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# Impact of crosslinked HA and its degradation products on skin and immune cells in an inflammatory environment

Benjamin Sanchez

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Soutenue à huis clos le 04/12/2020, par :  
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**Impact of crosslinked HA and its  
degradation products on skin and immune  
cells in an inflammatory environment**

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*« La physique mathématique et la biologie moléculaire sont la poésie d'aujourd'hui. Ce sont elles qui traduisent et qui façonnent le monde et elles soulèvent chez les jeunes gens l'enthousiasme qui venait hier des poètes. »*

*Jean d'Ormesson*

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# Résumé

L'acide hyaluronique (AH), un glycosaminoglycane majeur de la matrice extracellulaire, joue un rôle important dans un certain nombre de processus biologiques tels que la cicatrisation, le cancer ou le développement embryonnaire. Le catabolisme de l'AH génère des fragments jouant un rôle dans la migration, l'apoptose ou la régulation immunitaire. Le métabolisme des AH est un processus finement régulé, adaptant ses fonctions à son microenvironnement. En cas de dérégulation du métabolisme, l'accumulation de fragments d'AH semble être fortement liée à la gravité des pathologies inflammatoires chroniques. Néanmoins, de par ces propriétés rhéologiques et structurelles, l'AH, prévient la déshydratation et maintient la structure de la peau. Bien qu'il soit généralement considéré comme un matériau inerte et biocompatible, de rares cas d'effets indésirables tardifs ont été observés et c'est ainsi que nous avons émis l'hypothèse que les rares complications observées pouvaient être liées à un microenvironnement inflammatoire. Dans ce travail de thèse, nous avons évalué sur cellules dermiques, cellules immunitaires et sur modèle murin le potentiel inflammatoire et vasculaire de l'AH dégradé et non dégradé. Dans un microenvironnement inflammatoire, l'ajout de fragments d'AH sur les cellules endothéliales et fibroblastes dermiques n'aggrave pas la réponse inflammatoire au contraire des cellules immunitaires. Ceci suggère l'implication des macrophages M1 et cellules dendritiques matures à moindre échelle dans la transmission de l'inflammation au tissu. De plus, dans un modèle murin d'inflammation chronique bas grade nous avons montré in vivo que l'injection d'AH peut conduire à une sensibilité microvasculaire en réponse à l'acétylcholine. L'hypothèse est que cette sensibilité vasculaire est liée au recrutement des cellules immunitaires sur le site d'injection. Par ailleurs, nous avons mis en évidence in-vitro qu'en absence de cellules immunitaires, un microenvironnement inflammatoire vasculaire pouvait mener à une altération de la matrice extracellulaire. Ces résultats montrent une diversité dans les interactions cellules-cellules selon le degré inflammatoire du microenvironnement qui en présence de fragments de dégradation d'AH (i.e. au cours du vieillissement ou lésion) pourrait fragiliser le tissu cutané.

# Abstract

Hyaluronic acid (HA), a major glycosaminoglycan in the extracellular matrix, plays an important role in a number of biological processes such as wound healing, cancer or embryonic development. The catabolism of HA generates fragments that play a role in migration, apoptosis or immune regulation. HA metabolism is a finely regulated process, adapting its functions to its microenvironment. In the event of deregulation of the metabolism, the accumulation of HA fragments seems to be strongly related to the severity of chronic inflammatory pathologies. Nevertheless, due to its rheological and structural properties, HA prevents dehydration and maintains the structure of the skin. Although it is generally considered to be an inert and biocompatible material, rare cases of late side effects have been observed. Thus, in order to improve our understanding of the mechanisms that can lead to late complications, we hypothesized that the rare complications observed could be related to an inflammatory microenvironment. In this thesis work, we evaluated the inflammatory and vascular potential of degraded and non-degraded HA in dermal cells, immune cells and in a mouse model. In an inflammatory microenvironment, adding HA fragments to endothelial cells and dermal fibroblasts does not worsen the inflammatory response, unlike immune cells. This suggests the involvement of M1 macrophages and mature dendritic cells to a lesser extent in the transmission of inflammation to the tissue. In addition, in a mouse model of chronic low-grade inflammation we showed *in vivo* that injection of HA can lead to microvascular sensitivity in response to acetylcholine. The hypothesis is that this vascular sensitivity is linked to the recruitment of immune cells at the injection site. Furthermore, we demonstrated *in vitro* that in the absence of immune cells, an inflammatory vascular microenvironment could lead to an alteration of the extracellular matrix. These results show a diversity in cell-cell interactions depending on the inflammatory degree of the microenvironment, which in the presence of degradation fragments of HA (i.e. during aging or lesion) could weaken the skin tissue.

# List of publications and conferences

## Article published/submitted

### **“Impact of Human Impact of Human Dermal Microvascular Endothelial Cells on Primary Dermal Fibroblasts in Response to Inflammatory Stress”**

Benjamin.Sanchez, Linan Li, Joshua Dulong, Géraldine Aimond, Jérôme Lamartine, Guangrong Liu and Dominique Sigaucho-Roussel; April 2019; Frontiers in Cell and Developmental Biology

### **“Biological effect of hyaluronic acid products and wound healing”**

Benjamin Sanchez, Dominique Sigaucho-Roussel and Bérengère Fromy; Submitted in International Journal of Cosmetic Science

## Conferences

**“Impact of microvascular inflammatory cells on dermal fibroblasts behaviors”**, The European Tissue Repair Society Meeting (Munich, 2019)

**“Inflammation and aging: Focus on Intercellular communications impact on the skin”**, International Symposium of French Society of Cosmetology (Paris, 2019)

## Intitulé du laboratoire :

**Institut de Biologie et Chimie des Protéines (IBCP) UMR-5305 CNRS Laboratoire de Biologie**

**Tissulaire et Ingénierie Thérapeutique (LBTI) Equipe : Fonctionnalité et Dynamique du**

**Tissu Cutané Sous la direction de : Dr Dominique SIGAUCHO-ROUSSEL 7, passage du Vercors**

**69367 Lyon – cedex 07 France**

# List of abbreviations

ACh	Acetylcholine
BDDE	1,4-Butanediol Diglycidyl Ether
CCL2	chemokine (C-C motif) ligand 2
COX	Cyclooxygenase
ECM	Extracellular matrix
EDHF	Endothelium-derived hyperpolarizing factor
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HA	Hyaluronic acid
HARE	Hyaluronan Receptor for Endocytosis
HDMEC	Human Dermal Microvascular Endothelial Cell
HMW	High molecular weight
HPLC	High-performance liquid chromatography
HYAL	Hyaluronidase
ICAM-1	Intercellular Adhesion Molecule 1
IFN	Interferon
IGF-1	Insulin growth factor 1

IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
LED	Light-Emitting Diodes
LGI	Low-grade inflammation
LMW	Low molecular weight
LOX	Lysyl oxidase
LPS	Lipopolysaccharide
LYVE1	Lymphatic vessel endothelial hyaluronan receptor 1
MALS	Multi-Angle Light Scattering
mDC	mature Dendritic Cell
MMP	Metalloproteinase
MMW	Medium molecular weight
NO	Nitric oxide
NOS	Nitric oxide synthases
o-HA	Oligosaccharide of hyaluronic acid
PBS	Phosphate-buffered saline
PG	Proteoglycan
PGE2	Prostaglandin E <sub>2</sub>
PGI2	Prostaglandin I <sub>2</sub>
PMA	Phorbol 12-myristate 13-acetate
RHAMM	Hyaluronan-mediated motility receptor
RNA	Ribonucleic acid
TBP	TATA binding Protein

TGF	Transforming growth factor beta
TLR	Toll Like Receptor
TNF	Tumor necrosis factor
UV	Ultra Violet
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor

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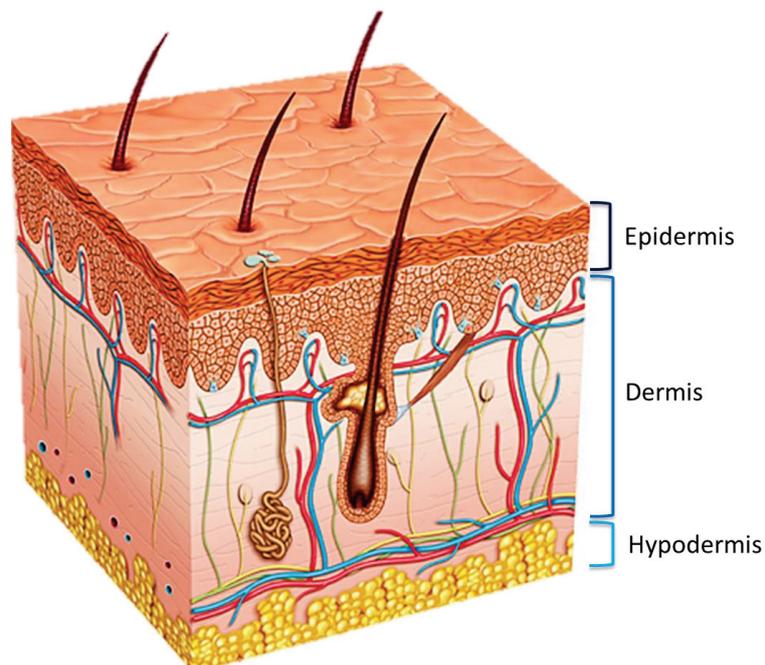
# INTRODUCTION

# The Skin

As a barrier between the external and internal environment of our body, the skin is a complex organ that has two purposes. The first is to ensure communication between our own body and the surrounding environment. And the second is to protect our organism from external aggressions. Covering approximately  $2\text{m}^2$ , the skin is the largest organ of the body with a weight over 5 kg (1). The skin is our first protector against external attacks like ultraviolet light, physical, chemical stresses and microorganisms but also prevents from dehydration (2).

Anatomically, the skin is composed of three communicating tissue layers, from the most superficial to the deepest layer: the epidermis and the dermis (which are separated by the dermal-epidermal junction), and the hypodermis (also called subcutaneous layer) (Figure 1).

All these three layers ensure essential functions as described below.



*Figure 1: Skin structure and its three layers (Adapted*

## 1.1 Skin ultrastructure: from the superficial to the deep layers

### 1.1.1 The epidermis

The epidermis is a pluristratified and keratinized squamous epithelium that constitutes the external structure of the skin. In contact with the external environment, it ensures a protective

function for the organism but also prevents loss of water. Its thickness of the order of a few hundred micrometers ( $\mu\text{m}$ ) varies according to the anatomical region. More than 90% of the epidermis is composed by epithelial cells called keratinocytes, the other epidermal cells corresponding to melanocytes, Langerhans and Merkel's cells (3, 4). The epidermis is divided into four strata defined according to morphology and keratinocyte positions. The basal layer is the deepest layer where keratinocytes division and differentiation start. Above this are suprabasal layers with exclusively differentiating keratinocytes starting with the stratum spinosum followed by the stratum granulosum and finishing with the stratum corneum the most superficial layer and as such has an important role in the barrier function of the skin (5) (Figure 2). The epidermis is constantly renewing itself. Other cell types involved in immunity, melanin production or touch are also present: Langerhans cells, melanocytes, and Merkel cells respectively. The basal layer of keratinocytes is attached to the dermal-epidermal junction which is a complex network of interconnecting proteins that provides the anchoring of the epidermis to the dermis. Besides ensuring the structural integrity of the skin, the dermal-epidermal junction allows the exchange of some molecules such as oxygen, nutrients and waste products between the dermis and the avascular epidermis (6).

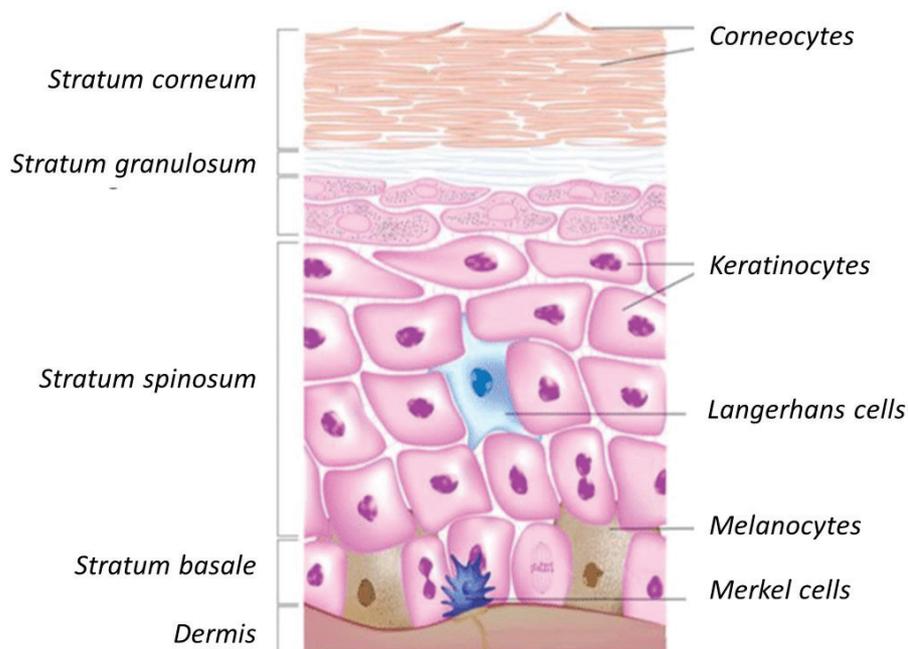


Figure 2: The epidermis (Extracted and modified (7))

### 1.1.2 The dermis

The dermis is a fibro-elastic connective tissue whose thickness varies according to its anatomical location. Its average thickness is between 1 and 2mm. It is composed of 2 parts:

- The superficial dermis or papillary dermis, which contains vertically oriented collagen fibers but also immune system cells, fibroblasts, blood capillaries and nerve fibers. It represents  $1/5^{\text{th}}$  of the dermis. The fibers and cells are bathed in a fibrillary gel-like substance. The superficial part of the dermis invaginates at the junction with the epidermis in the form of dermal papillae, increasing the contact surfaces with the epidermis and allowing better adhesion between these two layers. The dermal papillae contain nerve fibers, which can penetrate the basal lamina to go and innervate the epidermis or be connected to real nerve corpuscles in the dermis playing the role of tactile mechanoreceptors (Figure 3).
- The deep dermis or reticular dermis comprises collagen fibers organized in bundles and elastin fibers that intersect. It also contains arterioles, venules and nerve fibers. It represents  $4/5^{\text{th}}$  of the dermis and forms a much tighter network. The reticular dermis is poorly composed of fibrillary gel contrary to the papillary dermis (8) (Figure 3).

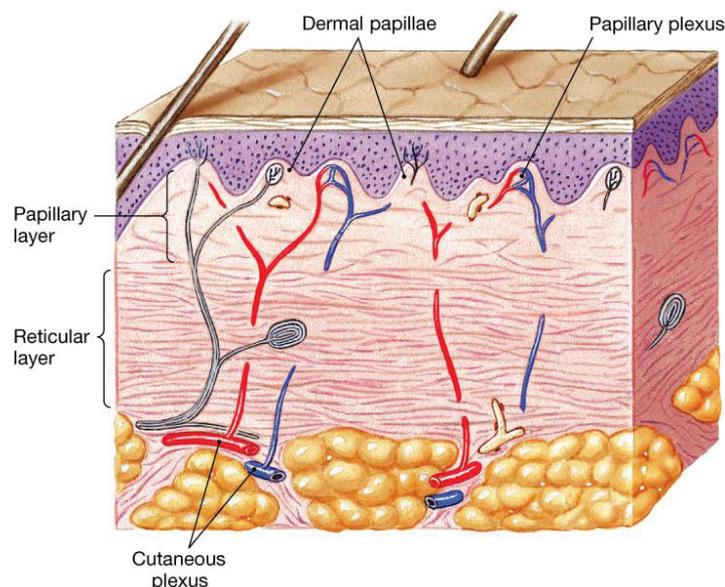


Figure 3: Dermis structure (Extracted from (9))

The fibrillary gel-like substance is composed of water, mineral salts and glycoproteins like proteoglycans and glycosaminoglycans (GAGs), of which 50% are hyaluronic acid.

