while HYAL2 was increased only by DNCB (Nikitovic et al., 2015). In this study, we aimed to investigate whether this upregulation is mediated, among others, by TLR4. Utilization of the anti-TLR4 antibody did not affect the basal levels of HYAL1 and HYAL2 expression (Fig. 6A, D). Furthermore, NCTC2544 cells were preincubated for 1 h with two concentrations of anti-TLR4 2 μg/ml and 4 μg/ml and then, exposed to PPD (30 μg/ml) and DNCB (2 μg/ml) for 24 h. Real-Time PCR verified the increase in HYAL1 levels upon PPD (Fig. 6B) ($p \leq .05$) and DNCB (Fig. 6C) treatments ($p \leq .01$), and HYAL2 upon PPD treatment (Fig. 6E) ($p \leq .01$) in accordance with a previous report (Nikitovic et al., 2015). Interestingly, the upregulation of HYAL isoform expression due to the allergens' action was annulled in the presence of anti-TLR4 at a concentration of 4 μg/ml ($p \leq .05$). The above data suggest that TLR4, at least partially, contributes to the contact sensitizer

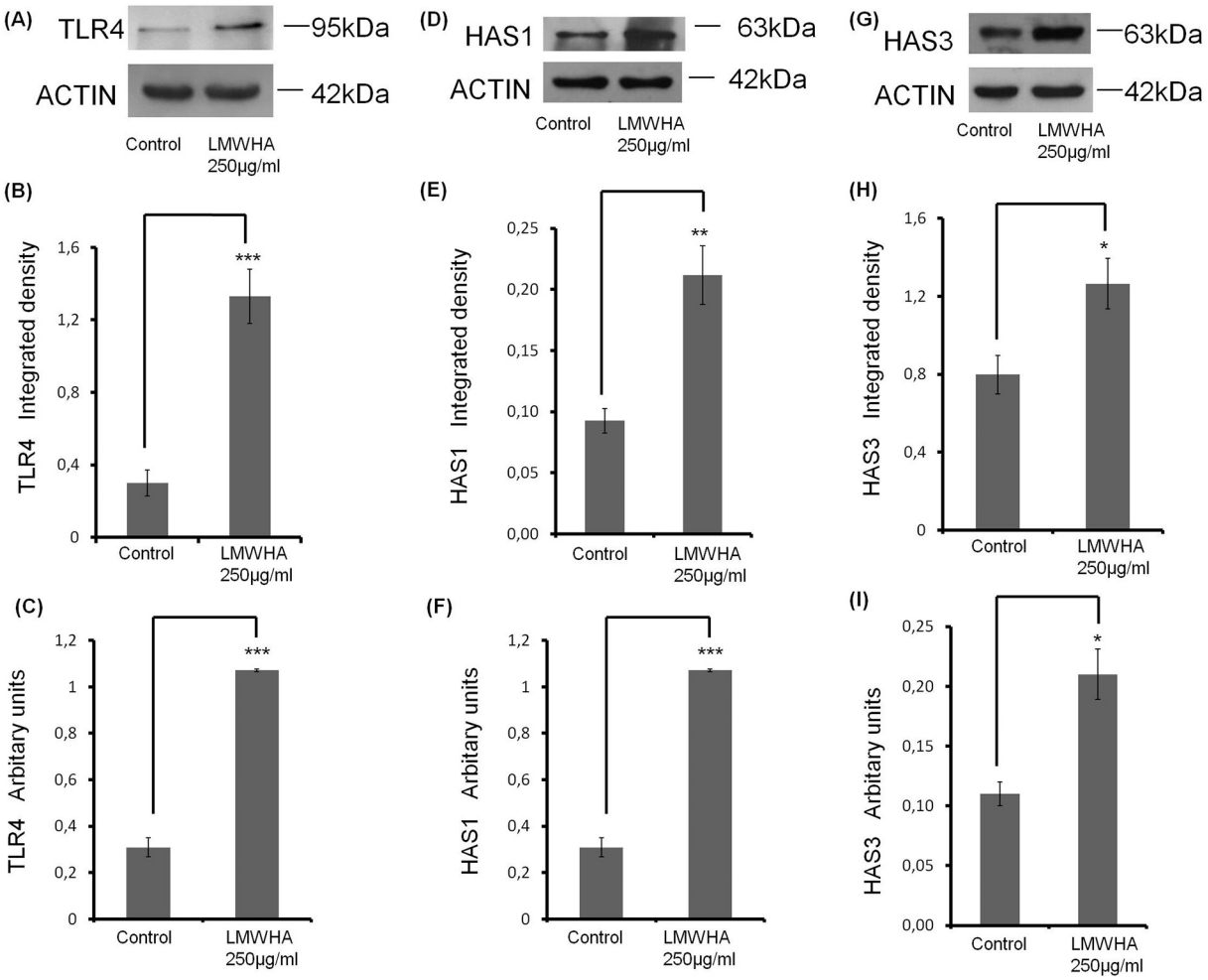

Fig. 3. Effect of LMWHA on TLR4, HAS1 and HAS3 expression. NCTC2544 cells were treated for 24 h with LMWHA 250 μg/ml before harvesting. (A), (D), (G) Representative blots of TLR4 (95 kDa), HAS1 and HAS3 protein (63 kDa) and actin (42 kDa) are presented. (B), (E), (H) TLR4, HAS1 and HAS3 protein bands were densitometrically analyzed and adjusted against actin. (C), (F), (I) TLR4, HAS1 and HAS3 mRNA expression was evaluated by Real-Time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p ≤ .05, ** p ≤ .01, ***p ≤ .001.

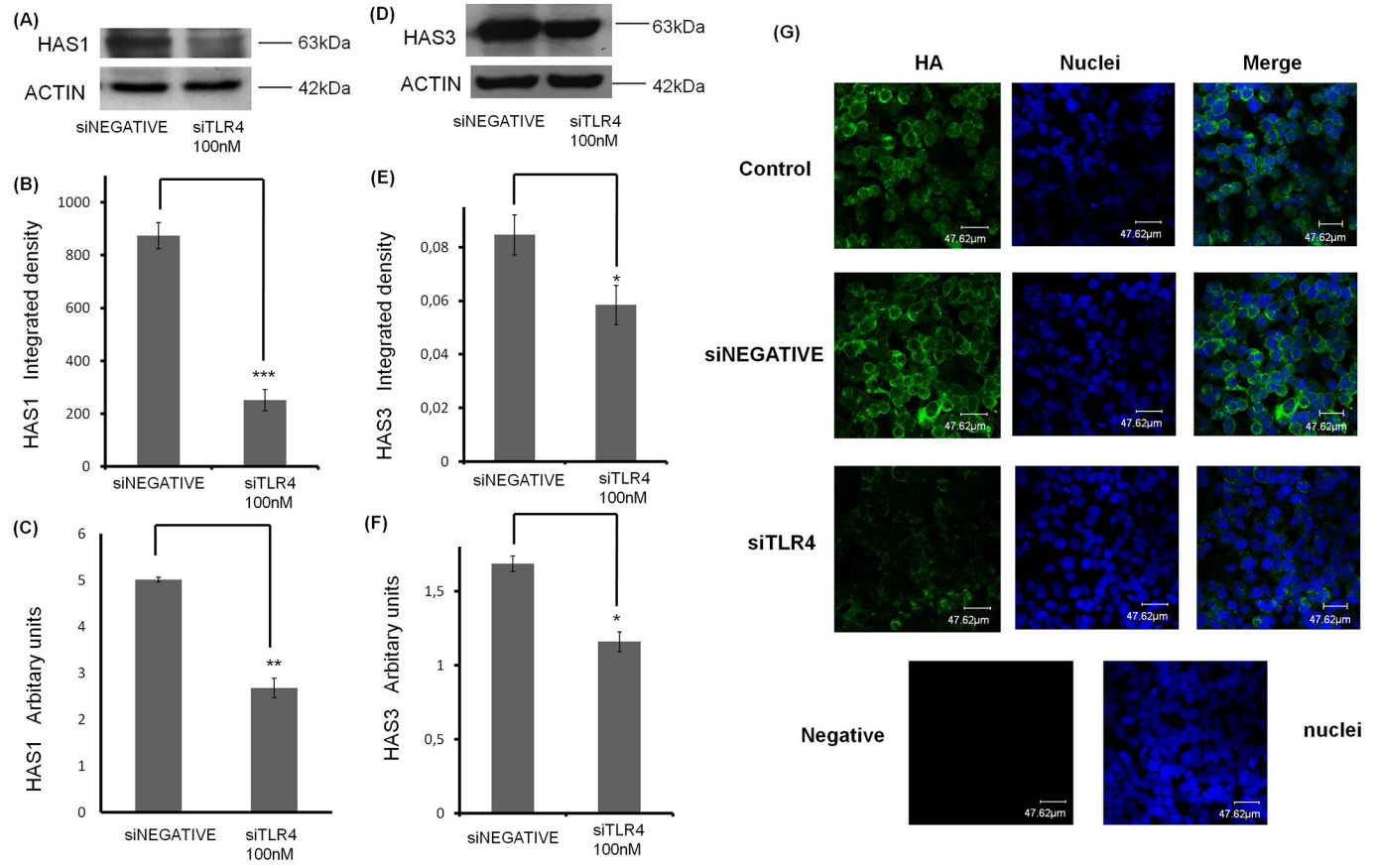

Fig. 4. Effect of TLR4 downregulation on HAS1 and HAS3 expression as well as on HA localization. NCTC2544 cells were transfected with 100 nM of siRNA specific for the TLR4 gene for 24 h before harvesting. A non specific RNA sequence was used as control (siNegative). (A), (D) Representative blots of HAS1 and HAS3 proteins (63 kDa) and actin (42 kDa) are presented. (B), (E) HAS1 and HAS3 protein bands were densitometrically analyzed and adjusted against actin. (C), (F) HAS1 and HAS3 mRNA expression was evaluated by Real-Time PCR. (G) HA was stained with a green fluorescent antibody and the nuclei were stained using TOPRO. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p ≤ .05, ** p ≤ .01, ***p ≤ .001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effect.

3.6. Effect of PPD and DNCB on NF-κB activation

Under inflammatory conditions, NF-κB is activated, which causes a multitude of cellular responses including TNF-α, IL-1 and IL-8 cytokine release (Hoesel and Schmid, 2013). In the present study NCTC2544 cells were treated with PPD (30 µg/ml) and DNCB (2 µg/ml) for two hours. In control experiments maximum activation of NF-κB, upon contact allergen treatment, was determined to be at the 2 h point (data not shown). LPS (500 ng/ml) was used as positive control as it is a well established activator of TLR4 and NF-κB (Kuzmich et al., 2017; Kim et al., 2018). Western blot analysis revealed a PPD- and DNCB- mediated NF-κB activation (p ≤ .05; p ≤ .01, respectively), as presented in Fig. 7. Furthermore, immunofluorescence demonstrated a translocation of NF-κB to the nucleus upon treating keratinocytes for 2 h with PPD and DNCB (Fig. 8). LPS, an established TLR4/ NF-κB trigger (Kuzmich et al., 2017), was utilized as positive control for NF-κB activation ($p \leq .001$).