Glycosaminoglycans give the dermis a high water retention capacity, more than  $\frac{3}{4}$  of the skin's water is stored in the dermis (10, 11).

The dermis is a matrix composed of different types of cells permitting intercellular communications to respond to internal and external stimuli.

The dermis gives the skin its suppleness, elasticity and tensile strength. It is a sophisticated and biologically active assembly that protects the body against mechanical injury, helps thermal regulation, allows water storage, brings nutrients to the epidermis and contains receptors for sensory stimuli (12, 13). It plays an important role in cellular immune traffic, regulation of vessel tone and local hemostasis.

In addition to these functions, the dermis plays a crucial role in the nutrition of the epidermis and in the healing process (14, 15). It is one of the actors, if not the major actor in maintaining the integrity of the skin.

#### *1.1.2.1 Extracellular matrix*

The extracellular matrix (ECM) is an assembly of macromolecules of proteins and carbohydrates, that bind together homologous or heterologous cells. The macromolecules of the extracellular matrix are classified into four categories: collagens, elastin, proteoglycans and structural glycoproteins. The proportion of these elements varies depending on the tissue.

##### *1.1.2.1.1 Collagen*

In the skin, the collagen fibers (mainly type I with 90% of total collagen and type III with 10% of total collagen) and elastin fibers are the main components of the matrix (16).

Collagen fibers make up 70% of the dry weight of the dermis and give its resistance to traction, tension, shear and other mechanical forces. Dermal collagen is a long-lived molecule of the extracellular matrix with a half-life of 15 years (17). The least represented fibrillary collagens are collagen V and IV. Nevertheless, they are essential to the maintenance and structure of the dermis and the structural integrity of the skin. Indeed, the collagen V determines the diameter of the collagen I fibers with which it is associated, and the collagen IV forms the framework of the dermal-epidermal junction and other basal membranes. During aging, including intrinsic aging and extrinsic aging (induced by external stimuli), it has been shown that the quantity and quality of collagen fibers were diminished, associated with a loss of network density (18). Furthermore, collagen network is finely regulated during life. The

collagen network is modulated during inflammation, homeostasis and immune responses (19). Nevertheless, dysregulation caused by chronological aging, pathologies or photo-aging led to metabolic dysregulation inducing complication such as fibrosis or skin structure alteration (20-22).

#### 1.1.2.1.2 Elastin

Elastin is the major component of elastic fibers; it is associated with micro fibrils composed of structural glycoproteins. In healthy human skin, the part of dermal elastic fibers is very low (2-5%) compared to the 70% part of collagen. The metabolic turnover of elastin is very slow, with a half-life at 70 years (23) . Elastin is synthesized in the form of a soluble precursor, tropoelastin. This 70 kDa protein is highly hydrophobic (24). The gene coding for the tropoelastin is regulated by many factors released during various processes such as development, inflammation and wound healing. In elastogenesis, it has been shown that IL-1 (Interleukin-1), TGF- $\beta$  (Transforming Growth Factor- $\beta$ ) or IGF-1 (Insulin Growth Factor 1) can increase the mRNA expression of the tropoelastin leading to increase the amount of elastic fibers (25), while some cytokines like TNF- $\alpha$  and IFN- $\gamma$  may decrease its activity (25, 26). However, certain enzymes (matrix metalloproteinase, elastase or serine proteases) can be activated in certain contexts such as cancer, aging, linked to the skin exposome or under the influence of UV rays and are then able to cleave elastin, causing a loss of elasticity (27, 28) .

#### 1.1.2.1.3 The Glycosaminoglycan's and proteoglycans

Glycosaminoglycans (GAGs) are a family of complex linear and unbranched polysaccharides. They share a common structural organization, based on the repetition of a disaccharide unit including a hexosamine (N -acetyl -glucosamine or N-acetyl -galactosamine) and an uronic acid (D-glucuronic acid or L-iduronic acid) or a neutral sugar (galactose) and may be variably sulphated.

The nature of the sugars forming these disaccharide units, as well as the types of glycosidic bonds involved, make it possible to define 6 types of glycosaminoglycans classified into two sub-families: glucosaminoglycans and galactosaminoglycans. In the extracellular matrix we can find some dermatan sulfate, chondroitin sulfate, heparan sulfate and hyaluronic acid.

Hyaluronic acid, also known as hyaluronan, is a non-sulfated glycosaminoglycan (GAG) non-protein compound with distinct physico-chemical properties of repeating  $\beta$ -1,4-D-glucuronic acid and  $\beta$ -1,3-N-acetylglucosamine units (29), is the most abundant glycosaminoglycans in the skin. Hyaluronic acid is a product naturally present in the body, especially in skin and cartilage tissues. Hyaluronic acid is biopolymer highly active in the metabolisms and more precisely in the joints and in the skin. Unlike elastin, HA has a dynamic turnover with a half-life in the skin between 24h and 48h and 1 to 3 weeks in the cartilage (30).

Its proportion and size differ according to the location of the hyaluronic acid (31, 32). Indeed, human skin contains approximately 400 to 500  $\mu$ g of HA /g of tissue and mostly in the dermis (33) while human articular cartilage has approximately 500 to 2500  $\mu$ g of HA /g of tissue (34). Furthermore, young skins have higher HA contents than older skin as observed with collagen network. The different sizes of HA and its fragments and their different biological role in the skin will be further described in the chapter “Hyaluronic acid to medical devices”.

Proteoglycans (PGs) belong to the family of glycoproteins. What they have in common is the presence of glycosaminoglycan chains covalently attached to a protein backbone during post-translational modification. Proteoglycans can absorb up to 1000 times their volume in water, directly impacting the volume and compressibility of the dermis. Due to their structure, PGs can bind to growth factors, cytokines and chemokines. They influence proliferation, differentiation, tissue repair and morphogenesis (35).

They include certain proteins that play a role in the diameter of collagen fibers such as decorin or versican interacting with microfibrils and hyaluronic acid (36).

### *1.1.2.2 Dermal cells types and functions*

The dermis hosts a multitude of different cell types. Dermal fibroblasts and immune cells, such as macrophages, dendritic cells and lymphocytes, are the main cell types in the dermis. Endothelial cells involved in blood and lymphatic microcirculation are also present.

#### *1.1.2.2.1 Dermal fibroblasts*

Fibroblasts have mesenchymal origins and are the most represented cell category in the healthy dermis. Dermal fibroblasts can come from different tissues localization such as

abdomen, foreskin, arms or breasts and have different phenotypes, behaviors and functions according to the embryonic origins (37). Their number is higher, and they are metabolically more active in the papillary dermis than in the reticular dermis. They are resident cells in the dermis producing and secreting molecules of the extracellular matrix (ECM) such as collagen, elastin, proteoglycan, glycosaminoglycan, but also matrix metalloproteinase (MMPs) or lysyl oxidase (LOX) responsible for the degradation, the stabilization and the remodeling of this matrix. Proteoglycan and glycosaminoglycan like hyaluronic acid plays a significant role maintaining extracellular matrix formation as support for the collagen and elastin network produced by dermal fibroblasts.

With their integrin-like membrane receptors, dermal fibroblasts interact with the extracellular matrix and receive messages from the skin cells that will have a direct influence on their metabolic activity. Directly in contact with keratinocytes and endothelial cells: they synthesize collagen type IV found in the basement membrane of the dermo-epidermal junction and the blood vessels as well as type VII collagen. And they also produce directly collagen type I and III and elastin fibers to form the ECM network of the dermis (38-40).

In addition to their synthetic role, they have anti-infectious and antiviral defense roles. Thus, they intervene in the skin's defense mechanisms via the secretion of chemoattractant (41). Fibroblast were commonly described as a simple resident as key player in the production of protein for the extracellular matrix but now they are considered as sentinel cells by the immune system (42, 43); and more particularly for microbial infection using CD40 ligand as the major activation antigen on fibroblasts. In addition to the synthesis of antimicrobial peptides, they can produce pro-inflammatory cytokines, such as TNF $\alpha$ , IL-6, IL-8, and IL-10; other chemokines, such as CCL2, CCL5, CXCL1, CXCL8 or CXCL10; and the growth factors granulocyte/macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) to induce and recruit inflammatory cells (43, 44).

Furthermore, their proliferation and activity are regulated by various growth factors, in particular IGF-I (Insulin Like Growth Factor I) and TGF-Beta (Transforming Growth Factor Beta), which stimulate the production of extracellular matrix constituents and are involved in the healing and tissue repair processes too (45).

#### 1.1.2.2.2 Dermal immune cells

##### 1.1.2.2.2.1 Macrophages

Macrophages in the dermis are derived from blood monocytes. Once fixed in tissues, macrophages have cytological aspects and functions distinct from those of monocytes. Resident macrophages are likely to persist for a long time within the same tissue. They act in acute and chronic diseases (46, 47). The first source is embryo-derived progenitors that seed the skin prenatally. The second and major source of dermal macrophages is circulating monocytes that differentiate once they reach the skin (41). Monocytes/macrophages, derived from myeloid precursors, represent a heterogeneous family in terms of morphology and localization, providing functions of phagocytosis and antigen presentation. They synthesize a very large number of enzymes to modulate the extracellular matrix in case of lesion or during healing process and are capable of secreting various inflammatory cytokines (48). Among all these functions, macrophages are the key player in the phagocytosis process, secreting cytokines and chemokines initiating the inflammatory process. Macrophages are plastic cells that have capacities to modulate their phenotype depending on the microenvironment (41). Nine different types of macrophages have been identified, covering large phenotypes and functions from acute and chronic inflammation to antioxidant function and atheroprotective effect in the case of atherosclerosis diseases. They can be activated in M1, M2a, M2b M2c, M2d, Mox, M(Hb), Mhem or M4 in vitro using different cocktail of cytokines, growth factors, hormones and chemokines (Figure 4) (49-52). Among all of these types, macrophages M1, which are pro-inflammatory, and macrophages M2a commonly known as anti-inflammatory are the most described and studied.

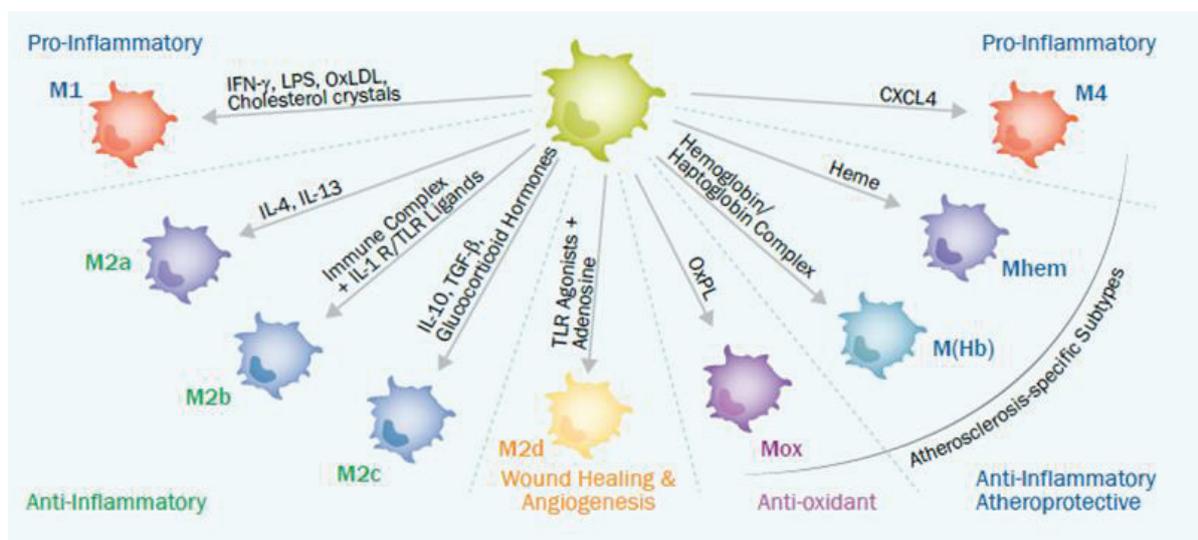


Figure 4: The Complex Biology of Macrophages: Origins, Functions, & Activation States, (R&D system)

Macrophages M1 express specific markers (CD68, CD11b, CD14 CD64) as well as molecules also expressed by dendritic cells (CD206, DC-SIGN, HLA-DR, factor XIIIa) that enable them to present antigens when interacting with T cells. M1 macrophages express iNOS and secrete inflammatory cytokines such as  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ , and  $\text{IL-6}$  (53) while M2 macrophages adopt an anti-inflammatory and/or pro-repair phenotype.

#### 1.1.2.2.2 Dendritic cells

Dendritic cells (DCs) in the epithelial interfaces are in an immature state, characterized by a high capacity for antigen capture and internalization (54, 55). Functional maturation of DCs can be induced by the presence of cytokines and pro-inflammatory chemokines in the environment, to then be able to present antigens and activate naive T cells. These maturation signals may come from viruses, bacteria or components, which act either directly by binding to DC receptors, such as toll-like receptors (TLRs), or indirectly through the production of pro-inflammatory cytokines in the microenvironment ( $\text{TNF-}\alpha$ ,  $\text{IL-1}\beta$  or  $\text{IL-8}$ ).

There are different subpopulations of dermal dendritic cells that have been isolated in human skin explants (56). Three subpopulations can be distinguished based on CD1a and CD14 expression:  $\text{CD1a-CD14-}$ ,  $\text{CD1a+CD14-}$  and  $\text{CD1a-CD14+}$ . The  $\text{CD1a-CD14-}$  cells constitute the classical population of dendritic cells of the dermis, the  $\text{CD1a+CD14-}$  cells certainly represent a

population of Langerhans cells leaving the epidermis, while the CD1a<sup>-</sup>CD14<sup>+</sup> population would appear to represent a precursor pool of dendritic cells of the dermis (57).

#### 1.1.2.2.2.3 Other cell types

In case of inflammatory process in the skin, other types of cells can be recruited: Polynuclear cells such as neutrophils, eosinophils and basophils (41). They have the ability to migrate into tissues in response to a chemotactic signal and to secrete a large number of inflammatory cytokines. Neutrophils are phagocytic cells that are involved in tissue damage from immunopathological reactions. Eosinophils are cytotoxic phagocytic cells that participate in the inflammatory response by early recruitment upon release of soluble mediators of inflammation (41). These cells are implicated in certain parasitic infections and allergic reactions. Basophils make up less than 1% of leukocytes in the blood and have high affinity receptors which, when activated, cause them to degranulate and release preformed mediators such as histamine and heparin (58). T lymphocytes are derived from hematopoietic bone marrow precursors differentiated in the thymus. They are mainly present in the lymphoid organs: the lymph nodes and the spleen. There are two subpopulations that are differentiated by the expression of CD4 or CD8 markers. Both have specific antigen receptors, the TCR (T cell receptor). The recognition of the antigen requires its presentation by MHC-I molecules for CD8<sup>+</sup> LTs and MHC-II molecules for CD4<sup>+</sup> LTs (41). LTs recruited into the skin are most often located just below the dermal-epidermal junction. Dermis present the properties to have many other cell types involved in extracellular matrix modulation, wound healing, bacterial and viral infections, hypersensitivity reactions or autoimmune diseases as described below (Table 1).

Immune Cell Type	Location in Skin	Inflammatory Functions	Functions during Wound Healing
<b>Mast cells</b>	Papillary and reticular dermis	Produce inflammatory mediators involved in allergic responses and asthma, and recruitment of immune cells Produce inflammatory cytokines and secrete histamine during contact-hypersensitivities	Induce collagen production (fibrosis) from fibroblasts
<b><math>\alpha\beta</math> T lymphocytes</b>	CD8+: Epidermis CD4+: Epidermis and papillary dermis	Induces antiviral state in the skin through IFN $\gamma$ mechanism T effectors produce cognate cytokines (i.e., IFN $\gamma$ , IL-4, IL-17) T <sub>regs</sub> suppress inflammatory monocytes and other autoreactive immune cells	Resolution of wound inflammation through T <sub>reg</sub> -mediated control of inflammatory monocytes
<b><math>\gamma\delta</math> T lymphocytes (DETCs and dermal)</b>	DETCs: Epidermis Other $\gamma\delta$ T cells: Papillary dermis	Produce IL-17 to induce $\beta$ -defensin expression from keratinocytes Protective against cutaneous <i>S. aureus</i> infection Involved in disorders such as psoriasis	Secretion of KGFs and IGF-1 to induce expansion of the epidermis
<b>Non-<math>\gamma\delta</math> CD1-restricted lymphocytes</b>	Papillary and reticular Dermis	Involved in defense against extracellular pathogens Suppress autoreactive cell activity in systemic lupus erythematosus	iNKT cells may be involved in wound healing by controlling inflammatory neutrophil populations
<b>B lymphocytes</b>	Reticular Dermis	Involved in delayed-type hypersensitivity reactions Involved in cutaneous autoimmune diseases via production of autoantibodies specific to components of the skin IL-10-producing B <sub>regs</sub> suppress autoreactive lymphocyte activation	N/D

Table 1: Skin's immune cells (Extracted from (41))

### 1.1.3 The hypodermis

The hypodermis is the deepest layer of the skin. It is an adipose tissue in continuity with the dermis, the difference between the two tissues is therefore marked by the progressive change in the nature and composition of the connective tissue (59). It is attached to the dermis by expansions of collagen and elastin fibers. It is innervated and vascularized by the numerous vessels and nerves that pass through it and reach the dermis. It acts as an interface between the dermis and the mobile structures below it, such as muscles and tendons. The hypodermis, predominantly composed of adipocytes (45), plays a key role in the storage and release of

lipids, managing the body's energy stock according to needs and supplies. It has an essential role in the skin's mechanical resistance to shocks and also has a cold protection function (60). Furthermore, it is an endocrine organ that synthesizes and secretes adipokines, which can act locally (autocrine or paracrine) or systemically and influence all other organs.

#### 1.1.4 Vascular microcirculation and endothelial cells

Blood distribution at the cutaneous level is ensured by arteries coming from the deep tissue, the hypodermis. The blood irrigates and nourishes the dermis by forming plexuses. The superficial vascular plexus is at the dermal-epidermal junction and the deep vascular plexus is in the dermis between the papillary and reticular dermis (Figure 5).

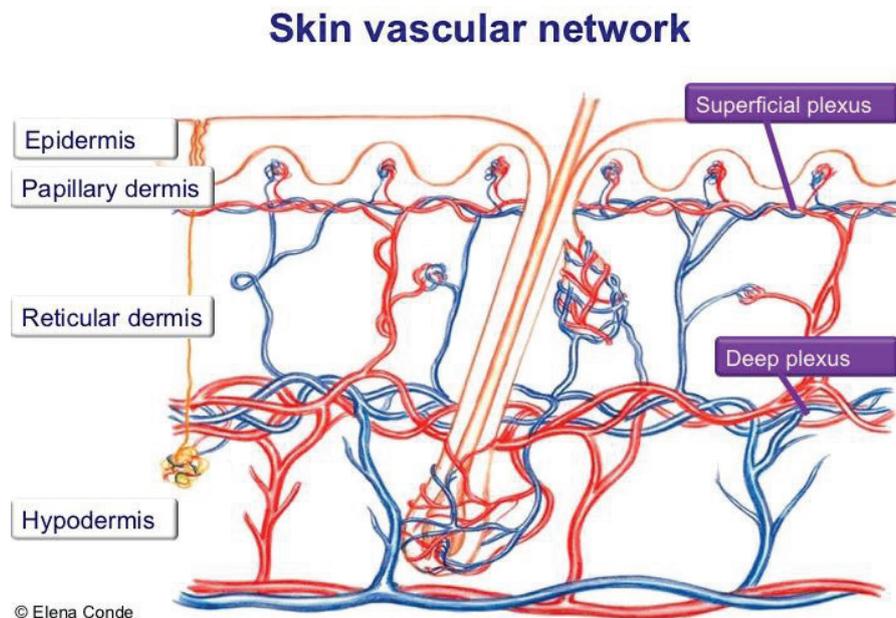


Figure 5: Skin vascular network (extracted from (61))

These two plexuses form a very complex vascular network that serves different purposes, including thermoregulation, nutrition, immune response and intercellular communication (62, 63).

The vascular endothelium, a monolayer of endothelial cells, forms the anatomical barrier between blood and tissue playing a major role in vascular function. The endothelial cells ensure the circulation and exchange of molecules between blood and interstitial fluid including gases, ions, proteins and also allow the crossing of cells through the phenomenon of

diapedesis. Indeed, they have a role in immunity, mainly through their control of leukocyte extravasation. They can express different adhesion molecules, which belong to the family of selectins (P-selectin, E-selectin) or immunoglobulins (ICAM1, VCAM1) that bind to integrin expressed following stimulation by cytokines or bacterial molecules like lipopolysaccharides (LPS). They also secrete lots of cytokines and chemokines allowing the recruitment of more competent immune cells at the site of inflammation (64). The endothelium has a large exchange surface and has many functions including the adaptation to the specific needs of the surrounding tissue. The endothelium is involved in vascular homeostasis but also in pathological processes such as thrombosis, inflammation or vascular remodeling (65, 66). Endothelial cells play an important role in the recruitment of leukocytes at the site of inflammation. The mechanisms contributing to this role involve the secretion of chemokines but also hyaluronic acid and oligosaccharides of HA present in endothelial cells (67, 68). The production of hyaluronic acid by the endothelial cells during the early stages of the inflammatory response is induced by pro-inflammatory cytokines such as TNF  $\alpha$  and IL1 $\beta$ , considered as the initiators of inflammatory process (69) and this process is essential in wound healing. Several experiments have suggested the role of HA in this phenomenon of leukocyte recruitment (67).

The endothelium also participates in the regulation of vascular tone through the secretion by the endothelial cells of vasodilator and vasoconstrictor molecules that will act at the level of the smooth muscle cell. Endothelial cells regulate blood pressure and blood flow by secreting vasodilatory factors such as nitric oxide (NO), prostacyclin, endothelium derived hyperpolarizing factor (EDHF) and vasoconstriction factors such as thromboxane, endothelin-1 or endoperoxides (70) (Figure 6).

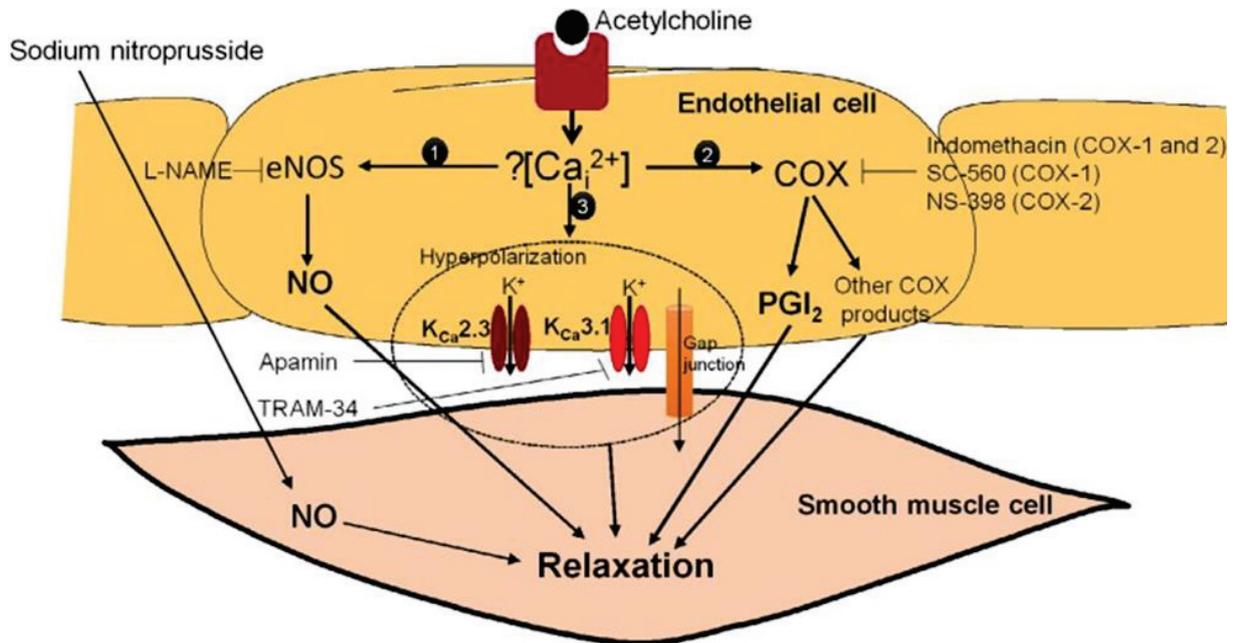


Figure 6: Acetylcholine-mediated vasodilation pathway (Extracted from (71))

The half-life of NO is very short and its action is therefore very localized. Many molecules can stimulate the endothelial cell to induce the synthesis of NO such as acetylcholine. When the endothelial cell receives this stimulus, this will lead to an increase in intracellular calcium. The calcium will form a complex with calmodulin leading to the phosphorylation of eNOS and thus allow the synthesis of NO. Cyclooxygenases (COX), COX-1 and COX-2, which are responsible for the synthesis of eNOS (72), are also involved in the regulation of vascular tone via the production of prostaglandin such as PGI<sub>2</sub> or PGE<sub>2</sub> (71).

# Hyaluronic acid to medical devices

## 1.2 Endogenous and exogenous HA functions

In 1934 Karl Meyer and John Palmer discovered hyaluronic acid, a combination of uronic acid and an aminoglycan, in the vitreous humour of eyes. It is by associating the terms uronic acid and the word hyaloid, from the greek "hyalos" meaning vitreous, that they proposed the term hyaluronic acid (HA) (73). It was only in the 1950s that HA structure and its properties were discovered (74-77). They have shown that HA was composed of a repeating disaccharide unit and they highlighted the sensitivity of HA to UV or X-ray inducing its complete degradation (77, 78). Each disaccharide has a molecular weight around 400 Da but depending on the number of repeating units in a polymer (Figure 7) it can reach to 20 MDa (79, 80). As mentioned above in the body, the proportion of HA and its size depends on the tissue and the type of synthesis, this part will be developed in HA metabolism part.

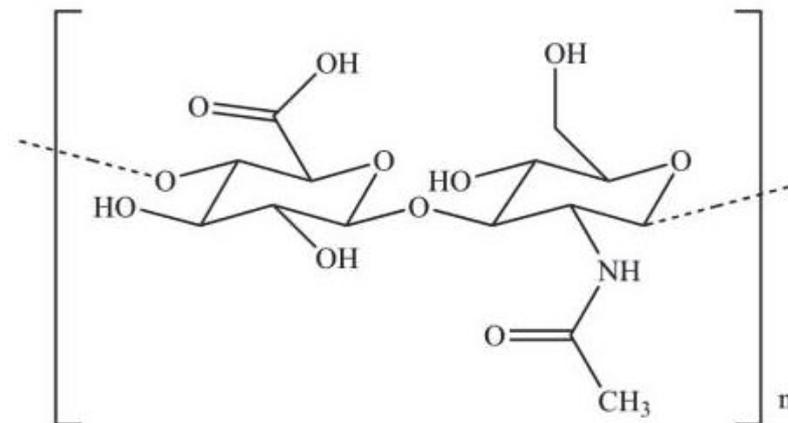


Figure 7: HA disaccharide of N-acetyl-D-glucosamine and D-glucuronic acid connected by  $\beta$  1,3-glycosidic bonds (29)

More than 50% of the total HA content of the whole body is located in the skin. Endogenous HA has other important functions in the skin but also in other tissues playing a role in organogenesis (81), angiogenesis (82), reactive oxygen species (83), cancer (84, 85), chondrocytes (86), lung injury (87), inflammation (88) and immune regulation (89). It exists in many forms of HA: circulating, associated within tissue and inserted into the extracellular

matrix. This hydrophilic molecule is used in the treatment of osteoarthritis, giving elasticity at the cartilage and increasing the viscosity of the synovial fluid using exogenous HA. Hyaluronic acid thus plays a valuable role in the joints as a lubricant and shock absorber. These various effects have been studied in recent years, highlighting the complexity of this regulation. The function and activities of hyaluronic acid depend on specific properties (Figure 8):

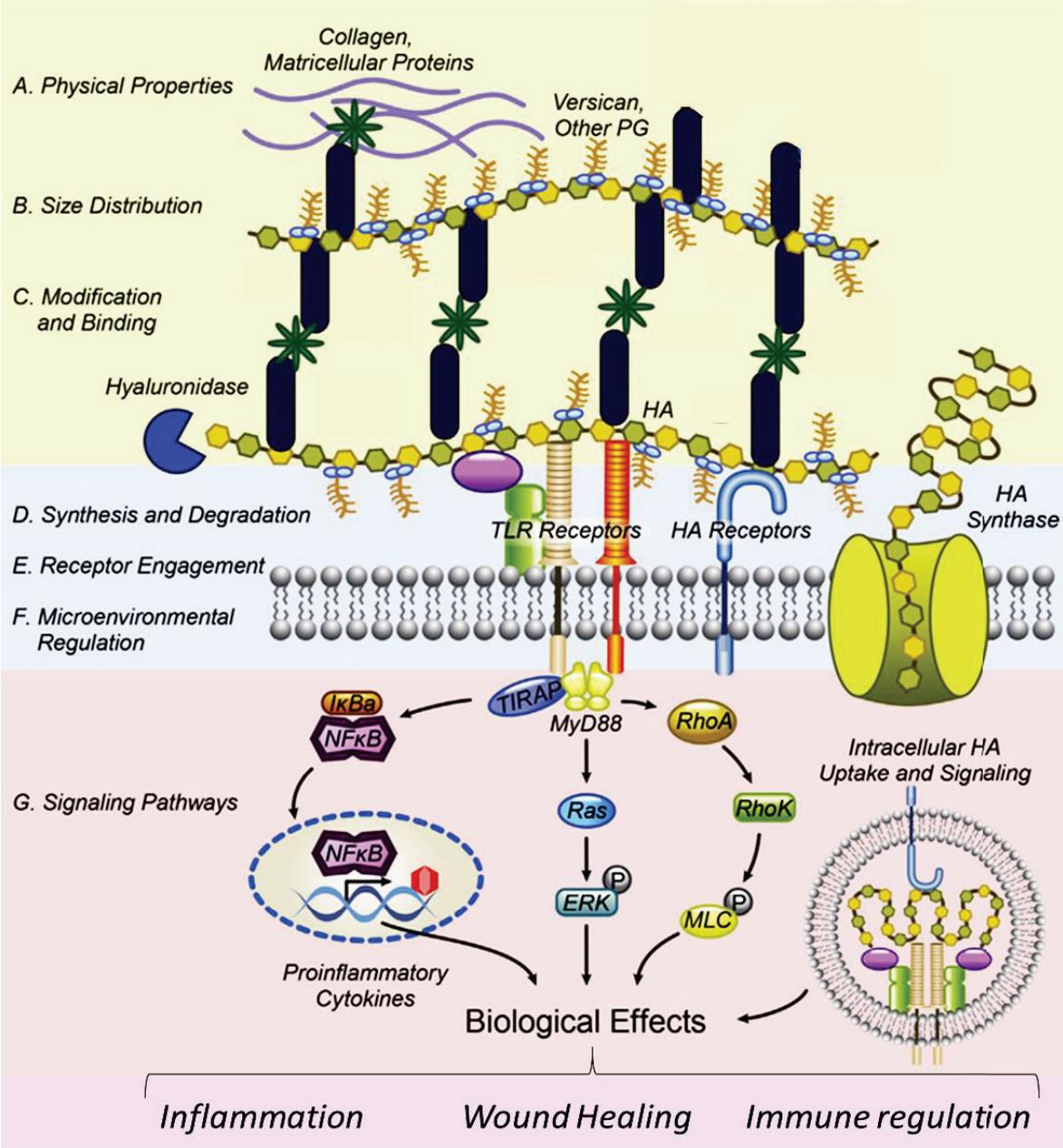


Figure 8: Hyaluronic acid functions and activities (Adapted from (90))

- Physical properties and chemical modifications (upper panel): size and molecular weight of HA and its distribution inducing different biological effect according to the

microenvironment (homeostasis or diseases), crosslinking patterns and macromolecular structure (Figure 8A and B).