3.7. Modulation of allergen mediated NF-κB activation by TLR4

Western blot analysis revealed that PPD- and DNCB-dependent NF-κB activation was attenuated upon blocking TLR4 with a specific neutralizing antibody (Fig. 9) (p ≤ .05). These data suggest that PPD- and DNCB dependent NF-κB is partly mediated through TLR4 downstream signaling.

3.8. The effect of LMWHA on NF-κB activation

In order to examine the direct effect of LMWHA on NF-κB activation, keratinocytes were exposed to 250 µg/ml of LMWHA. This approach resulted in enhanced NF-κB activation (p ≤ .05), as shown in Fig. 10A, B. On the other hand, a-acid, an inhibitor of HYALs activity and hence HA degradation, attenuated PPD and DNCB- mediated NF-κB activation ($p \leq 0{,}05$) (Fig. 10C, D). In control experiments, a-acid was found not to affect the basal levels of keratinocyte NF-κB activation. These data correlate well with the supposition that LMWHA directly participates in NF-κB activation in this cell-type.

4. Discussion

TLR4 downstream signaling has previously been correlated to ACD (Kaplan et al., 2012; Galbiati et al., 2014; Schmidt et al., 2016). Indeed, TLR4-deficient macrophages were shown to exhibit reduced cytokine expression upon activation (Jiang et al., 2011). Interestingly, mice lacking TLR4 were not sensitized by IL-12, whereas mice deficient in both TLR2 and TLR4 did not respond to contact allergens 2, 4, 6- trinitrochlorobenzene (TNCB), oxazolone and fluorescein isothiocynate (FITC) insult (Kaplan et al., 2012). Another contact sensitizer, nickel, also induced the proinflammatory cytokine IL-8 production, in endothelial cells whereas, the inhibition of MyD88, a TLR downstream mediator abrogated nickel-dependent IL-8 production (Schmidt et al., 2010). Likewise, the inhibition of TLR4 activity decreases the PPD and DNCB mediated IL-18 production (Galbiati et al., 2014), a well

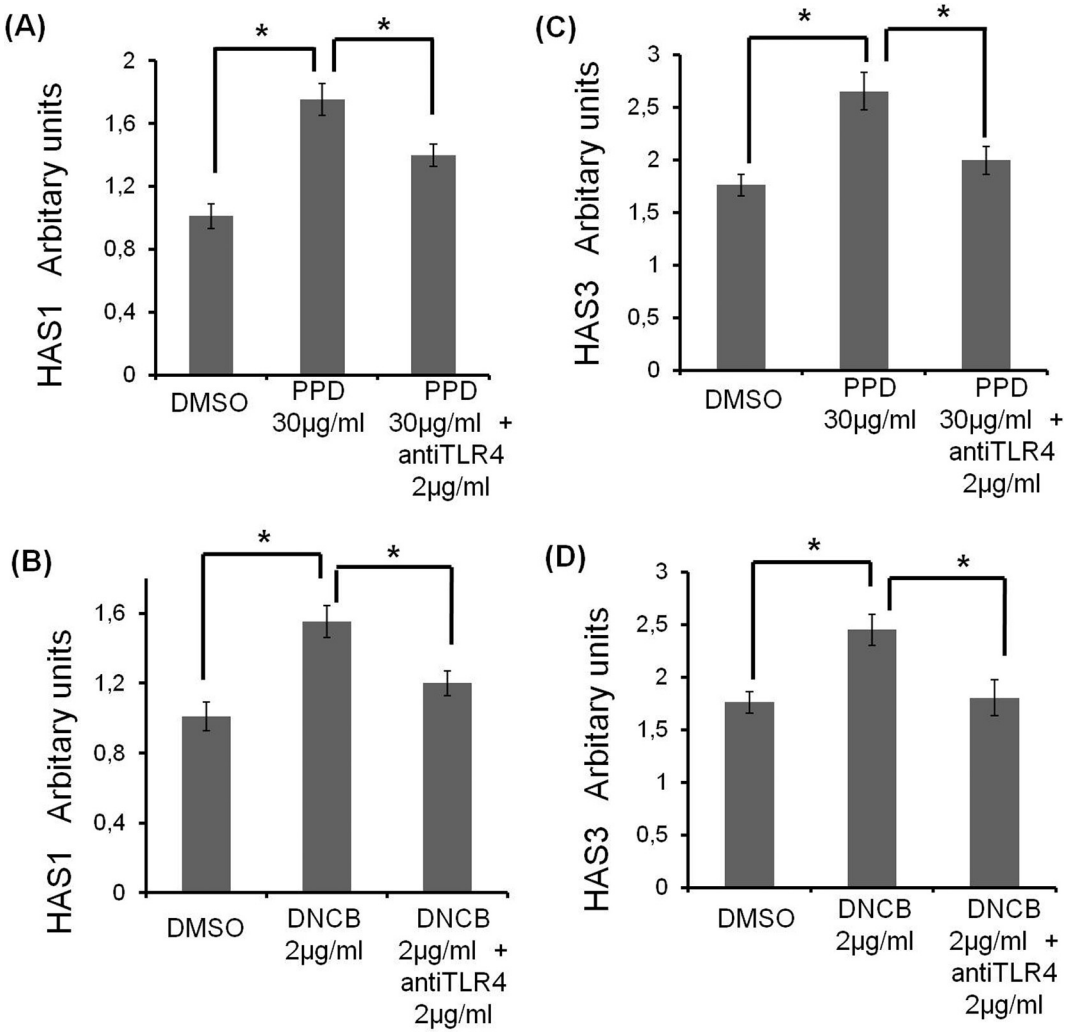

Fig. 5. Effect of blocking TLR4 on PPD and DNCB mediated HAS1 and HAS3 upregulation. NCTC2544 cells were treated for 1 h with a neutralizing antibody against TLR4 (2 µg/ml) and then with PPD 30 µg/ml and DNCB 2 µg/ml for 24 h, or just with PPD 30 µg/ml and DNCB 2 µg/ml for 24 h (DMSO – negative control), before harvesting. (A), (B) HAS1 and (C), (D) HAS3 mRNA expression was evaluated by Real-Time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance * $p \leq .05$.

established marker of contact allergen-induced keratinocyte activation (Corsini et al., 2009; Galbiati et al., 2011; Galbiati et al., 2018). In the present study, PPD and DNCB were demonstrated to increase keratinocyte TLR4 expression in a concentration dependent manner. These data are well in agreement with the previously shown contribution of TLR4 to contact allergen-induced IL-18 production (Galbiati et al., 2014) and suggest that TLR4 is able to affect the contact allergen mediated activation of keratinocytes. Feedback mechanisms between cytokine release and TLR4 expression have been proposed, as IL-33 in murine macrophages causes an upregulation of TLR4 levels (Espinassous et al., 2009). Likewise, macrophages infected by lactate dehydrogenase-elevating virus (LDV) exhibited increased TLR4 expression (Su et al., 2012). Indeed, LDV virus activates innate immune cells causing cytokine, including TNF-α, secretion to which the authors attributed TLR4 upregulation (Su et al., 2012). Moreover, in mouse articular chondrocytes, IL-1β treatment was shown to stimulate TLR4 expression (Campo et al., 2012).

LMWHA, in its role as a DAMP, is well established to affect innate immune system activation resulting in the release of inflammatory mediators (Jiang et al., 2005). In our previous study, we had demonstrated that LMWHA activates keratinocytes as determined by IL-18 production (Nikitovic et al., 2015). It is acknowledged that HA is a TLR4 ligand involved in immunomodulation (Jiang et al., 2011). Indeed, activation of TLR4 by HA was demonstrated in both *in vivo* and *in vitro* models (Vigetti et al., 2010; Campo et al., 2012; Watanabe et al., 2016). Previously, in HaCaT keratinocyte cell line, LMWHA (6-50 kDa) caused an upregulation of TLR4 expression (D'Agostino et al., 2017), likewise, demonstrated in the present study. Endogenous LMWHA is generated either by chemical degradation due to ROS or hyaluronidase enzymatic action (Agren et al., 1997; Galbiati et al., 2014). Noteworthy, in inflammatory conditions, ROS generation is increased whereas, it was demonstrated in murine skin that inhibiting ROS action with antioxidants reduces skin sensitization by allergens (Esser et al., 2012). The same authors had demonstrated that, the inhibition of HYAL isoform activity with aristolochic acid reduced contact sensitization. We have previously shown that PPD and DNCB increase the expression of HYAL isoforms, decreasing HA deposition to keratinocyte pericellular matrix by increased degradation of HMWHA to small HA fragments (Nikitovic et al., 2015).

HMWHA (200-2000 kDa) is produced by HAS 1 and 2 whereas HAS3 produces HA of 100-1000 kDa as recently discussed (Kavasi et al., 2017). Importantly, HAS enzymes are differentially expressed in pathological conditions (Yung et al., 2000; Calabro et al., 2002; Malaisse et al., 2014; Nikitovic et al., 2016; Passi et al., 2019). Expression of HAS2, which is the main synthase of fibroblasts and also expressed by keratinocytes (Itano et al., 1999), is increased by proinflammatory

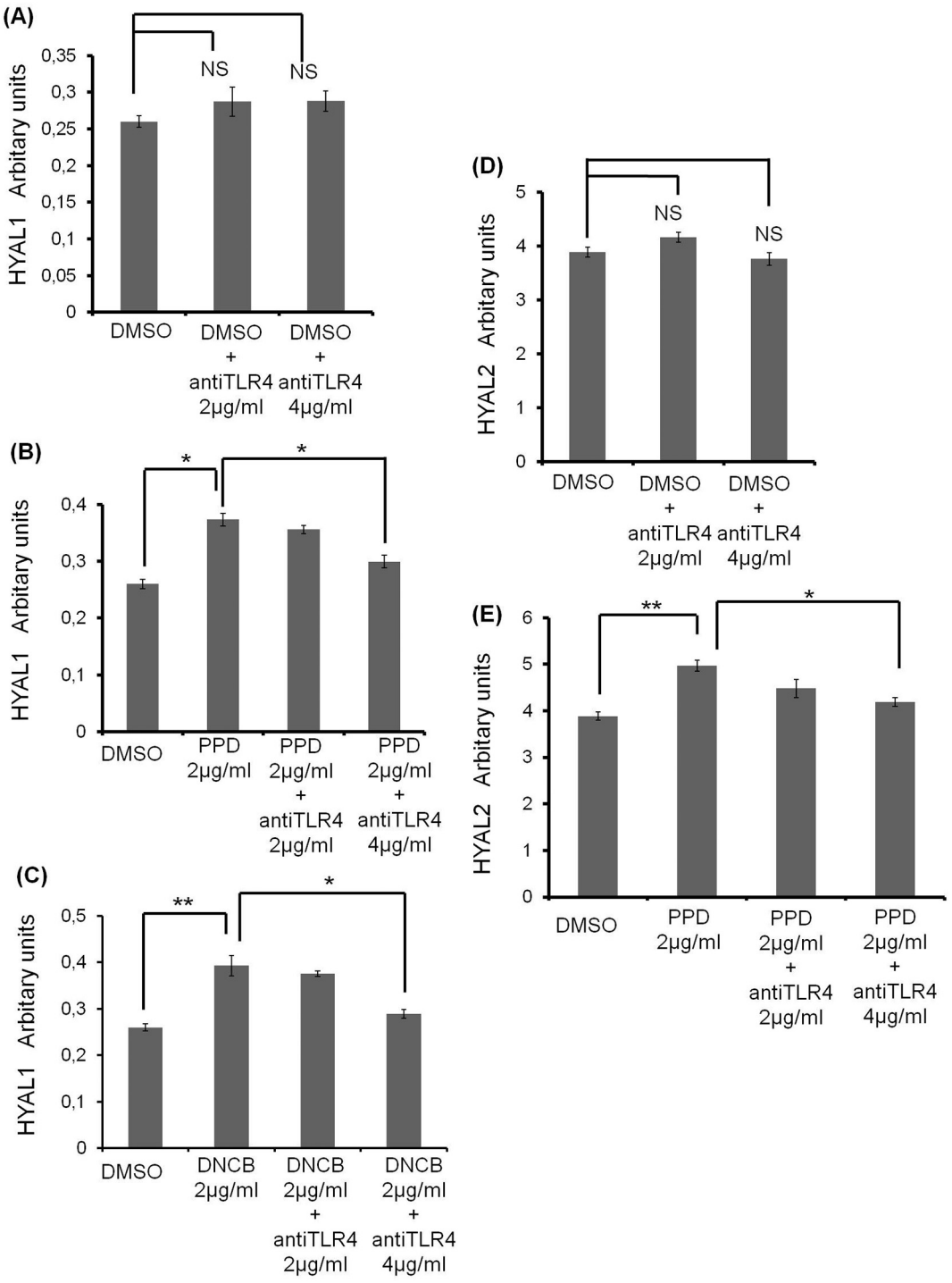

Fig. 6. Effect of a neutralizing antibody against TLR4 on PPD and DNCB mediated HYAL1 and HYAL2 upregulation. NCTC2544 cells were treated for 1 h with a neutralizing antibody against TLR4 2 µg/ml and 4 µg/ml and then with PPD 30 µg/ml and DNCB 2 µg/ml for 24 h, or just with PPD 30 µg/ml and DNCB 2 µg/ml for 24 h (DMSO- negative control), before harvesting. (A), (B), (C) HYAL1 and (D), (E) HYAL2 mRNA expression was assessed by Real-Time PCR. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *$p \leq 0{,}05$, ** $p \leq 0{,}01$.

cytokines (Malaise et al., 2014). HAS3, mainly expressed by keratinocytes (Kakizaki et al., 2008), has been demonstrated to play a role in skin inflammation (Barnes et al., 2012) and atopic dermatitis (AD) (Malaisse et al., 2014). In the present study, we demonstrated that the three HAS isoforms were upregulated upon exposure of keratinocytes to contact sensitizers. Furthermore, TLR4-deficient keratinocytes in this study exhibited significantly decreased HAS1 and HAS3 endogenous levels, implying a direct correlation between HA synthesis and TLR4-dependant signaling. Indeed, numerous endogenous ligands have been determined for TLR4 including Biglycan, CD138, α-crystallin A chain, β-defensin 2, endoplasmin, fibrinogen, fibronectin, heparan sulphate, HMGB1, HSP22, HSP60, HSP70, HSP72, hyaluronan, monosodium urate crystals, S100 proteins and tenascin-C among others as discussed by Yu et al. (2010). Some of these ligands *e.g.* fibronectin, laminin or tenascin-C are important components of the epidermal ECM and of the epidermal-dermal junction and known to be involved in the regulation of skin homeostasis (Wen et al., 2010; Abdou et al., 2012). Therefore, it is conceivable that the binding of these molecules to TLR4, under

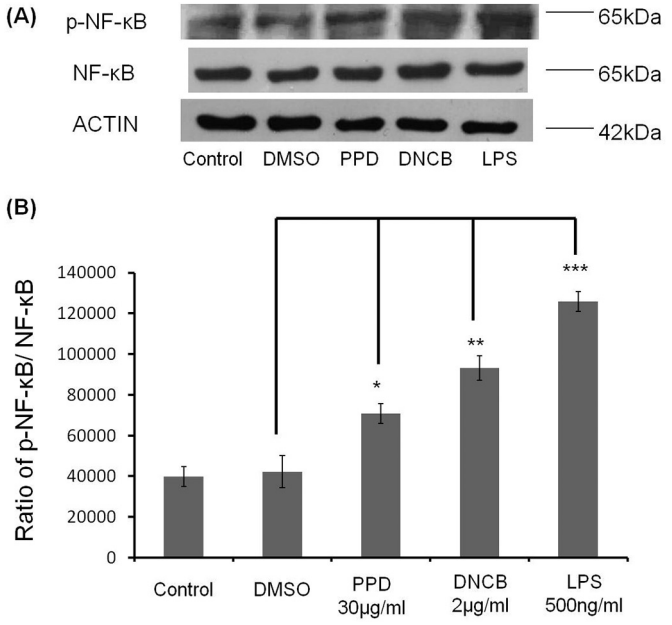

Fig. 7. Effect of PPD and DNCB on NF-κB activation. NCTC2544 cells were treated with PPD 30 μg/ml, DNCB 2 μg/ml and LPS 500 ng/ml as positive control, for 2 h (DMSO - negative control) before harvesting. (A) Representative blots of NF-κB (65 kDa), p- NF-κB (65 kDa) and actin (42 kDa) are presented. (B) NF-κB, p-NF-κB protein bands were densitometrically analyzed and adjusted against actin and the ratio of p-NF-κB/ NF-κB is presented. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p ≤ .05, ** p ≤ .01, ***p ≤ .001.

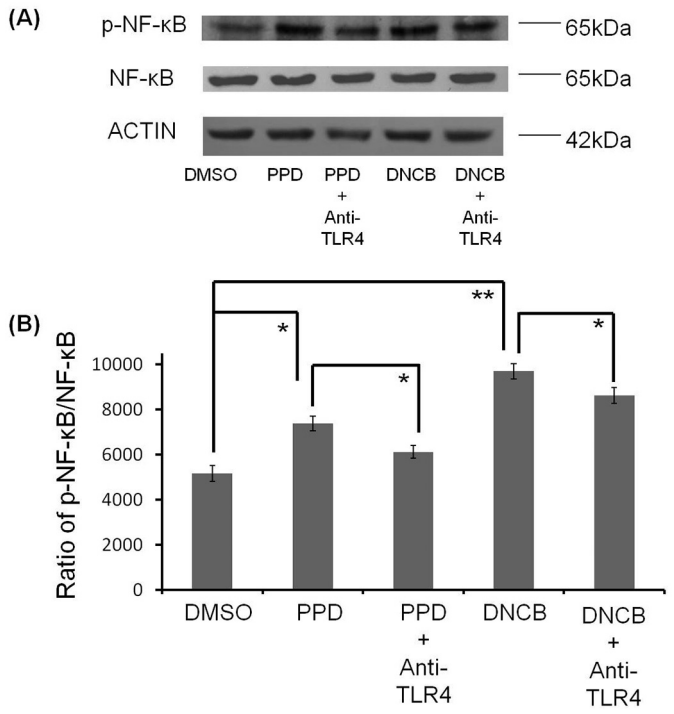

Fig. 9. Effect of blocking TLR4 on NF-κB activation. NCTC2544 cells were treated either with a neutralizing antibody against TLR4 (4 μg/ml) for 1 h and in continuation with PPD 30 μg/ml and DNCB 2 μg/ml during 2 h or just with PPD 30 μg/ml and DNCB 2 μg/ml for 2 h (DMSO - negative control) before harvesting. (A) Representative blots of NF-κB (65 kDa), p- NF-κB (65 kDa) and actin (42 kDa) are presented. (B) p-NF-κB, NF-κB protein bands were densitometrically analyzed, adjusted against actin and the ratio of p-NF-κB/ NF-κB is presented. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p ≤ .05, **$p \leq .01$.

physiological conditions, can mediate respective receptor downstream signaling, regulating the basal levels of HAS isoforms expression. Previously, HAS2 mRNA was shown to be increased upon treating endothelial cells with IL-1β and TNF-α inflammatory cytokines (Vigetti et al., 2010), suggesting that these mediators regulate HAS expression and HA synthesis. Moreover, as reviewed by Ghatak et al., 2015, TNF-α and IL-1β, whose expression is mediated by TLR4, have been demonstrated to affect HA deposition in several cell types. As earlier demonstrated, blocking of TLR4, results in decreased PPD and DNCB mediated IL-18 production (Galbiati et al., 2014). Moreover, in an *in*

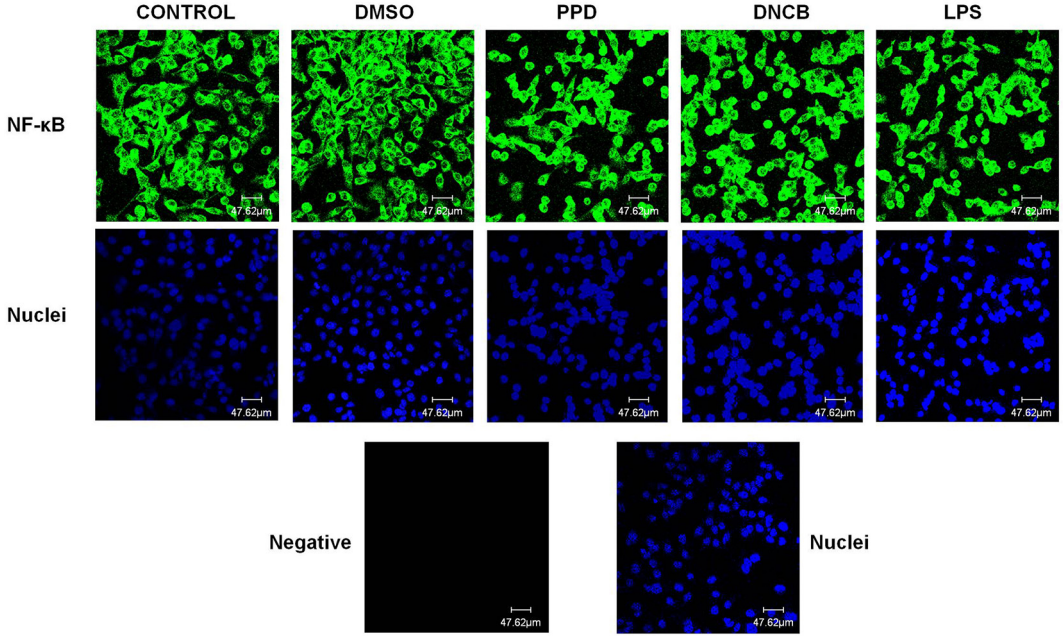

Fig. 8. Effect of PPD and DNCB on NF-κB cellular localization. NCTC2544 cells were treated with PPD 30 μg/ml, DNCB 2 μg/ml and LPS 500 ng/ml as positive control for 2 h (DMSO - negative control) before harvesting. NF-κB was stained with green fluorescent antibody and the nuclei were stained using TOPRO. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