- Metabolism of HA synthesis (HAS) and degradation (Hyaluronidase) and its microenvironment regulation with the receptors associated (example: TLRs, CD44 or RHAMM) (Figure 7D, E and F).
- Signaling pathway leading to biological effects such as inflammation, repair or immune regulation.

To counteract the signs of aging, like skin dryness, wrinkles and loss of tissues, the pharmaceutical industries developed medical devices using hyaluronic acid injection in aesthetic corrective. Hyaluronic acid is also used as treatment for cataract and osteoarthritis and many other joint disorders (91). In 2003, the FDA (Food and Drug Administration) has approved Restylane (Q-Med, Uppsala, Sweden) as injectable medical device. Since then, various other types of fillers have been approved by FDA for aesthetic use.

These commercial products use HA crosslinked with crosslinking agent to increase the longevity of HA and their rheological properties. The most common agent used and studied over 15 years in industry is the BDDE (1,4-butanediol diglycidyl ether) according to its biodegradability and stability. Contrary to endogenous HA which has a half-life of 24 to 48 hours in the skin, exogenous crosslinked HA can have a clinical duration that can reach or exceed 1 year (92). The interest of these fillers are their properties to counteract the loss of moisture and elasticity in the skin and the development of wrinkles improving skin quality and appearances.

### 1.3 Effect of HA and its degradation products

As described in Figure 7, physical properties and size distribution induce different signaling pathways leading to different biological effects. According to its high molecular weight, hyaluronic acid possesses structural effect allowing its association with the fibers of the extracellular matrix in the tissues. It contributes to the hydration of the extracellular matrix and tissues through its hydrophilic and viscoelastic properties due to its helical structure and negative charges. But during the different process of ECM remodeling, pathologies, aging or healing, hyaluronic acid can be degraded using specific enzymes named hyaluronidase and

some lysosomal enzymes. These enzymes can produce different range sizes of molecular weight of hyaluronic acid fragments, including oligomers of HA (o-HA) from 10 Da to 10kDa, low molecular weight of HA (LMW-HA) from 10kDa to 250kDa, medium molecular weight of fragments (MMW-HA) from 250kDa to 1000kDa and high molecular weight of HA (HMW-HA) from 1000 kDa to ~6000 kDa (93-95) (Table 2).

	<b>Homeostasis</b>		<b>Complication and diseases</b>	
	12000 kDa	1000 kDa	250 kDa	10 kDa
	<b>vHMW and HMW</b>		<b>MedMW and LowMW</b>	
				<b>Oligomers</b>
<b>Stromal/ Mesenchymal</b>	Cell contact inhibition	Increased cell migration Enhanced barrier function Reduced apoptosis	Cell differentiation <b>Enhanced ECM remodeling</b> <b>Increased cytokine release</b>	Inhibit synovial apoptosis <b>Increased cytokine release</b>
<b>Endothelial</b>	Unknown	Enhanced angiogenesis	<b>Inhibited angiogenesis</b>	<b>Increased cytokine release</b> Increased EC proliferation
<b>Immune</b>	Unknown	Inhibited cell recruitment Reduced cytokines release Anti-inflammatory effects	<b>Macrophages polarization</b> <b>Pro and anti inflammatory</b> <b>Immune recruitment</b>	<b>Monocytes and DC maturation</b> <b>Increased cytokine release</b> <b>Increase transmigration</b>
<b>Pathologies significance</b>	Unknown	Facilitates development Reduced scarring Inhibited vascular injury	Attenuated or exacerbate lung injury Pro and anti wound healing	Increase aortic remodeling Enhanced skin healing Inhibits vascular injury

Table 2: Biological and pathological significance of endogenous HA size (Adapted from (85))

Endogenous hyaluronic acid and its degradation products have different biological effects. The two main effects are its role in wound healing process and the second and most controversial is its role in regulating the immune response.

### 1.3.1 Wound healing role

Hyaluronic acid plays a role in the 3 phases of wound healing. During the first phase, specific hyaluronidases and lysosomal enzymes degrade hyaluronic acid into oligomers of variable sizes. The high concentration of oligomers, which interact with CD44, causes diapedesis of leukocytes and macrophages and stimulates the production of pro-inflammatory cytokines (96). During the proliferative phase, the increase in the concentration of oligomers allows the

organization and stabilization of the extracellular matrix that facilitates the migration of fibroblasts, leading to the formation of capillaries and a granuloma in the wound. During the remodeling phase, the cells produce native hyaluronic acid and it is this that binds to CD44 stopping the signaling pathway leading to stop the proliferation of endothelial cells.

### 1.3.2 Immune response regulation

It is unclear if these low molecular weight of hyaluronic acid fragments induce an inflammatory response or are a consequence of inflammation. It has been reviewed that oligomers and LMW of HA regulate the immune responses and are involved in migration of activated dendritic cells such as Langerhans cells (97). They are stimulators of pro-inflammatory cytokine synthesis through an immune-stimulant and antigenic effect. They act as pro-inflammatory mediators, stimulate Toll-like-receptor 4 (TLR4), activate the nuclear factor NF-kappa B in synergy with interferon  $\alpha$  (IFN $\alpha$ ), and lead to the synthesis of IL8 and IL10 (88, 98). They are also reported to become damage-associated molecular pattern molecules (DAMPs) inducing inflammatory responses via CD44 and TLR4 *in vivo* and *in vitro* (99). Nevertheless, these latter points are controversial. Indeed, it has been shown that the various sizes of hyaluronan from different sources do not induce inflammatory responses on *in vitro* and *ex vivo* murine macrophages and dendritic cells, assessed by TNF- $\alpha$ , IL-1 $\beta$ , IL-12, CCL3 and other markers productions (100-102). In addition the inflammatory responses observed was linked to unpurified samples and contamination of endotoxin presents inside the hyaluronidase used, even if samples were treated with polymixin B (102). Furthermore, no leukocyte recruitment was observed *in vivo* after lung instillation in response to HA (103).

Concerning high molecular weight of HA, it is considered as anti-inflammatory. By inhibiting the activation of the nuclear factor NF-kappa B in wound healing process, Baeva et al. observed that monocytes and macrophages under different inflammatory stimuli and in presence of high molecular weight of HA suppressed IL-1 $\beta$  production (100). Indeed, HA macromolecules mask the membrane receptors of the cells involved in the inflammatory reaction, thus preventing their activation and contributing to the anti-inflammatory action (94, 104).

## 1.4 HA metabolism

### 1.4.1 Intrinsic synthesis

Hyaluronic acid is a molecule whose constant synthesis and degradation allow a multitude of biological processes to be precisely modulated. It is a finely regulated process to maintain tissue regulation and homeostasis.

The mechanism of synthesis of hyaluronic acid is a fast and efficient process. This polysaccharide is synthesized mainly in the membrane of dermal fibroblasts but also to a lesser extent in the membrane of epidermal keratinocytes. Hyaluronic acid is produced mainly on the surface of the fibroblasts by extrusion into the extracellular matrix in association with numerous receptors such as CD44. Unlike other GAGs, hyaluronic acid is not synthesized in the Golgi apparatus but on the inner surface of the plasma membrane of the cell by transmembrane proteins: the HAS (Hyaluronan synthases). The intrinsic production of HA is provided by three mammalian hyaluronan synthases: HAS1, HAS2 and HAS3 (105, 106). The sequence homology between the three HA is very high (about 80%) but these enzymes differ in their stability and in the length of the HA chain produced. Indeed, HAS1 and HAS2 produce a hyaluronic acid twice as long as HAS3.

In this way, long linear chains of HA are formed on the cell surface and play an essential role in the cell microenvironment in keratinocytes, fibroblasts, endothelial cells and cells of the immune system (107).

It has been reported that HAS plays different roles in the development according to the different tissues or cells types, synthesizing different sizes for different functions (108, 109). The different activations of HAS have been reported *in vitro* to be modulated by cytokines, growth factor or chemokines in homeostasis and diseases like rheumatoid arthritis. For example, TGF- $\beta$  activates HAS1 while it suppresses HAS3 activities in synoviocytes (110); and IL-1 $\beta$  and TNF- $\alpha$  induces HAS2 mRNA on lung fibroblasts (111).

Intrinsic hyaluronic acid is finely regulated leading to different size and proportion of HA dependent of the tissue. Indeed, in healthy condition, HA in human synovial fluid has an average molecular weight around 6 to 7 MDa, in normal blood serum is mainly relatively low

from 100 to 300 kDa, while in human normal skin the average of molecular weight is around 4 to 6 MDa (31).

#### 1.4.2 Extrinsic synthesis

Hyaluronic acid can be obtained by tissue extraction or produced by bacterial fermentation. Tissue extraction can be carried out from tissues containing large amounts of HA such as the rooster crests. With tissue extraction from animals, 10 000–15 000 monomeric units per chain corresponding to an average of 4–6 MDa can be extracted (79). Nevertheless, these methods have drawbacks such as cost and difficulty in purification, which increases the risk of allergies and infections.

Recombinant HA production has emerged as an alternative using Gram-positive and Gram-negative bacteria as hosts, including *Bacillus* sp, *Lactococcus lactis* and *Escherichia coli* (112-114). Bacterial fermentation is the most common process to produce HA (115). HA is synthesized in the cell membranes and excreted into the media to facilitate manipulation and purification. With this technique, some risks remain with the existence of a possible contamination by endotoxins (116, 117). Bacterially produced HA can vary in molecular weight, producing low molecular weight of HA to very high molecular weight, depending on the monomeric units per chain (79).

Then, some chemical modifications are carried out in order to increase the half-life of HA in the body or modify its rheological properties according to the desired indications (Figure 9) (118, 119).

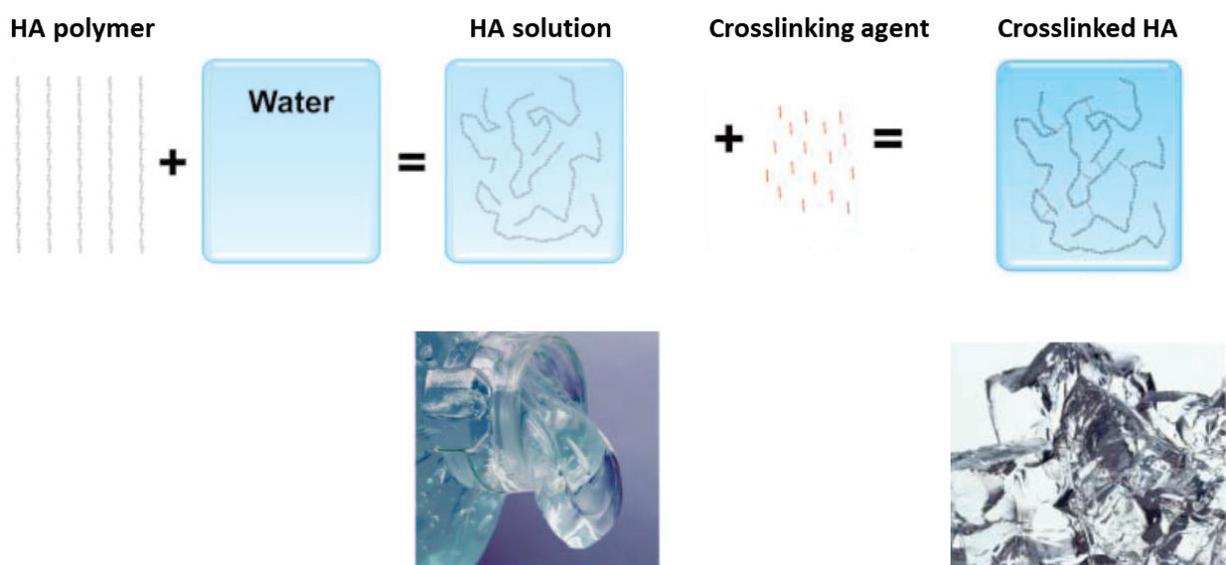


Figure 9: HA solution to crosslinked HA (Adapted from Tezel (79))

The main chemical modification is the use of crosslinking agents, inducing the formation of the gel producing a network of polymer, imposing a chemical and physical barrier to enzymatic and free radical breakdown. Indeed, enzymes and free radicals degraded then slowly crosslinked HA than native HA due to the large size of crosslinked HA and their difficulties to penetrate the gel (79).

#### 1.4.3 Degradation

In the human body, *in vivo* and *in vitro* experiments demonstrated that endogenous and exogenous HA were mainly degraded or altered inducing modulation of their physico-chemical properties and biological effects (77) according to different mechanisms:

- ⇒ Enzymatic degradation by hyaluronidases and  $\beta$ -glucuronidases and  $\beta$ -acetylglucosaminidases (120)
- ⇒ The action of hydroxyl radicals (121)
- ⇒ UV (122)
- ⇒ PH in solution (123)
- ⇒ Combination of sonication, ultrasound, H<sub>2</sub>O<sub>2</sub>, and copper Ion (124)
- ⇒ Heat (123, 125)

Hyaluronidases cleave the  $\beta$ 1-4 bond Hyaluronic acid and generate hyaluronic acid fragments on variable sizes. There are six genes in the human genome coding for hyaluronidases of varying tissue distribution (126). In humans, genes coding for hyaluronidases include HYAL-1, HYAL-2, HYAL-3, HYAL-4, HYALP1 and PH-20 and form two clusters on two different chromosomes (127). These enzymes are found in organs such as the spleen, skin, eyes, liver and in some body fluids.

HYAL-1 and HYAL-2 are the predominant isoforms to cleave HA and have ubiquitous expression. HYAL-2 begins by cleaving high molecular weight HA into intermediate sized fragments (about 20 kDa) that is mostly bound to the CD44 receptor. Its expression is limited to lysosomes. HYAL-1 cleaves the biopolymer into monosaccharides follow by lysosomal enzymes ( $\beta$ -glucuronidases and  $\beta$ -acetylglucosaminidases) leading to the formation of tetrasaccharides (800 Da) and then ending by single sugars formation (Figure 10) (127).

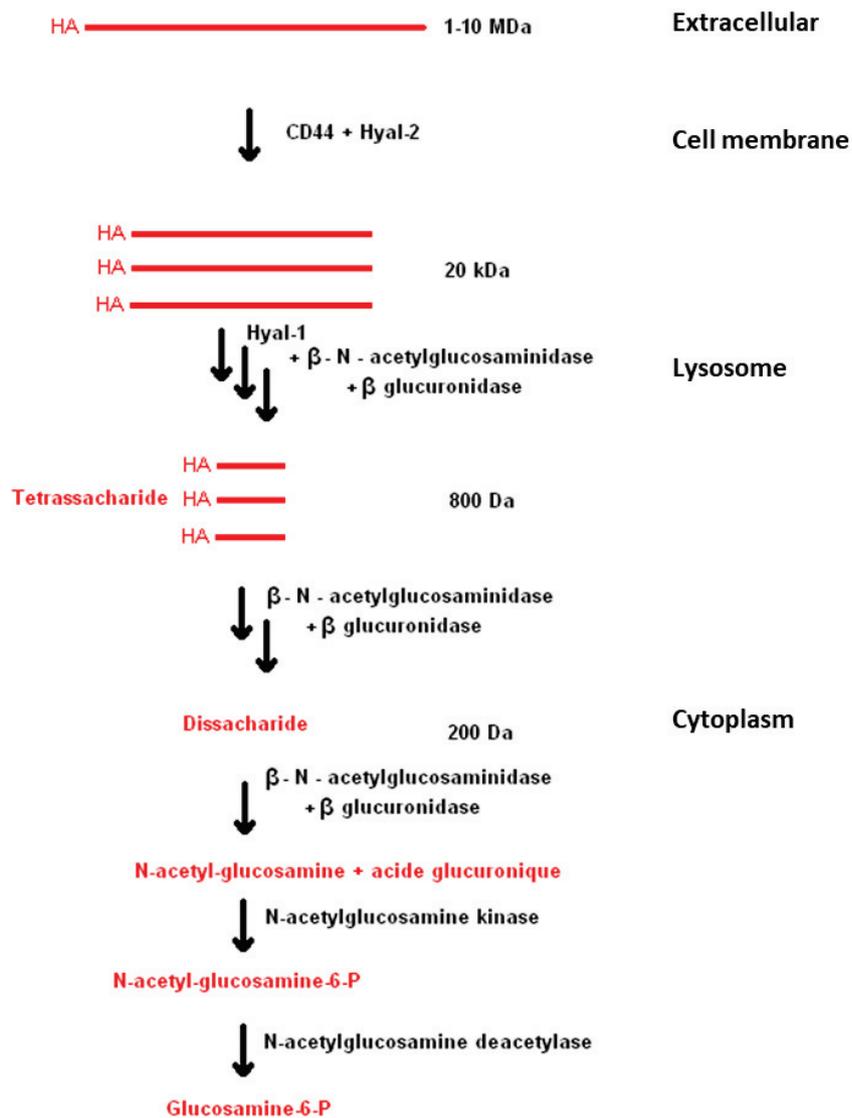


Figure 10: HA enzymatic catabolism (Adapted from (120))

The fragments of HA during the enzymatic process have been reported to induce transcription of MMPs (128) and stimulated endothelial cell activation (129) while oligomers (6 to 20 kDa) have been reported to be proangiogenic, pro-inflammatory and immune-stimulatory (130).

The role of HYAL3 in the degradation is not completely elucidated, but it seems to contribute to HA metabolisms by increasing the HYAL1 activity (131). PH-20 is a testicular enzyme encoded by the sperm adhesion molecule 1 (SPAM1), located on the human sperm surface and inner acrosomal surface. These enzymes are not totally specific to hyaluronic acid, HYAL4 can cleave chondroitin sulfate (127) for instance.

The progressive degradation of large polymers of hyaluronic acid by enzymatic reactions generates polymers of variable sizes. It has been reported that these different sizes of HA exert extremely diverse and often opposing biological functions. The mechanisms underlying this opposite signaling are still poorly defined but could involve competition for membrane receptors and induce inflammatory, immuno-stimulatory and angiogenic responses (132).

Nevertheless, hyaluronic acid can be degraded by other factors than enzymes like UV rays,  $\gamma$ , a combination of ultrasounds, ultrasound,  $H_2O_2$ , and copper Ion, by oxidation-reduction depolymerization (by hydrogen peroxide and or ascorbate) or by heat and/or under pressure (124, 133, 134).

The use of HA crosslinked as filler is also subject to be degraded. Crosslinking agents used to increase the HA stability in the skin have been shown not to interfere with the natural enzymatic process described above (135). In fact, it has been reviewed by De Boulle et al. 2013 that crosslinked HA have the same enzymatic, hydrolytic and oxidative degradation process as native HA in the body (92) .

## 1.5 HA Receptors

Secreted into the extracellular matrix, HA molecules and fragments are incorporated into the extracellular matrix and are capable of interacting with receptors in order to transmit different cellular signals and thereby influence the behavior of the cells, which could in turn modify their differentiation, migration and phenotypes (136, 137).

HA acts through its binding to various cellular receptors of the transmembrane type. CD44 is the main receptor, the most studied today. Its state of glycosylation is one of the elements conditioning its ability to bind with HA. The other receptors are: RHAMM, TLR (Toll-Like-Receptors) 4, HARE and LYVE-1.

### 1.5.1 CD44

CD44 is the major hyaluronic acid binding protein and its function has been extensively studied. CD44 is widely expressed in almost all types of cells in humans and mice. Within this family, there are many post-translational modifications that can modulate the affinity of CD44

to HA (138). The smallest isoform, without variant, is called "standard" CD44s and is expressed in most tissues. Its main role is that of receptor for hyaluronic acid. This molecule is involved in cell/cell and cell/matrix extracellular adhesion (139). It is involved in many biological processes, such as lymphocyte recruitment, hematopoiesis, embryogenesis and wound healing (140-142). 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, cancer and metastasis (143, 144) as well as cell migration (145). This receptor is essential for the inflammatory process in correlation with TLR4 in presence of lipopolysaccharides (LPS) (146-149). Furthermore, it has been shown that CD44 and its isoforms are overexpressed or involved in some inflammatory pathologies, such as cancer (150), asthma (151) or rheumatoid arthritis (152).

#### 1.5.2 RHAMM

The second major HA receptor is the Receptor for Hyaluronan Mediated Motility (RHAMM). RHAMM can be located on the cell surface, the cytoplasm, the nucleus, or the ECM. RHAMM is a functional receptor in many cell types including endothelial cells. In interaction with HA, RHAMM plays an important role in tissue injury and repair (153). Furthermore, it has been shown that RHAMM was not an absolute requirement for normal development (154) and RHAMM is not directly involved in the inflammatory process in presence of HA and HA fragments unlike CD44.

#### 1.5.3 Toll-Like Receptor 2 and 4

The TLRs are involved in the different inflammatory process. There are about 10 different TLRs. Among them, TLR2 and TLR4 have been identified as initiators of immune responses in presence of lipopolysaccharides, mycobacteria and gram-positive organisms (146, 155, 156).

Many types of cells have Toll-like receptors (TLRs) on their surfaces such as macrophages, dermal fibroblasts and endothelial cells (157). The activation of TLRs by binding to their natural ligand leads to signaling cascade resulting in the nuclear translocation of the NF- $\kappa$ B transcription factor, which modulates the transcription of different target genes. Thus, genes coding for pro-inflammatory chemokines and cytokines will be activated such as TNF- $\alpha$  and IL-6, resulting in the local recruitment of various cells such as polynuclear cells and macrophages.

The activation of TLRs also leads to the release of certain natural antimicrobial molecules and to an increased phagocytosis process of the cells.

Among all its interactions, TLR2 and TLR4 seem to be receptors of HA. Some studies show that in the absence of TLRs, and in the presence of HA fragments, there is no induction of cytokines and chemokines (158) and that HA oligomers induce dendritic cells maturation via TLR4 (130).

Del Fresno et al. showed that hyaluronic acid upregulates IL-1R-associated Kinase-M, a negative regulator of TLR signaling inhibiting IRAK4/1 complex, to deactivate human monocytes via CD44 and TLR4 (159). In a melanoma, small HA fragments stimulate expression of MMP2 and IL-8, leading to NF-κB activation, enhanced the motility of cells, in part in a TLR4-dependent manner (160).

Nevertheless, this part is controversial. In fact, there is no direct evidence that LMW of HA binds to TLR2 or TLR4, and some reports have suggested that the inflammatory response in the presence of fragments of HA was linked to samples contamination by endotoxin during the degradation process done *in vitro* using hyaluronidase (103).

#### 1.5.4 Other receptors

Other receptors are known to interact with hyaluronic acid such as: HARE and LYVE1 regulating lymphatic endothelial cells functions, Brevican and neurocan modulating neuronal development and brain tumor invasiveness and SHAP-1 and TSG-6 involved in osteoblastic differentiation, in fertility, in complement activation, injury and fibrosis (93). Below a summary of the principal HA receptor involved with HA (Figure 11).

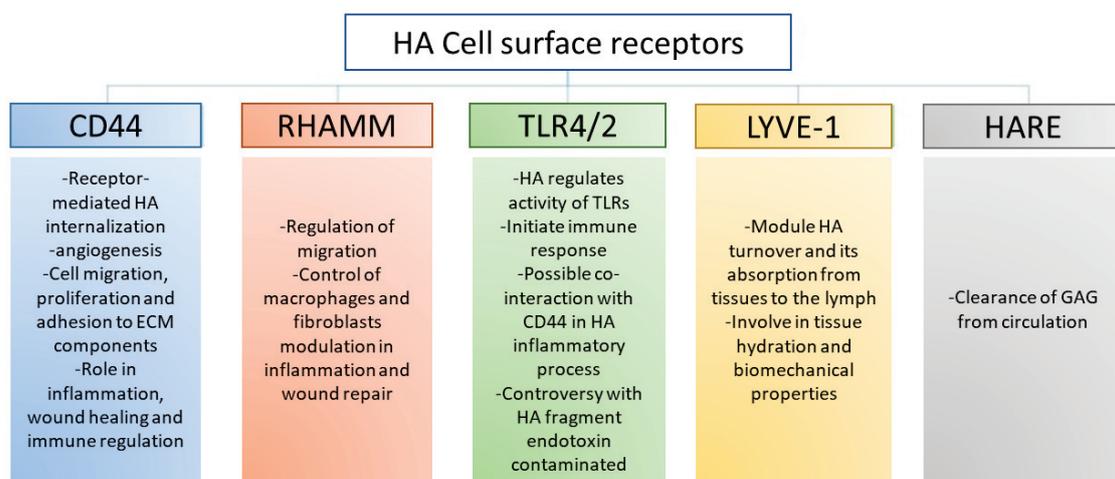


Figure 11: Summary of HA cell surface receptors and of the actions that they control when linked by HA (Adapted from (29))

## 1.6 HA as a medical device

### 1.6.1 History

With the pursuit of eternal beauty, injectable devices have been used over the last 100 years in order to counter the negative effects of aging. The first injectable devices were reported in 1893 by Neuber et al. with the autologous fat injection into the face following in the 20<sup>th</sup> century by the first injection of liquid paraffin by Gersuny (1844-1924). In the 1960s, liquid silicone became a product used as injected medical device for breast augmentation. But in 1964, Weiner showed that this type of injection induced granuloma and other complication inducing sequelae. Then collagen injection (Zyderm) has been approved by the FDA in 1981 to improve age-related wrinkles using bovine collagen, becoming the first facial filler approved for aesthetic. Injectable soft tissue fillers are classified by FDA on different characteristics, including site of placement (ie, dermal or subcutaneous), animal versus nonanimal derivation (eg, autologous, xenograft, semisynthetic, synthetic), and duration of effects. Meanwhile, hyaluronic acid was preferred as collagen because the latter present a certain number of complication and present a better and longer clinical correction (161, 162). Since its approval by the FDA in 2003 as aesthetic use, HA remains the most widely used material for soft tissue filler, combining safety, a long duration of clinical action and a capacity to modulate its rheological properties.

### 1.6.2 Crosslinked HA and its properties

Crosslinked hyaluronic acid is a biocompatible, viscoelastic, and hygroscopic compound (163, 164). One of the main properties of HA is its capacity to surround itself with water molecules, being a highly hydrophilic polymer, preventing dehydration and maintains skin structure. These properties can be modulated by the length, the molecular weight and the different degree of polymerization of HA (79) and give different rheological properties, clinical indications and durations depending on the crosslinking (119, 165). Their characteristics can be modulated depending on the cross linker used, its degree of crosslinking, the gel hardness, its consistency and the total HA concentration, which is the amount of HA per milliliter of finished product. All these characteristic modulations change the lifetime of HA in the skin (79, 166).

According to its capacity to have different rheological properties and actions, hyaluronic acid has been studied in many fields in order to understand its action on the organism, its metabolic role but also its interest in therapeutics and corrective aesthetics. Its properties give the possibility to develop a large variety of filler.

### 1.6.3 Complication associated with HA dermal filler

The demand for filler injections as aesthetic treatments is growing rapidly and complications are increasing with this growth in both the number of practitioners or nurses and the number of patients.

Serious complications are very rare and for the most part they are due to technical errors such as the injection technique, the depth of injection or an error in the choice of site to be injected, the volume injected or the lack of aseptic method, and some rare side effects are associated with the products: wrong indication, non-compliance contraindications, (167).

The reversibility of the dermal filling effect of HA is one of the reasons why this treatment mainly used. They correct wrinkles in a reversible manner, as a hypothetical medical error or complication can be remedied through the injection of hyaluronidase HYAL (such as Hylenex<sup>®</sup>, Halozyme Therapeutics), inducing the HA degradation (168). Two types of complications have been observed: the early adverse events occurring less than 24h to few weeks after injection and the delayed adverse events occurring 1 month to 1 year after injection (169, 170). All these complications have been classified and a management of Delayed-onset Adverse Reactions with the different approaches have been proposed by Heydenrych et al. in 2018 to avoid, limit or treat the different complications (170). The process to manage and limit complications is divided in 10 parts, reflecting the patient factors (evaluate the contraindication), the product used (Reversibility of the product, its characteristic), the procedure before and after injection (technical skills), and the algorithms and protocol to carry out in case of complications describing the different treatment for early or delayed complications (Table 3).