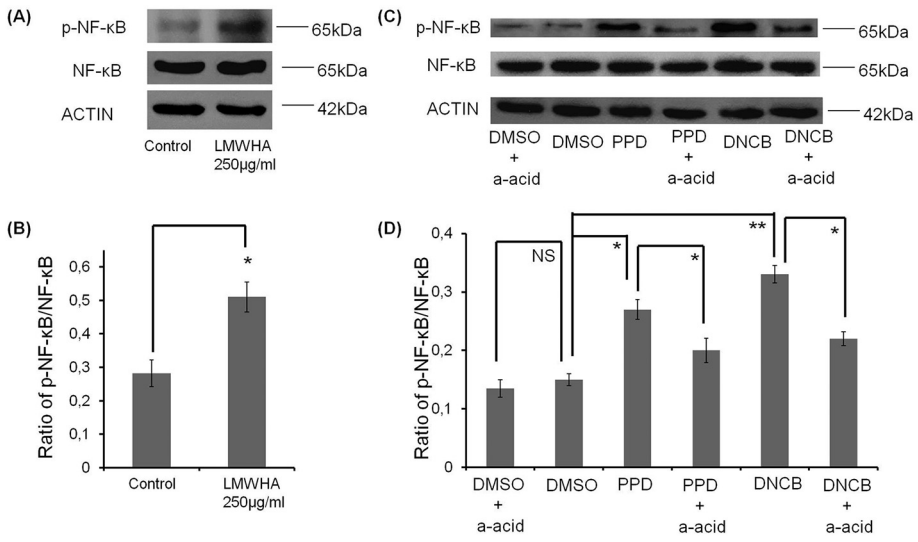

Fig. 10. LMWHA effect on NF-κB activation (A), (B) NCTC2544 cells were treated for 2 h with LMWHA 250 μg/ml before harvesting. (A) Representative blots of NF-κB (65 kDa), p- NF-κB (65 kDa) and actin (42 kDa) are presented. (B) p-NF-κB, NF-κB protein bands were densitometrically analyzed, adjusted against actin and the ratio of p-NF-κB/ NF-κB is presented. (C), (D) NCTC2544 cells were treated either with a-acid (40μM) for one hour and in continuation with PPD 30 μg/ml, DNCB 2 μg/ml for 2 h, or just with PPD 30 μg/ml and DNCB 2 μg/ml for 2 h (DMSO - negative control), before harvesting. (C) Representative blots of NF-κB (65 kDa), p- NF-κB (65 kDa) and actin (42 kDa) are presented. (D) NF-κB, p-NF-κB protein bands were densitometrically analyzed and adjusted against actin and the ratio of p-NF-κB/ NF-κB is presented. The results represent the average of three separate experiments in triplicate. Means ± SEM plotted; Statistical significance *p ≤ .05, ** p ≤ .01.

vivo model, TLR2 and TLR4 deficiency was accompanied by lower deposition of HA on the cell surface and decreased response to lung injury (Jiang et al., 2005). Likewise, in a mouse model, TLR4 deficiency was related to reduced oxidative stress and inflammation (Pushpakumar et al., 2017). In a separate murine TLR4 knock out model, a protective effect against brain injury was shown through decreased NF-κB activation (Yao et al., 2017).

Binding of LMWHA to the TLR4 receptor triggers downstream signaling which leads to NF-κB activation, as shown in dendritic cells (Kenny and O'Neill, 2008). Separate studies had likewise demonstrated that HA fragments induce NF-κB activation without specifying the mechanism(s) involved (Noble et al., 1996; Termeer et al., 2002). In the present study, well correlated to earlier findings, we demonstrate that treating keratinocytes with LMWHA enhances their NF-κB activation. Moreover, LMWHA was shown to mediate cytokine and chemokine production *via* TLR4 (Jiang et al., 2011). Indeed, TLR4 deficient macrophages, do not release inflammatory cytokines and chemokines in response to LMWHA stimulus (Jiang et al., 2005). Furthermore, small HA fragments also induce maturation and migration of dendritic cells through TLR4 activation a process important for the innate immune response (Termeer et al., 2002).

In this study, PPD- and DNCB-dependent increase in HAS and HYAL isoform expression was attenuated when utilizing a neutralizing antibody against TLR4. These data suggest that, blocking TLR4 downstream signaling results in lower HA synthesis, decreased HA degradation and attenuated inflammatory response. This is in accordance with previous studies where TLR4-deficient mice were shown to have attenuated ability to respond to an insult (Jiang et al., 2005; Bird et al., 2010). On the other hand, a recent study suggests that TLR4-deficient mice have increased susceptibility to develop atopic dermatitis (Lin et al., 2018). Noteworthy, it has been suggested that the inhibition of TLR4 downstream signaling is a promising therapeutic approach for inflammation-correlated pathologies (Shibata et al., 2017; Kuzmich et al., 2017; Tao et al., 2017).

The triggering of TLR4 leads to downstream NF-κB activation and is directly correlated to inflammatory responses (Hoesel and Schmid, 2013). Indeed, therapeutic approaches against inflammation target the NF-κB pathway (Huang et al., 2014; Gomes et al., 2015). Thus, DNCB treatment in mice induced increased TLR4 and NF-κB expression in rat skin, and cytokine, including IL-18, release. These effects were decreased by a flavonoid preparation which inhibits NF-κB *via* TLR4 (Xiao et al., 2018). Additionally, the cytokine IL-1β is able to activate NF-κB in mouse chondrocytes, with resultant upregulation of the cytokines' transcription (Campo et al., 2015). Oral exposure to diisononyl phthalate (DINP) and sensitization by FITC in mice, resulted in increased NF-κB activation, IL-18 release and an ACD like phenotype, which was attenuated by an NF-κB inhibitor (Kang et al., 2016). In the present study, PPD and DNCB treatments enhanced keratinocyte NF-κB activation, an effect mediated partly *via* TLR4 receptor, as blocking of TLR4 diminished the effect. In our keratinocyte model system LPS treatment was used as a positive control, due to its well established activation of TLR4/NF-κB signaling axis, upon binding of LPS to MD-2 an adaptor protein that binds directly to TLR4 (Kuzmich et al., 2017). Moreover, in a manner similar to the present study, LPS was shown to increase TLR4 and cytokine expression in HaCaT keratinocyte cell line (Kim et al., 2018). More importantly, it has previously been shown that, inflammatory cytokines increase HAS genes expression, whereas the inhibition of NF-κB ameliorated this effect (Kao, 2006; Vigetti et al., 2010). In the present study, a-acid, a specific inhibitor of HYAL activity and resulting HA degradation, diminished allergen-mediated NF-κB activation of keratinocytes. On the other hand, the exposure of keratinocytes to LMWHA resulted in enhanced NF-κB activation. These data in combination to the previously shown, attenuated keratinocyte activation by contact allergens upon a-acid treatment (Nikitovic et al., 2015), support our suggestion that allergen-dependent NF-κB activation and downstream inflammatory signaling, are partly executed *via* LMWHA effects.

In conclusion, we introduce a role for HA-TLR4 interaction in the process of contact allergen-induced keratinocyte activation. In the proposed mechanism exposure of keratinocytes to PPD and DNCB results in the increased ability of keratinocytes to produce but also to degrade HA. Released HA fragments bind TLR4 localized to cell membrane which results in downstream NF-κB activation and further cytokine production. The following upregulation of TLR4 expression is suggested to further facilitate release of bioactive HA fragments and sustain keratinocyte activation as summarized in Fig. 11. Further studies are needed to clarify the functional relationship of TLR4 and the circuit generating bioactive HA fragments, which may be useful in the design of a targeted treatment for ACD. Overall, our results provide further insights on the mechanism of action underlying contact allergen-induced keratinocyte activation, central for the acquisition of skin sensitization.

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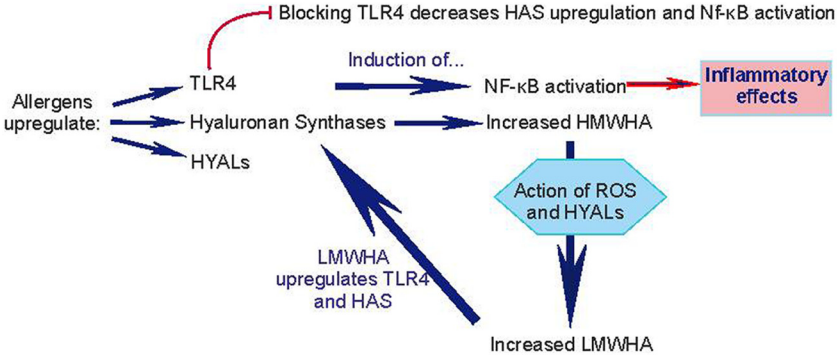

Fig. 11. The role of HA-TLR4 interaction in the process of contact allergen-induced keratinocyte activation. Contact allergens, in a manner dependent on TLR4/ NF-κB downstream signaling, induce TLR4, HASs and HYALs overexpression resulting in increased synthesis and degradation of HA. Released pro-inflammatory LMWHA fragments facilitate TLR4, HAS and HYALs overexpression and sustain keratinocyte sensitization.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2019.114632.

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Review

HA metabolism in skin homeostasis and inflammatory disease

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ABSTRACT

Hyaluronan (HA), an unsulfated glycosaminoglycan, is an important component of the complex extracellular matrix network which surrounds and supports cells in tissues. HA is detected in all vertebrate tissues, but the bulk of HA is produced and deposited in the skin. In this review we focus on the role of HA in skin-associated inflammatory disease and wound healing. Properties of HA are directly dependent on its molecular weight. Thus, high molecular weight HA (HMWHA) is deposited in normal tissues during homeostasis and promotes their stability whereas low molecular weight HA fragments (LMWHA), on the other hand, may arise from enzymatic or chemical activities. The degradation of HMWHA to LMWHA fragments, often leads to the generation of biologically active oligosaccharides with different properties and postulated functions in wound scar formation and inflammation. More detailed studies of HA involvement in skin-associated inflammatory disease may result in novel treatment modalities.