I. Patient	II. Product	III. Procedure	IV. Algorithms
<b>I. History</b>  <ul style="list-style-type: none"> <li>• Skin conditions</li> <li>• Systemic disease</li> <li>• Medication</li> <li>• Procedures</li> </ul>	<b>4. Reversibility</b>  <ul style="list-style-type: none"> <li>• Hyaluronidase</li> <li>• Dilution + dosage</li> </ul>	<b>7. Photographs</b>  <ul style="list-style-type: none"> <li>• Pre and post</li> <li>• Black background</li> <li>• No makeup</li> <li>• Rest and animation</li> <li>• Angles and lighting</li> <li>• SLR camera</li> </ul>	<b>Allergy/hypersensitivity</b> <ul style="list-style-type: none"> <li>• Early: vitals, adrenalin, IV</li> <li>• Late: cold compress</li> <li>• H1 + H2 antagonists</li> <li>• Oral corticosteroids</li> <li>• Propanolol, ibuprofen</li> </ul>
<b>Selection</b>  <ul style="list-style-type: none"> <li>• Skin + systemic</li> <li>• Pretreatment</li> <li>• Dental, vaccinations</li> <li>• Routine procedures</li> <li>• Dysmorphophobia</li> </ul>	<b>5. Product characteristics determine placement</b>  <ul style="list-style-type: none"> <li>• HA concentration</li> <li>• Cross-linking</li> </ul>	<b>8. Procedural planning and aseptic technique</b>  <ul style="list-style-type: none"> <li>• Technical flow</li> <li>• Everything at hand</li> </ul>  <ul style="list-style-type: none"> <li>• Skin: chlorhexidine/alcohol</li> <li>• Chlorhexidine mouthwash</li> <li>• Gloves + hand washing</li> </ul>	<b>Vascular events</b> <ul style="list-style-type: none"> <li>• HDPH</li> </ul> If visual disturbance occurs: a) Stop injecting b) Consult an ophthalmologist c) 90-minute timeframe <b>Infection</b> <ul style="list-style-type: none"> <li>• Oral antibiotics</li> <li>• IV antibiotics</li> <li>• Hyaluronidase</li> <li>• Surgical drainage</li> <li>• Laser</li> </ul>
<b>2. Assessment</b>  <ul style="list-style-type: none"> <li>• Beauty, aging, gender, ethnicity</li> <li>• Wants vs needs</li> </ul>	<b>6. Product layering</b>   <ul style="list-style-type: none"> <li>• HA over minimally biodegradable fillers</li> </ul>	<b>9. Injection anatomy</b>  <ul style="list-style-type: none"> <li>• Safety by depth</li> <li>• Danger areas</li> </ul>	<b>Late-onset nodules</b> <ul style="list-style-type: none"> <li>• Antibiotics</li> <li>• Hyaluronidase</li> <li>• Immunosuppressives</li> <li>• Tacrolimus</li> <li>• Pimecrolimus</li> <li>• Colchicine</li> </ul>
<b>3. Consent</b>  <ul style="list-style-type: none"> <li>• Informed consent</li> <li>• Procedure + complications</li> <li>• Financial consent</li> </ul>		<b>10. Technical knowledge</b>  <ul style="list-style-type: none"> <li>• Depth, placement</li> <li>• Speed</li> <li>• Needle vs cannula</li> <li>• Aspiration vs movement</li> </ul>	

Table 3: Plan to avoid filler complications (Adapted from (156))

### 1.6.3.1 Early complications

Ecchymosis, hematoma, edema are the common early complications observed with no gravity and resorbable in time. Edema can be post-traumatic with immediate post-injection. More frequent in certain areas HA such as the lips, it disappears in less than a week. But edema can also correspond to an immediate hypersensitivity reaction (169).

Vascular complication is exceedingly rare, it's one of the most serious related to an intra-arterial injection that can lead to necrosis of the skin tissue. Nevertheless, the risk of vascular accident would be approximately 0.05%. The main treatment is the use of hyaluronidase in this case, upon injection, hyaluronidase diffuses widely into the subcutaneous tissue and dissolves intravascular hyaluronic acid (167). Some early complications can appear after few days or weeks such as bacterial infection, inflammatory and non-inflammatory nodules (167).

### *1.6.3.2 Delayed complications*

Late-onset inflammatory reactions are rare complications. Late-onset nodules (4 weeks to > 1-year post-treatment) most likely occur due to inflammatory or biofilm-related causes (171). These nodules could be related to prolonged mechanical irritation with the product or to the presence of harmless bacteria before injection. The binding of non-virulent infectious agents at the time of the procedure may result in an immune response via the TLRs (169, 170).

Furthermore, these different late-onset adverse effects could be explained by different process. Some HA that are highly cross-linked such as Restylane® or have large or short particle sizes have a slow resorption (12-18 months), which would favor chronic encapsulation reactions. This is due to the isolation and limitation of foreign body migration that cannot be rapidly eliminated by phagocytosis or enzymes. The organism thus proceeds to the formation of a capsule of monocytes and macrophages secreting pro-inflammatory cytokines leading to the formation of more or less inflammatory nodules or granulomas (167, 172). Nodules are likely to be due to product misplacement or migration and associated chronic immune-inflammatory reaction and possibly low-grade bacterial infection (173). Nevertheless, another aspect is poorly understood link to the difficulty to diagnose auto-immune diseases on patients. In fact, filler treatments are not recommended in active autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, or mixed connective tissue disease (169).

**Among these auto-immune diseases, a new concept is emerging. These different auto-immune diseases have in common a low-level inflammatory microenvironment called low-grade. However, it has not been identified whether the autoimmune disease occurs due to low-grade inflammation or whether the low-grade inflammation is related to the disease.**

# Objectives

Delayed adverse effect of HA-based fillers are poorly understood, but with the increasing number of treatments, and thus the increasing number of delayed adverse effects observed, there is a real need to more investigate the causes and pathways that leads to these late adverse events. The scope of this work was to evaluate in partnership with AbbVie (Legacy Allergan) whether the onset of these late complications was related to the degradation HA based fillers products with the formation of low molecular weight of HA and/or related to an inflammatory microenvironment that could induce inflammatory or vascular reaction with the products. Because of the differences in adverse event rate observed with two different crosslinking technologies products, i.e. HYLACROSS vs. VYCROSS part of this evaluation was also to compare HA-based fillers that were manufactured using these 2 technologies.

We hypothesized a possible altered phenotype leading to these rare complications. We focused on low-grade inflammation, which is poorly studied. Inflammatory microenvironment could induce inflammatory or vascular reaction with the product persistent inflammatory state could affect microvascular network and dermal skin metabolism with a focus on the potential inflammatory responses to low molecular weight of crosslinked HA. The objectives was to asses inflammatory effect on immune cells, preconditioned dermal skin cells and in an inflammatory *in vivo* model.

**In order to achieve these goals, this thesis work is divided in three parts:**

**Part I:** The characterization size of hyaluronic acid fragments, to produce low molecular weight of fragments using human hyaluronidase endotoxin free.

**Part II:** Assess the effect of low-grade inflammation on vascular network and its impact on dermal skin cells. Follow by dermal skin cells and immune cells responses to different range size of fragments coming the two technologies in an inflammatory microenvironment.

**Part III:** Evaluate *in vivo* the vascular modulation in response to HA and its degradation products in a low-grade inflammation murine model.

## Materials and methods

The "materials and methods" section presented here is written in the form of detailed protocols to facilitate the understanding of the experiments and the reproduction of the methods by the future students and members of the host team.

## 1.7 *In vitro* experiments

### 1.7.1 Immune cells from THP-1 lineage

#### **Material for THP1 and Macrophages M1 culture**

Complete culture medium: RPMI 1640 supplemented with 1% W/S (5mL), 10% inactivated BSF (50mL), 4.5 g/L glucose, 10 mM HEPES (5mL), 1.0 mM sodium pyruvate (500 uL) and supplemented with 0.05 mM 2-mercaptoethanol (500 uL). Cells: THP-1 ATCC (TIB-202) Acute Monocytic Leukemia Human.

#### **Complements:**

- Flask T25 (25cm<sup>2</sup>) and/or T75 (75cm<sup>2</sup>) and/or 6-well plate
- RPMI 1640 (Roswell Park Memorial Institute medium) (500mL) (Thermofischer #11875093)
- HEPES BUFFER 1M (Thermofischer #15630056)
- 2-MERCAPTOETHANOL (Thermofischer #31350010)
- SODIUM PYRUVATE 100 mM (Thermofischer #11360039)
- D-glucose (powder) (Sigma-Aldrich #D9559-100G)
- PMA (Sigma-Aldrich #P8139-1MG) aliquot at 1mg/mL (50uL)
- LPS via aliquot at 5mg/mL (40uL)
- IFN gamma via aliquot at 100 pg/mL (5ug/mL)

#### **Procedure for culture cells**

- ⇒ Upon receipt, examine the culture for macroscopic evidence of any microbial contamination. Use an inverted microscope and carefully check for evidence of microbial contamination.
- ⇒ Incubate the flask for several hours at 37°C. Once the temperature is equilibrated, remove the entire contents of the flask and centrifuge at 125 x g for 5 to 10 minutes. Resuspend the cell pellet in 10 ml of medium.

- ⇒ From this cell suspension, count the number of cells using a Malassez cell. In a 96-well plate, dilute 50 $\mu$ L of cell suspension at  $\frac{1}{2}$  with 50 $\mu$ L of trypan blue.
- ⇒ Adjust the cell density of the suspension to  $4 \times 10^5$  viable cells/mL.
- ⇒ Incubate at 37°C at 5% CO<sub>2</sub>. Culture of THP1 should not exceed  $1 \times 10^6$  cells/mL. After thawing the ampoule, it is recommended to culture THP1 in 75cm<sup>2</sup> flasks.
- ⇒ The medium should be changed every 2 days.

#### **Procedure for frozen cells**

- ⇒ Thaw the cells by gentle agitation in a water bath at 37°C. To reduce the possibility of contamination, keep the cap out of the water. Thawing should be rapid (no more than 2 minutes).
- ⇒ Remove the vial from the bath as soon as the contents have thawed and decontaminate with 70% ethanol. All operations from this point on should be carried out under sterile conditions.
- ⇒ Transfer the contents of the bottle to a centrifuge tube containing 9.0 ml of complete culture medium RPMI 1640. Centrifuge the 15mL Falcon tube containing cells and medium at 125 x g for 5-7 minutes.
- ⇒ Remove the supernatant
- ⇒ Resuspend the cell pellet with complete medium and inoculate into a 25 cm<sup>2</sup> culture flask.
- ⇒ It is important to avoid excessive alkalinity of the medium during cell recovery. It is suggested that before adding the contents of the ampoule, the complete culture medium be placed in the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).
- ⇒ Incubate the culture at 37°C 5% CO<sub>2</sub>.

#### *1.7.1.1 THP1 to M1 polarization and mature Dendritic cells*

##### **Macrophages M1:**

To differentiate and polarize THP1 into M1 macrophages,

- ⇒ Seed 200,000 cells/mL are into a 6-well plate (4mL) or T25 flask (8mL) and
- ⇒ Stimulate cells with 100 ng/mL PMA.
- ⇒ Incubate for 24 hours to cause THP1 differentiation

- ⇒ Cells adheres becoming macrophages M0.
- ⇒ Wash cells twice with PBS.
- ⇒ Stimulate cells with 100 ng/mL of LPS, 100 ng/mL of PMA and 20 ng/mL of IFN- $\gamma$  for 66h.
- ⇒ Remove the medium.
- ⇒ Wash cells twice with PBS.
- ⇒ Add complete medium for 24h.
- ⇒ Macrophages polarize into M1 (174).

M1 phenotypes is then evaluate on gene expression M1 Markers such as CD68, CD64, TNF- $\alpha$ , IL-6, IL-8 and IL-1 $\beta$  to validate the inflammatory phenotypes.

### **THP1 to mature dendritic cells**

To differentiate THP1 into Mature Dendritic Cells (mDC) according to Berges et al. 2005 (175):

- ⇒ 800,000 cells are inoculated in a 6-well (4 mL) plate
- ⇒ Add 200 ng/mL of IL-4, 100 ng/mL of GM-CSF, 200 ng/mL of ionomycin (Sigma) and 20 ng/mL of TNF- $\alpha$ .
- ⇒ Pay attention to use medium FBS-free.
- ⇒ Incubate cells for 4 days and change medium with new fresh medium completed every 2 days.
- ⇒ After 4 days, wash the cells twice with PBS
- ⇒ Add medium supplemented with 10% SVF.
- ⇒ The morphotype of the dendritic cells can be kept for 2 weeks.

Mature dendritic cells (mDC) phenotypes is then evaluated on gene expression mDC Markers such as CD206, Transglutaminase 2 (TGM2), TNF- $\alpha$ , IL-6, IL-8 and IL-1 $\beta$  to validate the inflammatory phenotypes and according to Berges et al. 2005 (175).

### 1.7.2 Effect of crosslinked HA and degradation product on M1 and mDC

The objective of this experiments was to evaluate the inflammatory reaction to vehicle, LMW of VYC-15L, VYC-15L, LMW of HYC-24L+, HYC-24L+ and LPS as positive control for 24h, 48h and 72h. Three different range sizes of low molecular weight of fragments were analyzed for each filler. These 3 ranges were obtained by a short time degradation (30 minutes), medium time degradation (2 hours) and a long-time degradation (6 hours) by human hyaluronidase. The

cytokines release by mDC and macrophages M1 were then quantified by Luminex assay on IL-8, TNF- $\alpha$ , IL-1 $\beta$  and proinflammatory chemokine CCL5/RANTES mainly studies in inflammation with HA fragments.

#### *1.7.2.1 Enzymatic degradation of HA*

##### **Objective:**

Volbella (VYC-15L) and Ultra Plus XC (HYC-24L+) gels are degraded by enzymatic degradation (human hyaluronidase named HYLENEX at 150 IU/mL) for 30 minutes, 2 hours or 6 hours and then analyzed by SEC-MALS to determine the different range size of HA fragments obtained.

##### **Sample preparation**

- ⇒ In a 10 ml glass tube
- ⇒ Weigh 400 mg of each gel
- ⇒ Add 1 000  $\mu$ l of PBS + 100  $\mu$ l of enzyme solution
- ⇒ For these first two steps, make sure to weigh the gel and introduce the solutions directly into the bottom of the tube, avoid putting any on the walls.
- ⇒ Do not shake the vortex, only by hand.
- ⇒ Place the tube in the water bath at 37.0 °C for the degradation time defined for this tube (30 minutes (short time degradation), 2 hours (medium time degradation) or 6 hours (long time degradation)).
- ⇒ At the end of the degradation time, remove the tube from the bath.
- ⇒ Put in an oil bath, immediately, for 10 minutes at 100°C to inhibit the action of the enzyme.
- ⇒ Remove from the bath and allow to return to room temperature in a cold-water bath.

#### *1.7.2.2 HPLC analysis*

High-performance liquid chromatography (HPLC) allows separation, purification, identification, and quantification of one or more compounds from a HA solution. HA fragments produce after degradation were analyzed by steric exclusion chromatography (steric exclusion column: Shodex OHPak SB-806M-HQ reference F6429106 and a precolumn Shodex OHPak SB-G 6B reference F6709430) (HPLC with refractometer) by Allergan, France.

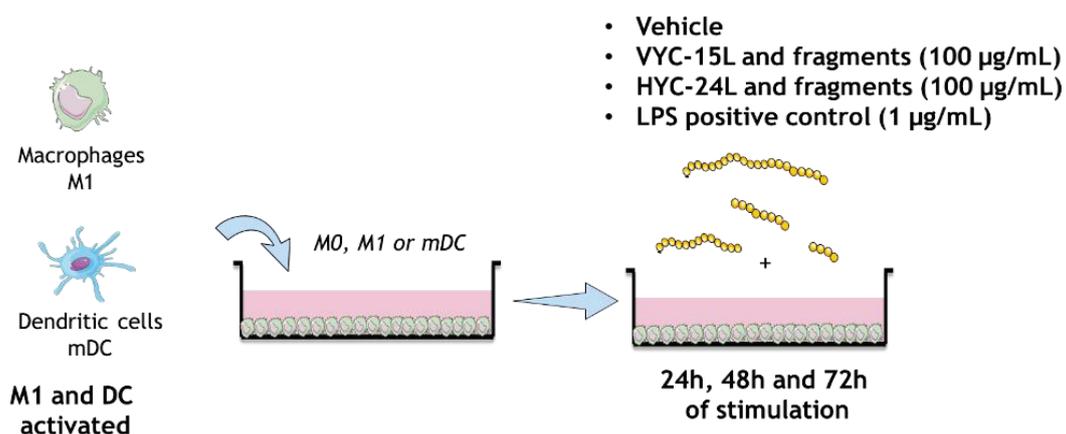
The analyses were performed with an Alliance Waters 2690 HPLC and using a Dawn Heleos 2 Light Scattering Detector (MALS) from Wyatt Technology.

HPLC permits to have the different molecular mass distribution (kDa), the different size of HA fragments obtained (kDa), the different polydispersity and the percentage of degradation of each condition.

### 1.7.2.3 Cells stimulation with HA products

#### Protocol:

- ⇒ One day before, HA fragments are prepared following “Enzymatic degradation of HA” protocol
- ⇒ After cells differentiation into mDC and polarization into macrophages M1, cells are washed twice with PBS.
- ⇒ Prepare solution at 100  $\mu\text{L}/\text{mL}$  of HA for each condition
- ⇒ Prepare LPS solution as positive control at 1  $\mu\text{g}/\text{mL}$  in order to induce an inflammatory response on immune cells.
- ⇒ Immune cells are then stimulated during 24h, 48h or 72h according to the following conditions:



#### Immune cells stimulation with HA

- ⇒ After 24h, 48h and 72h of stimulation, recover the supernatant of each conditions.

- ⇒ Collect and centrifuged at 10000  $\times g$  for 10 min at 4°C the conditioned medium recovered
- ⇒ Recover the supernatant purify, in a new Eppendorf tube and store at -80°C until analysis by Luminex assay.

### 1.7.3 Isolation and culture of the dermal fibroblasts

Human dermal fibroblasts were provided by the Cell and Tissue Bank (Hospital Edouard-Herriot, Lyon, France). Normal breast and abdominal human skin tissue explants were obtained from the surgical discard of anonymous healthy patients with informed consent of adult donors in accordance with ethical guidelines (French Bioethics law of 2004) and declared to the French research ministry (Declaration no. DC-2008-162 delivered to the Cell and Tissue Bank of Hospices Civils de Lyon). Exclusion criteria were related to positive test result for hepatitis B or C, or HIV as well as obesity history

Fibroblast isolation is performed according to the following protocol:

- ⇒ Transfer biopsy into falcon 50 mL and add Betadine® to tubes.
- ⇒ Incubate for 15 minutes at RT
- ⇒ Prepare solution composed by dispase at 20U/mL (Dispase II; Roche diagnostics) + 5mL of Penicillin/Streptomycin (P/S) (10 000U/mL) (Gibco) + 5 mL of Trypsin (0.02 mg/mL) (Gibco) and 7,6mL of Dubelcco's Modified Eagle's Medium (DMEM; Gibco)
- ⇒ Transfer the biopsy into petri dish and wash with Phosphate-Buffered Saline (PBS) (Gibco)
- ⇒ Remove adipose tissue
- ⇒ Place the biopsy, epidermis side up, and add the solution previously prepared with the dispase overnight at +4°C.
- ⇒ The next day, separate the epidermis from the dermis.
- ⇒ Place biopsies into 6-well plates
- ⇒ Lightly soak the dermis with one or two drops of Fetal Bovine Serum (FBS), this will allow better adhesion of the skins.
- ⇒ Incubate at 37°C 5% CO<sub>2</sub> for one night.
- ⇒ Add 1 ml of DMEM supplemented with 10% of FBS and 1% of P/S.
- ⇒ Incubate at 37°C 5% CO<sub>2</sub>.

- ⇒ The next day add 2ml of medium.
- ⇒ The medium is then changed every 2 days.
- ⇒ According to this protocol, fibroblast should begin to emerge from the explants after 3-4 days of culture to colonize the wells.

Human dermal fibroblasts were cultured in a medium consisted in a ratio 1 :1 of DMEM with Glutamax and F-12 medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% FBS (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and 1% penicillin/streptomycin (Gibco Life Technologies). Every 2 days, medium is changed until used. Dermal fibroblasts were used below 20 population doubling level.

#### 1.7.4 HDMEC culture

Human dermal microvascular endothelial cells (HDMEC) from adult donors (Promocell Heidelberg, Germany) were cultured at 37°C 5% CO<sub>2</sub> in the endothelial cell medium from Promocell composed by Dulbecco's Modified Eagle's Medium (DMEM) containing heparin 90µg/ml, hydrocortisone 1µg/ml supplemented with 2% Fetal Bovine Serum (FBS), 0.4% Endothelial Cell Growth Supplement (Promocell) and Epidermal Growth Factor (recombinant human) at 10ng/ml. Complete medium was supplemented with 1% penicillin/streptomycin (Gibco, Life Technologies, Carlsbad, CA, United States) at 37°C and 5% CO<sub>2</sub>.

HDMEC must be seeded between 5000 and 10 000 cells/cm<sup>2</sup>. Pay attention to this cell type because the phenotype of the cells can change rapidly. In case of morphotype change, thaw a new ampoule. Do not exceed 10 population doubling level.

#### 1.7.5 Preconditioning skin cells

To induce an inflammatory phenotype on HDMEC and dermal fibroblast, different inflammatory stimulations have been performed.

##### *1.7.5.1 Effect of HDMEC condition medium on dermal fibroblast behavior*

The first inflammatory stimulation on HDMEC was performed, using an inflammatory cocktail composed by a mix of cytokines: recombinant human IFN-γ, recombinant human TNF-α, and recombinant human IL-1β (Promokine; Heidelberg, Germany) at 10 IU/mL or 100 IU/mL for 24h. The following protocol has been performed for the study of the "Impact of Human

Dermal Microvascular Endothelial Cells on Primary Dermal Fibroblasts in Response to Inflammatory Stress” published in April 2019:

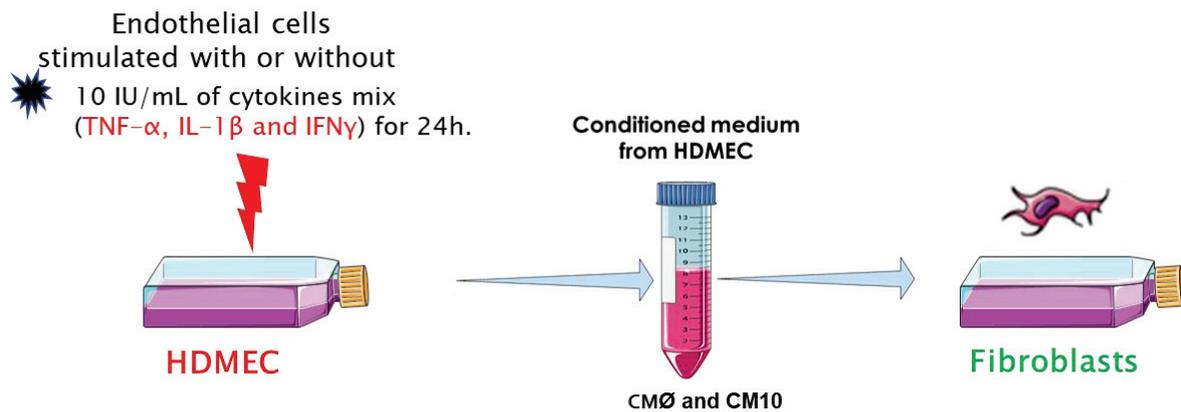
- ⇒ Seed HDMEC in a 75 cm<sup>2</sup> flask ( $4 \times 10^5$  cells/flask) 24 h before stimulation.
- ⇒ Extemporaneously, prepare the inflammatory cocktail at 10IU/mL
- ⇒ Wash the cells twice with HEPES buffer (Sigma)
- ⇒ Stimulate HDMEC with or without mix for 24h.
- ⇒ Conditioned media were then collected and centrifuged at  $10000 \times g$  for 10 min at 4°C.
- ⇒ Supernatant of each stimulation experiment was then collected and stored at -80°C until used.

The different condition media are named CM $\emptyset$  as control, CM10 as conditioned medium produced with 10 IU/mL cytokines mix (Figure 12) and CM100 produced with 100 IU/mL cytokines mix.

#### **Dermal cells stimulation**

- ⇒ After abdominal extraction, seed dermal fibroblasts in 6 well plates (200 000 cells/well) during 24 h for cell adhesion.
- ⇒ Wash the cells twice with PBS
- ⇒ Stimulate dermal fibroblasts with the different CM obtained (CM $\emptyset$  or CM10) during 48 h at 37°C 5% CO<sub>2</sub>.
- ⇒ Remove the medium
- ⇒ Wash the cells with fresh PBS
- ⇒ Lyze cells with the appropriate lysis buffer (RNA extraction or protein extraction)
- ⇒ For immunofluorescence, please refer to “Immunofluorescence” protocol.

Results were obtained from 3 independent experiments from 3 donors of fibroblasts.



### Effect of HDMEC condition medium on dermal fibroblast behavior

#### Human 3D full thickness skin models

- ⇒ Human 3D skin models are provided by LabSkin Creation
- ⇒ Do not use the skin before 41 days post maturation
- ⇒ Change the medium by CM100 for 5 days
- ⇒ Do not let the skin dry
- ⇒ At day 5, remove the medium and wash with PBS twice.
- ⇒ Put the 3D skin model on RNA later at -80°C until RNA extraction.
- ⇒ RNA extraction protocol is the same as “Mouse skin RNA extraction”

#### 1.7.5.2 Effect of crosslinked HA and degradation product on HDMEC and dermal fibroblasts

The second inflammatory stimulation on HDMEC and dermal fibroblasts, is the use of condition medium produced by macrophages M1 (CM M1). Dermal cells have been stimulated during 48h with complete medium of macrophages, diluted at 1/10 and 1/100 (Figure 12). The last dilution factors have been considered as a low-grade inflammatory condition.

#### Protocol:

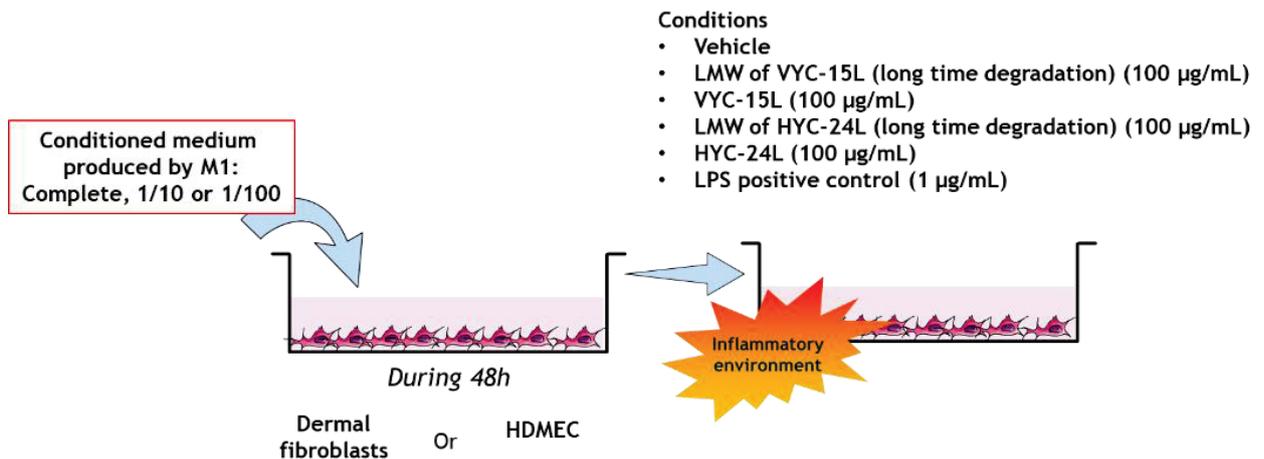
- ⇒ Seed HDMEC and dermal fibroblast (from breast biopsies) into 6 well plates ( $5 \times 10^5$  cells/well) for 24 h before stimulation.
- ⇒ Wash the cells with HEPES (for HDMEC) or PBS (for dermal fibroblasts)
- ⇒ Stimulate the cells with the three different concentration of condition medium produced by macrophages M1 for 48h.
- ⇒ After 48h of stimulation, remove the medium and wash cells.

- ⇒ Let the cells in rest phase for 24h.
- ⇒ Prepare the stimulation with the appropriate medium for each cell type according to the following table:

Conditions
1) Vehicle
2) LMW of VYC-15L (Long degradation time) at 100 µg/mL
3) VYC-15L at 100 µg/mL
4) LMW of HYC-24L (Long degradation time) at 100 µg/mL
5) HYC-24Lat 100 µg/mL
6) Positive control LPS (1µg/mL)

#### Dermal cells with crosslinked HA stimulation

- ⇒ Stimulate cells with the different condition for 72h (Figure 12)
- ⇒ After stimulation, wash cells 3 times with PBS or HEPES. Pay attention to remove all HA products.
- ⇒ Lyze cells with the appropriate lysis buffer (RNA extraction or protein extraction (See sections “Biochemical and molecular biology techniques”).



#### Effect of crosslinked HA and degradation product on HDMEC and dermal fibroblasts

## 1.8 In vivo and ex vivo experiments

These experiments were performed on healthy adult C57BL6/JRj male mice from Charles River. All experiments were conducted in accordance with European Union recommendations for animal experimentation and approved by Rhone-Alpes ethics committee and then by the Ministry of Research - Project Authorization Service (agreement #18529).

As soon as the mice arrived, they were randomly separated in cages with 5 animals per cage with access to water and food (animal facility EU0608 Lyon - agreement C 69 387 0601). Special effort was made to minimize the number as well as the stress and suffering of mice used in this study. Before experimentation, animals were acclimatized for 1 week in a regulated environment with a constant temperature of 24°C and a day/night cycle of 12h/12h. The animals were 6 weeks old with an approximate weight of 20 g at the beginning of the experiments.

### 1.8.1 Induction of a Low-grade inflammation induction

To study the effect of crosslinked HA in a low-grade inflammatory phenotype in mice, we developed a LPS model using super low dose of lipopolysaccharide (LPS). Inspired by the studies of Guo and Baker to develop the model (176, 177), 25 mice were used to validate the model, 5 mice treated with PBS (control) and 20 mice treated with LPS (low-grade inflammatory phenotype).

#### **Protocol**

##### **a) Treatment LPS vs PBS (6 weeks)**

- ⇒ Prepare the solution of LPS with stock solution at 1 mg/mL.
- ⇒ Dilute with PBS stock solution to obtain 5 ng /kg of LPS Solution (E. Coli O26:B6 (L2654)) for approximately 100 µL per i.p. injection (2 injections per mouse)
- ⇒ Use insulin syringe to perform intraperitoneal (i.p.) injections in mice (after 1-week acclimatization period)

The intraperitoneal injections of PBS for control group and LPS for low-grade inflammatory group were performed 3 times per week for 6 weeks. The mice were daily monitored throughout the process to ensure that no complications or pain occurred during treatment. Every week, mouse body weight was measured.