Contents

Abbreviations: HA, Hyaluronan; LMWHA, low molecular weight hyaluronan; HMWHA, high molecular weight hyaluronan; ECM, extracellular matrix; CD44, cluster of differentiation 44; TLR, Toll like receptor; DAMP, damage associated molecular pattern.

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1. Introduction

1.1. Hyaluronan metabolism and skin homeostasis

1.1.1. Extracellular matrix (ECM)

The extracellular matrix (ECM) is a complex network of molecules secreted by the cells, with an inherent self-assembly ability, that supports and regulates specificity in tissue structure and organization. The ECM components participate in a variety of biological functions by activating specific signaling pathways, including proliferation, adhesion, cell motility (Afratis et al., 2012; Kouvidi et al., 2014; Tzanakakis et al., 2014), angiogenesis and vasculogenesis, wound healing as well as inflammation (Badylak, 2002; Nikitovic et al., 2016). This vital network is mainly composed of collagens, elastin, proteoglycans (PGs), glycosaminoglycans (GAGs), fibronectins as well as laminins (Kresse and Schonherr, 2001; Jarvelainen et al., 2009).

1.1.2. Hyaluronan (HA)

Hyaluronic acid (HA), a fundamental ECM component, is a linear non-sulfated GAG composed of repeating units of N-acetyl-glucosamine (GlcNAc) and glucuronic acid (GlcA) (Weigel et al., 1997). In contrast to other GAG types, HA is not attached to a core protein (West et al., 1997), nor has it sulfated groups (Tammi et al., 2008). Uniquely, this GAG is synthesized at the plasma membrane instead of the Golgi apparatus as reviewed by Weigel et al., in 1997. Philogenetic studies demonstrated that HA initially arose about 500 million years ago in lower chordates (Vigetti et al., 2014; Weigel and DeAngelis, 2007; DeAngelis, 2002).

HA is detected in all vertebrate tissues, but the bulk of HA is produced and deposited in the skin especially in the dermis, where it is synthesized by dermal fibroblasts (Stern and Maibach, 2008). In fact, approximately 50% of the total HA in the body is located at the skin (Meyer and Stern, 1994). Noteworthy, epidermal keratinocytes also produce HA with HA being deposited extracellularly to the spinous and granular layers, while at the basal layer it is located intracellularly (Stern and Maibach, 2008). This GAG, with high rate of turnover (West et al., 1997), has a wide range of functions. Thus, it plays an important role in tissue homeostasis, facilitating the formation of intercellular gels, while its interactions with other ECM components, such as proteoglycans, facilitate proper tissue organization (Evanko et al., 2007). HA regulated signaling is, additionally, important for cell attachment, migration, proliferation, as well as embryogenesis (Li et al., 2007; Rauhala et al., 2013). Moreover, the biological activities of HA have been related to wound healing progression through their modulation of fibroblast and epithelial cell response (Itano and Kimata, 2002; Jiang et al., 2005; Zoltan-Jones et al., 2003) and also to development of several types of cancer (Karousou et al., 2014; Nikitovic et al., 2015). Importantly, even early reports demonstrated that HA participates in the regulation of the immune response and the resulting inflammation (McKee et al., 1997). Thus, in several tissues HA, through the formation of covalent bonds reacts with the heavy chains of inter-α-inhibitor (IαI), a reaction catalyzed by tumour necrosis factor-stimulated gene-6 (TSG-6) which is suggested to exert a positive impact on the resolution of inflammation (Tan et al., 2011). In fact, a reduction of TSG-6 levels is suggested to contribute to the abnormal formation of scar tissue in keloid lesions (Tan et al., 2011).

Properties of HA are directly dependent on its molecular weight. Thus, high molecular weight HA (HMWHA) is deposited in normal tissues during homeostasis and promotes their stability (Noble, 2002). Indeed, interactions between HMWHA and other macromolecules of the ECM play an important role in ECM structural organization as well as in out-in signaling. HMWHA polymers suppress angiogenenesis, immuno-response and inflammation (Stern et al., 2006; Kim et al., 2008) as well as cellular differentiation through intercellular interactions or ligand-receptor binding (Stern et al., 2006).

Low molecular weight HA fragments (LMWHA), on the other hand, may arise from enzymatic or chemical activities (Jiang et al., 2011; Corsini et al., 2013). These truncated products may be generated during HA synthesis, or during HA degradation initiated either by hyaluronidases (HYALs) and through chemical reactions triggered by reactive oxygen species (ROS) (Soltes et al., 2006; Corsini et al., 2013). The degradation of HMWHA to LMWHA fragments, often leads to the generation of biologically active oligosaccharides with different properties and functions (Noble, 2002; Karousou et al., 2014). Thus, small HA fragments contribute to wound scar formation (West et al., 1997), inflammation, immunostimulation and angiogenesis, in contrast to even smaller fragments, tetrasaccharides that can ameliorate these effects (Stern et al., 2006).

1.1.3. Hyaluronan Synthases (HAS)

HA synthesis is carried out by Hyaluronan Synthases (HAS) *de novo* (Weigel and DeAngelis, 2007). In other words, HA synthesis does not require a primer, but only UDP-GlcNAc, UDP-GLcUA sugars and Mg^{2+} (Weigel and DeAngelis, 2007). The HAS enzymes are glycosyl transferases, with the molecular weight of approximately 63 kDa (Weigel et al., 1997). Three HAS isoforms have been identified in mammals and their transcriptional levels are tissue or cell dependant as well as affected by cell microenvironment (Stern et al., 2006). Importantly, HAS enzymes engage in the synthesis of HA chains of various molecular weights on the inner side of the plasma membrane (Itano et al., 1999) which are concurrently secreted to the ECM (Weigel et al., 1997). This mechanism differs from the other GAG secretion, as GAG chains are extruded by exocytosis after they are synthesized (Evanko et al., 2007). Specifically, HAS1 has been reported to produce HMWHA of 200–2000 kDa, HAS2 produces HMWHA in the same range with a predisposition for the higher range of the spectrum, whereas HAS3 is responsible for smaller fragments (100–1000 kDa) of HA (Itano et al., 1999; Itano and Kimata, 2002). Importantly, a fine modulation of both the HA synthesis rate and molecular weight has been suggested (Baggenstoss et al., 2017; Viola et al., 2015a).

Noteworthy, while fibroblasts which produce the bulk of HA in humans, utilize mainly HAS2, keratinocytes engage equally HAS2 and HAS3 enzymes (Itano et al., 1999; Pasonen-Seppanen et al., 2003). Generally, in vertebrates it was demonstrated that HAS activity, especially of HAS2, may be stimulated by the presence of a variety of growth factors and cytokines, such as keratinocyte growth factor (KGF), epidermal growth factor (EGF), tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β) and bone morphogenenetic protein (BMP) superfamilies (Spicer and Tien, 2004). Furthermore, it was recently shown that growth factors including TGF-β1, FGF-2, EGF, and PDGF-BB facilitate HA deposition of skin fibroblasts by stimulating HAS expression, with the molecular size of secreted HA being determined by the expression of "Hyaluronan-binding Protein Involved in HA Depolymerization, KIAA1199" (Nagaoka et al., 2015). Importantly, aged fibroblasts significantly decrease their HA deposition due to decrease in HAS expression mainly as regarding the expression of HAS2 (Terazawa et al., 2015).

In respect to epidermis, it has been suggested that HAS3 is the most abundant HAS enzyme of the epidermis (Kakizaki et al., 2008). Moreover, KGF was shown to significantly stimulate HAS 2 and 3 expression and activity (Karvinen et al., 2003).

On the other hand, Malaisse et al., show that during keratinocyte

differentiation, HAS1 is upregulated, implying a role for HA accumulation. Moreover, HAS3 seems to play a role in atopic dermatitis (AD) (Malaisse et al., 2014), and skin inflammation in general (Barnes et al., 2012). Earlier reports suggest that the expression of HAS2 and HAS3, are positively correlated to keratinocyte proliferation and differentiation as well as epidermal thickness. Indeed, inflammation-dependent growth factors (e.g. EGF and TGF-β) strongly enhanced HA synthesis due to up-regulation of HAS2 and HAS3 expression (Karvinen et al., 2003; Pasonen-Seppanen et al., 2003).

Interestingly, the HAS enzymes have a different dependence on the UDP- GlcNAc substrate concentration. Thus, HAS1 activity seems to be heavily dependent on UDP- GlcNAc sugars availability, in contrast to HAS2 and HAS3 (Rilla et al., 2013). In fact, HAS1 exhibits negligible activity at low GlcNAc concentration, HAS2 activity is enhanced at high substrate availability and HAS3 is the most active enzyme of the HAS family even at minimum GlcNAc concentration (Rilla et al., 2013). Additionally, HAS2 is stabilized in the membrane via *O*-GLcNAcylation a post translational modification that occurs only at high UDP-GlcNAc concentration (Moretto et al., 2015).

Moreover, Spicer and Tien, suggest that *in vivo* HAS3 activity may also be affected by substrate availability. Even though a considerable amount of HA is produced by epidermal keratinocytes its function in the epidermis is not clearly defined. Indeed, recently it has been suggested, in reconstructed epidermal model, that HA levels are not immediately associated with both the proliferation and the differentiation of human epidermal keratinocytes (Malaisse et al., 2016).

1.1.4. Hyaluronidases (HYALs)

Enzymatic degradation of HA is mediated through the activities of HYALs, enzymes that cleave HA with high affinity. Six mammalian HYAL genes have been detected in the human genome (Stern, 2008), establishing a specific enzyme family with Hyal 1 and 2 and PH-20 being the most active (Stern and Jedrzejas., 2006). To begin with, HYAL2 is anchored to the cell surface through glycophoshatidyloinositol or is located to the lysosome (Lepperdinger et al., 2001) and degrades high molecular HA into smaller fragments of approximately 20 kDa (Lepperdinger et al., 1998; Harada and Takahashi, 2007). These fragments are internalized via endosomes, transported to lysosomes where HYAL1, located to lysosomes, degrades HA into smaller fragments, mainly, tetrasaccharides (Harada and Takahashi, 2007). Moreover, PH-20 is also glycophoshatidyloinositol-anchored on the plasma membrane and has HMWHA as a substrate (Spicer and Tien, 2004).

An interesting fact in the evolution of HA enzymes, is that some metazoans exhibit hyaluronidase activity, even though they had not developed the appropriate enzymes for HA synthesis. This can be explained, however through the fact that the majority of HYALs may also use as a substrate chondroitin sulfates (Spicer and Tien, 2004). It should be noted that human HYALs were hard to study due to the difficulties of purifying and characterizing them or measuring their activity. Moreover, they are in very low concentrations and have very high and, in the absence of detergents, unstable specific activities (Stern and Maibach, 2008).

1.1.5. HA receptors

The main HA receptor is cluster of differentiation 44 (CD44), a cell membrane glycoprotein occurring in diverse isoforms, as a result of alternative splicing and post-translational modifications (Naor et al., 2002). It is noteworthy that CD44 is encoded by a single gene with 10 constant and ten variant exons (Screaton et al., 1992) and the arising protein can accept the addition of GAG chains (Stern and Maibach, 2008). Moreover, post-translational modifications may influence the receptor's affinity for HA (Tammi et al., 1998). Thus, in epidermal keratinocytes interleukin-1β strongly reduces CD44 Ser-325 phosphorylation which significantly modulates the HA-binding ability of the receptor (Jokela et al., 2015). CD44-HA binding is important not only for intercellular interactions, but also for interactions between the cells and the ECM and affects numerous functions, including inflammation, wound healing and metastasis (Bajorath, 2000) as well as cell migration (Thomas et al., 1992). Importantly, CD44 is highly expressed both in the dermal and epithelial compartment of adult skin under the condition of normal tissue homeostasis (Wang et al., 1992; Brown et al., 1991). It has been demonstrated that CD44 contributes significantly in the maintaining of the keratinocyte percellular matrix (Pasonen-Seppanen et al., 2012). Recently, it has been discussed that specific CD44-HA binding regulates keratinocytes functions and differentiation (Bourguignon, 2014). Specifically, CD44-HA interactions have been shown to facilitate keratinocyte adhesion and (Bourguignon et al., 2004). Interestingly, the major CD44 isoform detected in the epidermis compartment of the adult skin is a heparan sulfate proteoglycan (epican) infrequently expressed in physiological tissues (Tuhkanen et al., 1997). The characteristic heparan sulfate glycosylation bestows to this CD44 isoform the capability to specifically bind and present growth factors (Bennett et al., 1995). Moreover, CD44 is suggested to determine the proper assembly of tight junctions facilitating thus, epidermal barrier function (Kirschner et al., 2011).

The second major HA receptor is the receptor for hyaluronan mediated motility (RHAMM) an, also, variable protein, due to alternative splicing with cell specific expression of isoforms (Cheung et al., 1999). RHAMM can be located on the cell surface, the cytoplasm, the nucleus or the ECM. It is important that the cell-surface RHAMM expression is not very high in normal tissues, in contrast to several types of cancer (Nikitovic et al., 2016), implying its' putative use as a prognostic marker (Wang et al., 1998; Assmann et al., 2001). Cytoplasmic RHAMM binds to parts of the cytoskeleton, including actin filaments, podosomes, the centrosome, microtubules, and the mitotic spindle (Assmann et al., 1999; Turley et al., 2002; Maxwell et al., 2005), while cell-membrane RHAMM is capable of interacting with other HA receptors, such as CD44, affecting MAPK (ERK1,2) signal transduction pathway (Turley et al., 2002; Hamilton et al., 2007). RHAMM is a receptor of major significance for cell growth (Mohapatra et al., 1996), motility and transformation (Hall et al., 1994; Kouvidi et al., 2011, 2016). In fibroblasts, transforming growth factor β (TGF-β) is implicated in the expression of HA pathway components, including RHAMM, affecting cell motility (Samuel et al., 1993). There are conflicting reports as regarding the expression of RHAMM in skin as RHAMM expression was not detected in immortalized keratinocytes (Hasova et al., 2011) whereas dermal fibroblasts are established to express this receptor (Croce et al., 2003).

The Toll-like receptors (TLRs) were primarily identified in drosophila and subsequently homologous genes were recognized in humans. TLRs are a family of transmembrane type I proteins with a wide variety of ligands and functions. They are characterized by a leucine-rich repeat (LRP) domain at the outer side of plasma membrane and a carboxyl-terminal Toll-interleukin I receptor (TIR) domain (Sandor and Buc, 2005). The activation of keratinocyte TLRs upon stimulation by pathogen-associated molecular patterns (PAMPs) initiates a cascade of signaling events, mainly through the NF-κB signaling pathway, leading to the expression of various genes involved in host defense (Goodarzi et al., 2007; Lebre et al., 2007). TLRs recognize not only PAMPs, but also respond to self molecules produced after cell damage or death, i.e. DAMPs (Dusio et al., 2011) with LMWHA being defined as DAMP (Scheibner et al., 2006). It is suggested that TLR activation during skin inflammation processes

may proceed via endogenous ligands (Martin et al., 2008). Toll-like receptor 4 (TLR4) seems to recognize small fragments of HA during immune responses (Termeer et al., 2002). In fact, several recent *in vitro* or *in vivo* studies imply a direct correlation of HA and TLR4 in the process of immune system activation (Watanabe et al., 2016; Hirabara et al., 2013; Campo et al., 2011, 2012).

Another receptor related to HA is intercellular adhesion molecule-1 (ICAM-1) (Yasuda, 2007), a glycosylated transmembrane protein of 80–114 kDa with a protein core of 55 kDa (Dustin et al., 1986). ICAM-1 has been shown to affect some HA-related signaling pathways, for instance, matrix metaloproteinase (MMP) production in fibroblasts (Hiramitsu et al., 2006), inhibition of LPS-induced cytokine production in macrophages (Yasuda, 2007), inflammatory response in mouse epithelial cells (Oertli et al., 1998). Upon treatment of keratinocytes with the extracellular toxin streptolysin O, which is produced by certain streptococci and is characterized by ability to damage cell membranes, a strong upregulation of both CD44 and ICAM-1 was established (Mamber et al., 2011).

2. Discussion

2.1. HA and keratinocyte differentiation

A question which, up to date, remains unclear is whether HA plays a role in keratinocyte differentiation. Thus, early reports argued that EGF and TGF-β-dependent modulation of HA synthesis, due to altered expression of HAS2 and HAS3, correlates well with epidermal keratinocyte proliferation, epidermis thickness, and differentiation stage (Pasonen-Seppänen et al., 2003).

Furthermore, Passi et al. (2004) supported the contribution of HA in the differentiation process based on the observation that HA prevents terminal keratinocyte differentiation. In the same study it was, additionally, demonstrated that exogenously enhanced enzymatic degradation of HA can induce epidermal differentiation. In agreement with this report, previous studies (Tammi and Tammi, 1991; Wells et al., 1991) had identified lower HA concentration at the upper layers of the epidermis in latter phases of cellular differentiation. Indeed, HAS2 and 3 expressions are sensitive to KGF regulation which facilitates keratinocyte migration and wound healing and on the other hand inhibits the terminal differentiation of these cells (Karvinen et al., 2003).

It is noteworthy that after disruption of the keratin layer in mice with acetone, increased HA was observed in all epidermal layers. Enzymatic degradation of the elevated HA led to a thinner epidermis, but surprisingly, not due to increased proliferation. Instead, differentiation markers were overexpressed and the number of the terminally differentiated keratinocytes was increased, suggesting that lack of HA triggers differentiation (Maytin et al., 2004). On the other hand, it was shown that HA-CD44 interaction with Rac1-PKN gamma contributes significantly to cortactin-cytoskeleton function which is obligatory for keratinocyte intercellular adhesion and cell differentiation (Bourguignon et al., 2004). Moreover, it was suggested that in addition to facilitating keratinocyte differentiation, HA-CD44 interactions regulate the permeability of the epidermal barrier (Bourguignon et al., 2006).

Furthermore, HA-CD44 binding was shown to support differentiation, as CD44 knockout mice exhibited lower expression of differentiation markers, as well as thinner epidermis and reduced keratinocyte proliferation rate (Bourguignon et al., 2006). Likewise, treatment of human cultured keratinocytes with exogenous HA, increased differentiation markers (Bourguignon et al., 2006).

Kage et al. suggest that treatment of keratinocytes with HA tetrasaccharides enhanced cellular differentiation, presumably due to demonstrated increased CD44 phosphorylation (Kage et al., 2014). However, it wouldn't be safe to generalize the fact that endogenous HA in the epidermis supports differentiation, as these authors suggest that this occurs only by exogenous application. To sum up the above, exogenous addition of HA enhances keratinocyte differentiation as well as the decrease of endogenous HA. Thus, cautiously, a dose-dependent effect could be hypothesized, where at very low or very high HA concentrations, differentiation is enhanced. However, a more recent study on a RHE model, suggests that exogenous HA has no effect on keratinocyte differentiation, as the mRNA expression levels of major differentiation markers, including keratin 10 and filaggrin were not altered (Malaisse et al., 2016). Moreover, neither abrogation of endogenous HA deposition via HYALs addition affected cellular differentiation (Malaisse et al., 2016). Thus, the mechanism of HA involvement in keratinocyte differentiation remains unclear. Indeed, contradictory results could be due to differences of experimental procedures and methods.

2.2. Wound healing and HA

HA has been shown to play an important role in wound healing even from early reports (Longaker et al., 1991). Interestingly, even in these early studies discriminate effects of HA in foetal versus adult wound healing process were demonstrated (Longaker et al., 1991; Mack et al., 2003). The wound healing process is established to consist of four temporal phases e.g. (i) homeostasis (ii) inflammation; (iii) proliferation and migration and the final (iv) remodelling phase as previously discussed (Singer and Clark, 1999; Ghatak et al., 2015).

Importantly, shortly after the wound healing process is initiated, the wounded area is characterized by elevated levels of HA and high influx of inflammatory cells, leukocytes and neutrophils as comprehensively reviewed by Stern and Maibach (2008). HA is initially produced by migrating keratinocytes at wound edges (Monslow et al., 2009) and subsequently by proliferating and migrating fibroblasts. Shortly after a wound infliction or onset of inflammation, LMWHA is generated as a result of ROS or HYALs action. Upon recognition and binding of LMWHA fragments, by TLR2 and TLR4, intracellular downstream signaling pathways are initiated which result in cytokine production such as IL-6, TNF-α and IL-1β production. These, in turn, enhance transcription of HAS genes and subsequent HA synthesis resulting thus in a feedback loop which facilitates the inflammatory response (Ghatak et al., 2015). In addition, HB-EGF is, also, produced at a wound site, causing HA production in neighboring cells. Notably, an interesting crosstalk between keratinocytes and migrating fibroblasts is the production of keratinocyte growth factor (KGF or FGF-7) by fibroblasts, which binds to the respective FGFRIII2b receptor expressed by epidermal keratinocytes, and induces their increased proliferation (Werner et al., 2007). In the adult wound healing, after the initial maximum HA deposition, an instant decrease is observed, upon the appearance of proliferating fibroblasts at the spot of the wound, which facilitates chondroitin sulphate and collagen deposition. Indeed, it seems that the decrease of HA concentration, triggers keratan (KS) and dermatan (DS) sulfate production in the Golgi apparatus (Ghatak et al., 2015). Then, KS and DS proteoglycans assist in collagen polymerization, necessary for wound closure (Ghatak et al., 2015). In the adults the described process of wound “closure” usually results in the formation of scar –fibrous tissue (Stern and Maibach, 2008).

In contrast, a fetal wound is not only healed rapidly, but the healing process is executed with no scar formation during the first six months of fetal intrauterine development (Colwell et al., 2003). In the fetus, HA deposition is similarly increased at the beginning of the wound healing, but its levels remain elevated until the

termination of the "scar free fetal repair process" (Longaker et al., 1991; Mast et al., 1991). A credible explanation for secondary decrease of HA deposition are the higher levels of HYALs in the adult wound fluid, as compared to fetal, in the sheep model, which would result in higher HA degradation rate (West et al., 1997). In fact, from third to sixth month of pregnancy in humans, HA levels are particularly elevated in amniotic fluid (Dahl et al., 1983). Indeed, it is worth mentioning that fetal amniotic fluid contains HMWHA (Sawai et al., 1997; Mackool et al., 1998) and the addition of HYAL enzyme results in scar formation (West et al., 1997). Importantly, it is believed that ammonic fluid plays a major role in the absence of scar formation, due to sterile conditions, the abundance of nutrients and growth factors and the ideal temperature which it provides (Gao et al., 1994). Indeed, *in vitro* experiments revealed higher wound healing rates for mouse epithelial oral cells' cultures treated with amniotic fluid (Takigawa and Shiota, 2007). Moreover, amniotic fluid has been demonstrated to assist re-epithelialisation on skin wounds, in a manner potentially similar to HA (Nyman et al., 2013). Interestingly, double knock out HAS 1/3 mice show faster wound closure, increased migration of leukocytes and enhanced ECM restoration (Mack et al., 2012). However, whether "faster is better" is currently under examination as elegantly discussed by Maytin in a recent review article (Maytin, 2016). The putative HA involvement in wound healing is schematically presented in Fig. 1.

2.3. HA metabolism in inflammation –related skin disorders

Even though HA is an important component of the skin ECM, its putative role in inflammation –related skin disease is still not fully understood. In the case of acute eczema where the main characteristic is spongiosis of the epidermis, a strong increase in keratinocyte mRNA HAS3 expression was demonstrated. This was correlated to enhanced deposition of HA to the ECM, dowregulation of cadherins and diminishment of epidermal keratinocyte cohesiveness which was accompanied by water inflow (Ohtani et al., 2009). Moreover, in patient skin biopsies HAS3 protein was also found to be overexpressed, presumably due to action of proinflammatory factors (Barnes et al., 2012). Similar findings were established in an *in vivo* model, as mice deficient in HAS1 and 3 exhibited increased inflammation and facilitated wound repair (Mack et al., 2012). Importantly, there seems to be a feedback mechanism between HA synthesis and CD44 expression in the skin. Thus, in CD44-deficient mice there is excessive accumulation of HA both in the dermis and epidermis compartments with suppressed keratinocyte growth response. These modulations were associated to disabled skin topic inflammatory response and attenuated tissue repair (Kaya et al., 1997). Indeed, a positive correlation between CD44 expression and uptake of the overplus of HA was suggested in an early study which examined HA clearance (Culty et al., 1992). Interestingly, the same mechanism was implicated in the resolution of inflammation in a mice pneumonia model (Teder et al., 2002).

Atopic dermatitis (AD), otherwise referred to as atopic eczema, is the most common skin inflammation with genetic, immunological and autoimmune implications (Madhok et al., 2015). In biopsies from AD patients HAS3 expression was likewise shown to be enhanced, while HAS1 was downregulated (Malaisse et al., 2014). Moreover, these authors suggest that HAS1 is the major HAS responsible for HA deposition by keratinocytes under physiological conditions. This is not in accord with studies suggesting that HAS3 action accounts for HA synthesis in keratinocytes, while HAS1 is mainly responsible for fibroblast HA synthesis (Sayo et al., 2002; Yamada et al., 2004). Moreover, Malaisse at al., postulate that the balance in HA levels achieved by specific expressions of distinct HAS isoforms may be a key point in discriminating keratinocyte pathologic conditions. In earlier studies it was demonstrated that pro-inflammatory cytokines including IL4, IL13, INF-γ (Sayo et al., 2002), growth factors such as EGF and KGF (Karvinen et al., 2003; Pasonen-Seppanen et al., 2003) as well as retinoic acid

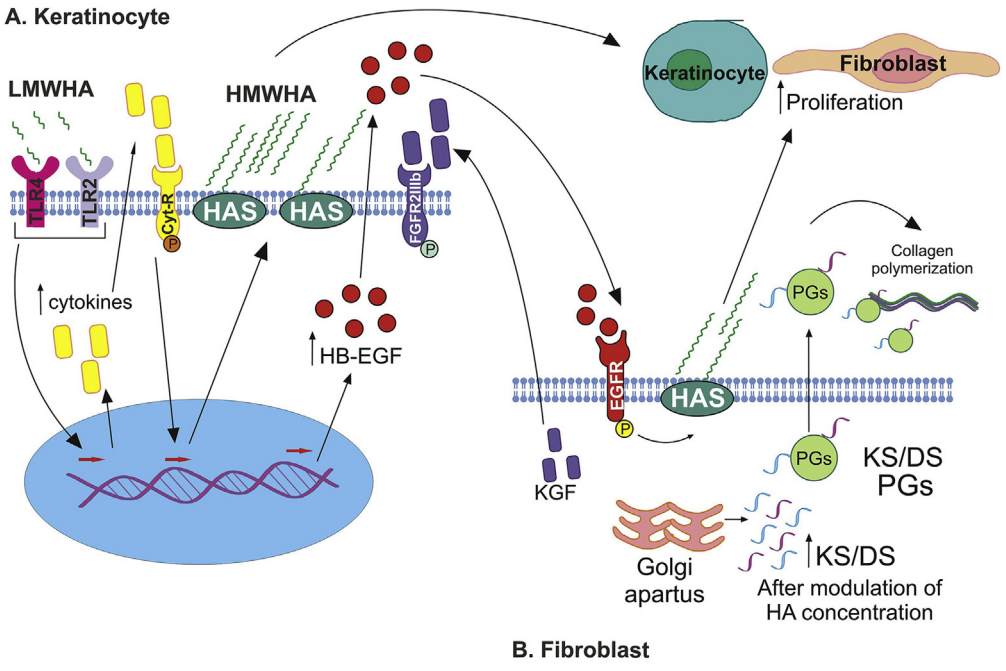

Fig. 1. HA involvement in the processes of wound healing and the associated keratinocyte and fibroblast crosstalk. (A) Inflammatory cells released cytokines and generated LMWHA fragments activate keratinocytes to produce IL-6, TNF-α and IL-1β, which in turn enhance HA synthesis partly through HB-EGF action. The resulting HA-mediated feedback loop facilitates the inflammatory response including fibroblast migration and proliferation. (B) Crosstalk between keratinocytes and migrating fibroblasts is the production of keratinocyte growth factor (KGF) by fibroblasts which binds to the respective FGFRIII2b receptor expressed by epidermal keratinocytes, and induces their increased proliferation. In the adult wound healing, after the initial maximum HA deposition, an instant decrease is observed, which triggers keratan (KS) and dermatan (DS) sulfate production in the Golgi apparatus. KS and DS proteoglycans assist in collagen polymerization, necessary for wound closure and scar formation.

(Saavalainen et al., 2005) have been suggested to enhance both HAS2 and HAS3 expression. In accordance with these findings the increase of EGF levels in patients with AD, correlates well with the shown enhancement of HAS3 expression in the RHE model (Malaisse et al., 2014).

Allergens are known to be present in/on objects of daily use. Irritant and allergic contact dermatitis (CD) are the unwanted side effects of exposure to chemicals of various origins (Peiser et al., 2012), A very common cause for allergic contact dermatitis are antimicrobial preservatives used in cosmetics, medicines or food products, likewise parabens (Wilkinson et al., 2002; Sasseville, 2004). An illustrative example is exposure to methyl paraben. Thus, biopsies of forearm skin revealed partially hydrolysed methyl paraben after its daily application. In fact, the frequency of use was directly correlated to its concentration in the stratum corneum due to the slow hydrolysis rate of this molecule (Ishiwatari et al., 2007). Long term treatment of epidermal keratinocytes with methyl paraben resulted among other in decreased expression of HAS1 and 3 and collagen type IV while at the same time it enhanced the expression of HSP27 and involucrin (Ishiwatari et al., 2007), well established to be differentiation markers in normal human epithelial keratinocytes (NHEK) (Kindas-Mugge and Trautinger, 1994; Robinson et al., 1996). Finally, Ishiwatari et al. (2007) postulate a link between methyl paraben accumulation and the differentiation and aging of the keratinocytes (Ishiwatari et al., 2007). Furthermore, the involvement of HA in the initiation of sterile skin inflammation by contact sensitizers like 2,4,6-trinitrochlorobenzene (TNCB), was demonstrated in a mice model when pre-treatment with Pep1, attenuated contact sensitization (Martin et al., 2008). Pep1 is a HA-binding peptide, whose binding affinity for HA is suggested to depend on HA conformation state (Zmolik and Mummert, 2005). Indeed, HA is postulated to be a key molecule in pathways of contact dermatitis (CD) as skin sensitization was abolished by pre-treatment of the skin with antioxidants or aristolochic acid, an established hyaluronidase inhibitor (Esser et al., 2012). These authors suggest that organic contact sensitizers initiate release of ROS and a subsequent breakdown of HA to pro-inflammatory LMWHA fragments in the skin. Furthermore, in an *in vitro* model of human keratinocytes, contact allergens were shown to stimulate HYAL1 and 2 expressions, which correlated to HA degradation as well as to IL-18 production (Nikitovic et al., 2015). Importantly, IL-18 is a well established biomarker of keratinocyte activation, selectively upregulated by contact allergens (Corsini et al., 2009; Galbiati et al., 2011). Modulation of HA production, by HYAL or aristolochic acid pre-treatment, resulted in a significant reduction of contact allergen-induced IL-18 production (Nikitovic et al., 2015). Earlier, increased oxidative stress has likewise been associated to CD-related alterations in ECM constitution (Esser et al., 2012; Corsini et al., 2013). These authors, therefore suggest, that the generated LMWHA fragments can act as a DAMPs in the process of keratinocyte activation (Esser et al., 2012; Corsini et al., 2013; Nikitovic et al., 2015). This would suggest that keratinocyte activation is dependent on HA-size in a non-linear manner (Fig. 2).

Apart from keratinocytes, dendritic cells (DCs) are also affected by alterations of HA metabolism. In fact, HYAL1 overexpressing mice, with the resulting increased HA degradation, presented enhanced DCs migration from the skin, potentially due to increased generation of LMWHA fragments (Muto et al., 2014). In the same model, a notable observation is that HA degradation resulted in faster antigen presentation and activation of immune response. Specifically, HYAL1 overexpression before the application of an allergic antigen, results in the suppression of immune response, while simultaneous HYAL1 overexpression and antigen application,

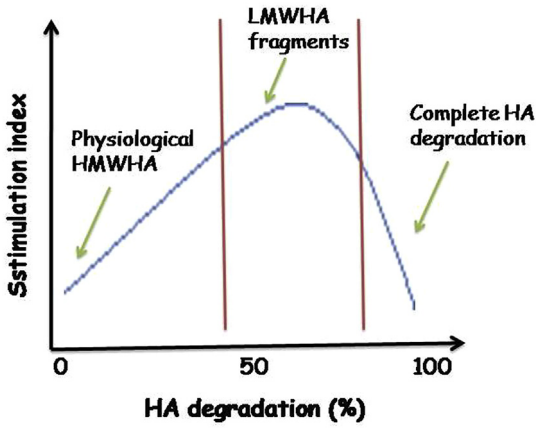

Fig. 2. This figure is a hypothesis based on results from Nikitovic et al. (2015) where it is postulated that HA in a size-dependent but a non-linear manner regulates keratinocyte activation which is presented as stimulation index.

lead to a faster response (Muto et al., 2014). The role of HA in skin inflammatory processes is schematically presented in Fig. 3.

2.4. UV irradiation and HA

It has been widely established that ultraviolet irradiation (UV) exerts a wide scale of harmful effects on human skin, from simple sunburn to skin cancer and early aging. The main cause of these unwelcome effects is cell death of epidermal cells, mainly keratinocytes, or immune responses of the same cells, leading to inflammation. As keratinocytes play major role in inflammation, and consequently in various dermatoses, it should be taken into account that upon exposure to UVB irradiation they react by producing inflammatory cytokines such as interleukins. Therefore, immune system activation is followed by ECM remodeling, including collagen degradation, and causing among other the premature aging of skin (Hasova et al., 2011).

UV irradiation has the ability to penetrate the epidermis and reach the dermal layer, causing numerous deleterious reactions, including oxidative stress, mutations or skin aging (Nichols and Katiyar, 2010). Interestingly, long-term exposure to UV has been shown to reduce HA in the dermis, due to lower HAS expression and progressive inactivation of fibroblasts (Dai et al., 2007). The other ECM component responsible for the youthful appearance of skin is collagen and it is also degraded by UV exposure (Nichols and Katiyar, 2010).

The participation of HA in mechanisms of UV induced cell damage appears to date ambiguous. The key role of HA in keratinocyte response to UV and its potential use as a therapeutic agent were recently implicated by Hasova et al., These authors demonstrate a rapid reduction of CD44, TLR2, HAS2 and HYAL2 expressions after short UVB exposure, indicating strong effects of UVB on HA signaling pathway. Moreover, the UV induced inflammatory cytokine production, as well as TGF-β1 production of keratinocytes, closely correlated to inflammatory response, were attenuated by HA treatment. Finally, HA also ameliorated the UVB disrupted cell viability, setting the potential therapeutic use of HA in UV induced skin disorders in picture (Hasova et al., 2011). However, in other studies (Averbeck et al., 2007; Kakizaki et al., 2008), cell viability was not altered after keratinocyte UVB exposure. Moreover, NHEK keratinocytes demonstrated increased HAS2 and HAS3 expression several hours after irradiation treatment, presumably due to an endogenous mechanism for the protection against UV (Kakizaki et al., 2008). Similarly, increased expression of cytokines related

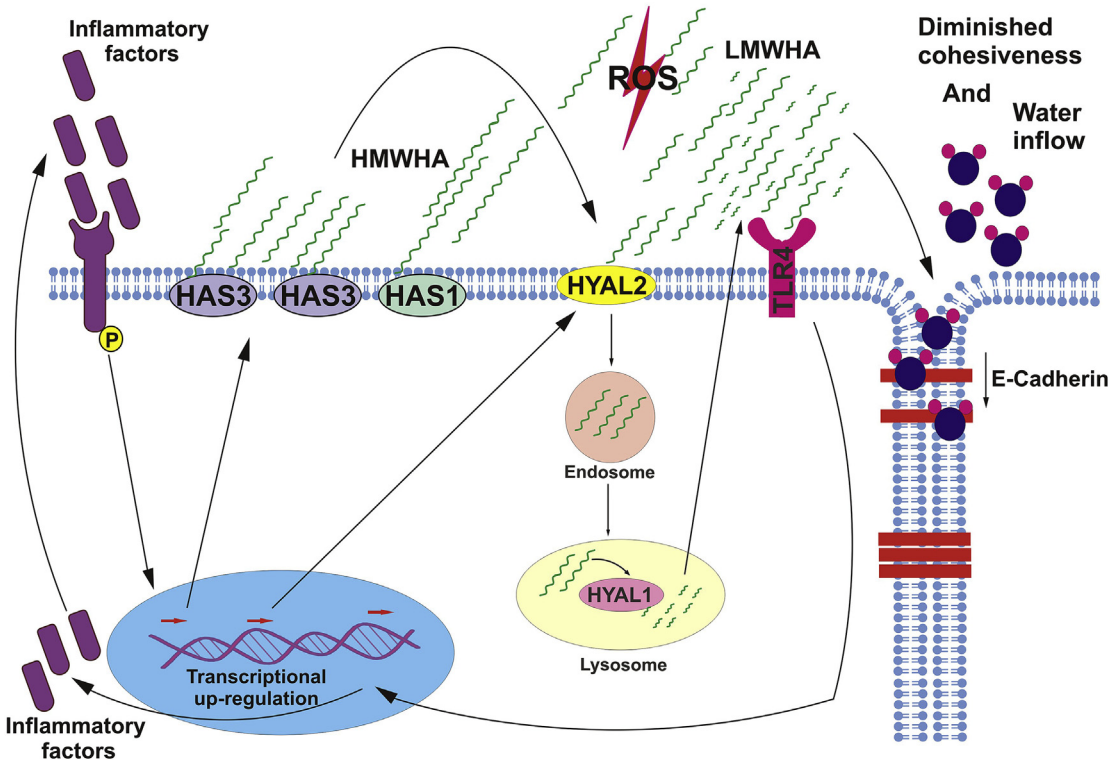

Fig. 3. The role of HA in skin inflammatory processes. Inflammatory mediators upregulate HA synthesis and degradation resulting in LMWHA generation which modulates the inflammatory response. Enhanced deposition of HA to the ECM, leads to dowregulation of cadherins and diminishment of epidermal keratinocyte cohesiveness which may be accompanied by water inflow and distrurbance of the epidermal barrier. Water molecules are designed as a combination of oxygen (blue) and hydrogen (pink) circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to HAS production was observed. Consequently, epidermal response to UVB seems to be time-dependant, as HA production is decreased a few hours after irradiation and increased 24 h later (Averbeck et al., 2007; Kakizaki et al., 2008; Rauhala et al., 2013). Discrepancies in expression levels of HA related proteins or time differences of certain processes could be due to different irradiation intensities used in each study or differences between keratinocyte cell lines. To conclude, UV radiation clearly affects HA metabolism as was observed by differential expression patterns of HA pathway proteins, but further studies are needed to fully determine the role of HA in UV-related cytotoxicity.

2.5. HA in treating skin disorders

HA as an important component of the skin, has been suggested or is currently applied for the treatment of several skin disorders. To begin with, atopic dermatitis occurs in a remarkable number of patients (Pacha and Hebert, 2012) accompanied by lesions (Lee et al., 2016) and visible skin changes including thickening, redness, oozing, xerosis crusting, blistering scaling, and color change (Kessel and Goldenberg, 2016). Patients have diminished amounts of ceramide in the skin; therefore, ceramide containing emollients are used as treatment aiming to increase the effectiveness of skin barrier. Simple emollients relieve the symptoms, while ceramide based ones aim to restore the damaged lipid bilayer components by modulating the structure of the stratum corneum (Pacha and Hebert, 2012). Indeed, hyaluronan-ceramide products aim to take the next step and transport active substances through the epidermis to the dermis (Spencer, 1988). Moreover, there are positive initial responses in HA gel use against facial seborrheic dermatitis, potentially due to LMWHA ability to stimulate immune response in the skin (Schlesinger and Powell, 2014).

In addition, taking advantage of the wound healing ability of HA, some creams designed for treating severe wounds contain HA (Milpied et al., 2011). However, combination of other ingredients incorporated into the creams, together with the easier absorption by the traumatized skin, may cause side effects, such as ACD (Milpied et al., 2011). HA may be a promising substance for wound healing, but there is still a long way to go until a proper combination of substances is defined for healing creams, targeting a minimum immune response.

HA is, also, very popular in cosmetic surgery (Viola et al., 2015b), used as skin filler for wrinkles or to restore the diminished volume that comes with aging. HA-fillers are known as safe and non-immunogenic, consequently they are widely used (Fertig et al., 2016; Maytin, 2016; Bogdan Allemann and Baumann, 2008). However, in some cases HA-filler injections have been reported to cause ecchymosis, edema, erythema, pain (Fertig et al., 2016), or even delayed immunological reactions (Bitterman-Deutsch et al., 2015).

Moving on, a very common skin disease, actinic keratoses (AK) may also be treated with a diclofenac/HA mixture. AK occurs as a lesion with enhanced likelihood of turning into squamous cell carcinoma, thus, an appropriate treatment is highly important. Diclofenac/HA gel targets the problematic cells and has demonstrated remarkable rates of effectiveness without side-effects and seems to be very promising for long-term treatment (Ulrich et al., 2014).

3. Conclusion

HA, a fundamental ECM component is a linear non-sulfated GAG composed of repeating units of N-acetyl-glucosamine (GlcNAc) and glucuronic acid (GlcA). HA is detected in all vertebrate tissues, but

the bulk of HA is produced and deposited in the skin especially in the dermis, where it is synthesized by dermal fibroblasts and epidermal keratinocytes. This GAG has been implicated in a plethora of biological functions including the modulation of wound healing, inflammation and the immune response. This is mediated by key signaling pathways regulating body homeostasis including growth factor and cytokine action. The skin layer as the body external barrier is prone to injury and inflammation. This review discusses the role of HA in the processes of skin wound healing and inflammation as well as the possibility of using HA to treat several skin disorders. The up to date knowledge supports a key role of HA in these processes cautiously proposing that the effects are dependent on HA size and concentration. However, the complexity of the processes and the sometime ambivalent endpoints strongly stress the need for further study.

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