##### **b) Collection samples**

- ⇒ After 6 weeks treatment (PBS or LPS), anaesthetize the mouse under isoflurane on a warm plate at 35°C. Place the mouse in a 4% isoflurane induction chamber with an air flow rate of 2L/min and then under a 2% isoflurane mask with an air flow rate of 0.5L/min (TEMSEGA, Pessac, France).
- ⇒ Prepare the syringe with 23G needle with heparin sodium solution to avoid coagulation and erythrocytes lysis during the cardiac puncture.
- ⇒ Take blood (around 1 mL) from the heart, preferably the ventricle, which can be accessed either via the left side of the chest or through the diaphragm. Blood should be withdrawn slowly to prevent the heart collapsing. If blood does not appear immediately, withdraw 0.5 cc of air to create a vacuum in the syringe.
- ⇒ Collect then blood samples (volume?) using blood collection tubes (BD Vacutainers, Type 3: Lithium heparin)
- ⇒ Put the samples directly into ice and no more than 1 hour until plasma extraction
- ⇒ To collect plasma, centrifuge the tubes 15 min at 3000 rpm (1500×g), 4°C.
- ⇒ Collect the plasma from the top. The plasma is ready for analysis. If necessary, store at -20°C. Analyze plasma using Luminex assay.
- ⇒ Collect skin samples using bio-punch 8mm of diameter from the lower back of mice (depilated under isoflurane 2 days before)
- ⇒ Put Immediately the skin samples into cold RNA later (Sigma R0901), then stabilize samples at +4°C for up to 2 weeks or indefinitely at -80 °C until RNA extraction

### 1.8.2 Depilation

To study the skin by iontophoresis and to inject intradermally the different products, its necessary to remove all the back hair.

However, to study the vascular and inflammatory responses on skin mice, they were all depilated exactly 2 days before the intradermal injections to avoid any inflammation reaction due to depilation protocol.

### Protocol

- ⇒ Anaesthetize the mouse under isoflurane on a warm plate at 35°C. Place the mouse in a 4% isoflurane induction chamber with an air flow rate of 2L/min and then

under a 2% isoflurane mask with an air flow rate of 0.5L/min (TEMSEGA, Pessac, France).

- ⇒ Shave carefully the back hair closely
- ⇒ Spread Veet Cream over the shaved area and massage to distribute the cream evenly over the back.
- ⇒ Stop the contact with the skin for up to 30 seconds to limit inflammation.
- ⇒ Remove all cream with a moistened compress.
- ⇒ Dry the skin
- ⇒ Replace the mouse in its cage in the animal facility

### 1.8.3 Intradermal injection

All mice received different injections according to table 1 below 24h and 72h after the end of their treatment (PBS for control group and LPS for low-grade inflammatory group).

Different injections	
1) Volbella (VYC-15L)	} In control group (PBS) and low-grade inflammatory group (LPS)
2) LMW of VYC-15L (Long degradation time)	
3) UltraPlus (HYC-24L)	
4) LMW of HYC-24L (Long degradation time)	
5) Vehicle (use to degraded HA)	
6) Positive control LPS at 5 µg/mL	

**Different intradermal injection conditions**

### Protocol

- ⇒ LMW of HA (induced with 6 hours degradation) and vehicle solution have been prepared one day before (Enzymatic degradation of HA protocol)
- ⇒ Anaesthetize the mouse under isoflurane on a hot plate at 35°C. The animals are placed in a 4% isoflurane induction chamber with an air flow rate of 2L/min and then under a 2% isoflurane mask with an air flow rate of 0.5L/min (TEMSEGA, Pessac, France).
- ⇒ Prepare insulin syringe with the different solution non diluted except for LPS as positive control at 5 µg/mL.

- ⇒ Gently fold the skin of the lower back with forceps and gently insert the needle slowly in parallel to the skin to inject intradermally.
- ⇒ Do not exceed 20  $\mu\text{L}$  per injection
- ⇒ Check that a bubble has formed after injection to validate the correct injection
- ⇒ Pinch for a few seconds where the needle is retracted to prevent any liquid exit
- ⇒ Monitor the mice afterwards to prevent them from scratching on the injected area or being attacked by other mice.

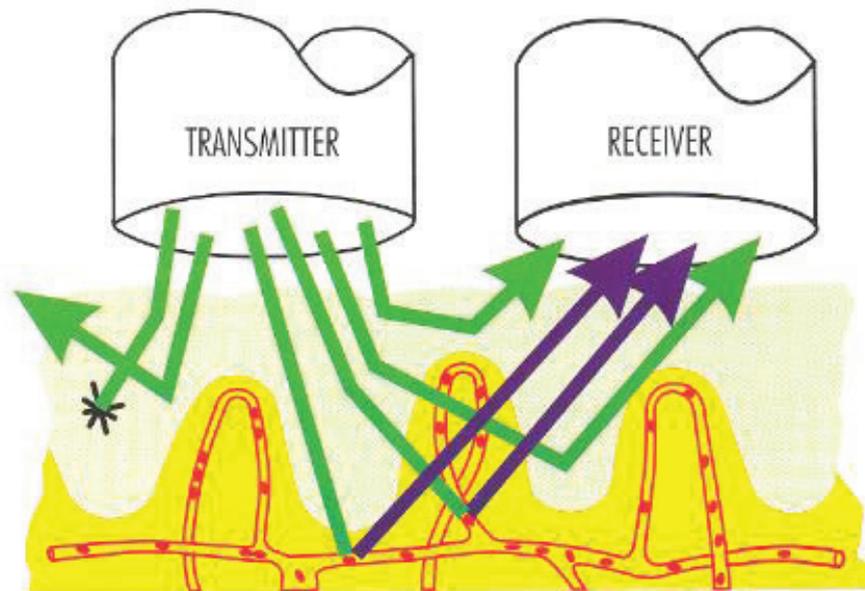
#### 1.8.4 Study of cutaneous vascular functionality on the back area

When assessing cutaneous vascular functionality, the animal is placed under general anesthesia. All mice were anaesthetized with thiopental ( $65 \text{ mg}\cdot\text{kg}^{-1}$ , i.p.). A stabilization period of 10 minutes is observed after anesthesia. The animal is then placed in an incubator (MP4SI, Mediprema) allowing control of the ambient temperature and maintenance of a stable skin temperature. The skin temperature is measured using a skin probe connected to a thermocouple (Physitemp BAT-12, Physitemp instruments Inc.) and is maintained at  $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  to limit temperature-related changes in blood flow.

Systolic blood pressure (SBP) is measured before and after blood flow measurements by a non-invasive tail system to verify cardiovascular stability during experiments. The systolic blood pressure is defined as the average of these measurements. The pressure and temperature measurements are recorded and processed using a multi-channel acquisition system (AcqKnowledge, Biopac Systems Inc.).

#### 1.8.5 Blood flow measurement

The Doppler laser permits to study skin microcirculation by measuring variations in blood flow. The Helium-Neon laser, with a wavelength of 780nm, emits monochromatic red light. This light beam of high intensity, unidirectional photons is emitted by a photon emitter/sensor where changes in blood flow are measured. The Doppler laser technique is based on the principle of the Doppler effect: the frequency of the photon is changed when it encounters a moving element. As a result, the emitted and reflected beams have different frequencies.



**The principle of laser Doppler flowmetry (Extracted from Perimed, Järfalla, Sweden)**

A transmitter produces laser light that is reflected in all directions without changing its wavelength when it meets fixed elements. When the light meets red blood cells, the wavelength of the reflected photons and therefore their frequency will be changed. The frequency varies proportionally to the speed of the red blood cells. The reflected laser light returns to the sensor and is transmitted by an optical fiber for analysis.

The Doppler laser probe is coupled with iontophoresis to record the blood flow responses induced by a pharmacological substance.

**1.8.6 Iontophoretic delivery of acetylcholine**

In this study we focused on the endothelial integrity of the vessels studied by the endothelium-dependent vasodilation in response to ACh. This approach allowed us to evaluate whether the different crosslinked hyaluronic acids and their fragments could induce vascular modulation. It has been shown that vasodilation in response to ACh administration predominantly involves the production of NO in humans and rats (178, 179). ACh iontophoresis provides information on the endogenous vascular function of NO. However, some studies have demonstrated the involvement of COX in ACh-induced vasodilation (179).

Iontophoresis is a non-invasive experimental tool allowing the diffusion of positively or negatively charged molecules through the skin (or other tissues) by the application of an electric power (180). This technique, coupled with the Doppler laser, permits to study

variations in cutaneous blood flow linked to the administration of locally delivered pharmacological substances (181). Measurements are carried out in the back area injected with different conditions (Table 3). A direct current increases the penetration rate of ionized compounds through the epidermal barrier and the study of the effect of pharmacological substances on skin microcirculation. Iontophoresis permits to study both dependent and independent endothelium vasodilatory responses (182) in response to pharmacological molecules in order to verify the integrity of the vasodilatory function of cutaneous microcirculation (183, 184).

The iontophoresis probe consists of an adhesive bonded to the surface of the Doppler laser probe (Periflux System 5000, Perimed AB, Järfälla, Sweden) and an iontophoresis patch is attached to the probe using a sticker. The foam of the patch is then soaked with 0.1 mL of a 2% ACh solution (Sigma-Aldrich, Steinheim, Germany,). The assembly is installed on the lower back of the mouse at the injected area and is connected to the corresponding stimulator (anodal current 0,1 mA).

#### 1.8.7 Collection skin samples and extraction

After sacrifice, two bio punch of 8 mm of diameter were carried out at lower back, at the site injected with HA products. One for histological analysis and the other was cut in half to protein and RNA extraction (See section “Biochemical and molecular biology techniques”).

Note that, site use for iontophoresis was recovered for histological analysis while the other site was recovered for protein and RNA extraction.

#### 1.8.8 Histology

Histological sections and labeling are performed by Novotec (Bron, France) in the framework of a collaboration. Skin samples are taken and fixed in AFA (CARLO ERBA Reagents S.A.S., Val de Reuil, France) for 24 hours at room temperature or at 4°C for a longer period and then included in paraffin. Sections of 5µm are marked with a Hematein Eosine Saffron (HES) stain for tissue morphology analysis. Vessels were labeled with two antibodies: anti-Col IV and anti-CD31 to assess vascular density. Mean area of vessels in each condition have been quantified using ImageJ. Two images of each skin section per mouse and per condition were used for the analysis.

## 1.9 Biochemical and molecular biology techniques

### 1.9.1 Endotoxin quantification by EndoSafe PTS

The solution used to degrade HA into fragments (Vehicle) and the Low molecular weight obtained after 6h of degradation were quantified by Endosafe PTS (Charles River). The Endosafe®- PTS (Portable Test System) is a rapid, point-of-use test system which is comprised of a test cartridge along with a hand-held spectrophotometer. The PTS provides quantitative LAL test results in approximately 15 minutes. For this test we have used cartridge with 0.01 EU/mL sensitivity (PTS2001F).

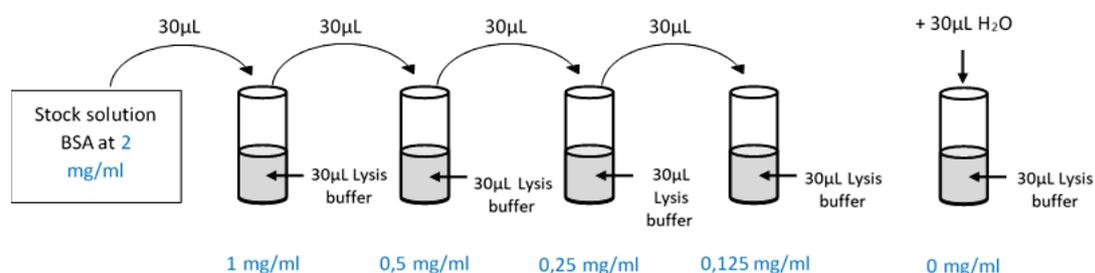
#### Protocol

- ⇒ Samples are diluted at 1/100. A positive control of LPS is used and diluted at 1/100000 (from stock solution at 1mg/mL).
- ⇒ Pay attention to used water certified endotoxin free.
- ⇒ Do not use cotton-filled pipette tips.
- ⇒ Before samples analysis, test the LAL water reagent endotoxin quantification with a new cartridge.
- ⇒ Inject 25µL of samples into the cartridge.
- ⇒ Start the read.

### 1.9.2 Protein assay protocol

Protein Assay with Pierce BCA Protein Assay Kit:

Standard range with albumin (stock at +4°C, Western-blot lab):



- ⇒ In a 96-well plate, dispense 25µL/well of each range point or sample to be tested.
- ⇒ Prepare the reaction mix A+B with B at 1/50th (for 10 wells: 40µL B + 1960µL A)
- ⇒ Dispense A+B mix: 200µL/well

- ⇒ Put a film on the plate and incubate 30min at 37°C.
- ⇒ Remove the film and remove the bubbles with a needle.
- ⇒ Reading the plate at 560nm

### 1.9.3 Western Blot

The following antibodies were used for WB : Rabbit polyclonal anti-collagen I (Novotec, Bron, France), Rabbit polyclonal anti-elastin antibody (Abcam, Cambridge, Royaume-Uni), Rabbit monoclonal anti-pro-LOX antibody (D8F2K, Cell Signaling), Rabbit polyclonal anti-β-actin antibody (ab8227, Abcam Cambridge, Royaume-Uni) and Rabbit polyclonal anti-CD44 (Abcam, ab157107). As secondary antibody Goat anti-rabbit IgG, HRP-linked antibody for western blot (Cell signaling).

#### Protocol

##### 1) Preparation of plates and electrophoresis system

- Alcohol plates
- Mount the plates on the rack

##### 2) Preparation of acrylamide/Bis acrylamide gels 37.5/1

- For the composition of migration and separation gels, generally, 10% acrylamide for medium size protein (60kDa) is recommended but decrease the concentration for a larger or increase for a smaller protein.
- NB: after having poured the migration gel, add 200µl of isopropanol to smooth the surface. After polymerization, remove the isopropanol.

##### 3) Sample Preparation

- Prepare samples to the desired concentration
- Add 1X of the SB charge buffer (SB stock at 5X, you have to add 25µL of DTT before use)

##### 4) Migration

- Denature the proteins by heating them to 95°C for 5 min.
- Migration buffer: TG-SDS 1X (stock TG-SDS 10X to be diluted in distilled water)
- Size marker: Precision Plus Protein Standards, Kaleidoscope (Bio-rad)
- Put 10µl size marker

- Migrate at 90V for 20 min to concentrate the samples in the concentration gel and then once in the migration gel increase to 150V.
- Allow to migrate until the deposition blue is at the bottom of the gel.

#### **5) Transfer**

- Cold transfer buffer: TG Ethanol 20% (stock TG 10X to be diluted in distilled water + Ethanol 20% (v/v))
- Soak the PVDF (polyvinylidene fluoride) membrane for 30 seconds in Ethanol under the fume cupboard to activate and waterproof it.
- Close the assembly
- Make the transfer in a cool place at 4°C
- Transfer at 220mA for minimum 1h

#### **6) Blocking**

- Block and wash buffer: TBS 1X-Tween 0.1% (stock TBS 10X to be diluted in distilled water)
- Prepare extemporaneously 20ml of TBS 1X-Tween 0.1% buffer with 5% milk.
- Incubate for 1 hour at room temperature.

#### **7) Primary antibody**

- Dilute the primary antibody in a solution of TBS 1X-Tween0.1% + milk 2%.
- Incubate overnight at 4°C or 2 hours at room temperature.

#### **8) Secondary antibody**

- Wash 3 times during 5 min in TBS 1X-Tween0.1% buffer (Tween reduces non-specific interactions)
- Dilute the secondary antibody in TBS 1X-Tween0.1% solution.
- Incubate for 1 hour at room temperature with agitation.
- Wash 5 times 5min in TBS1X-Tween0.1% buffer, with shaking.

#### **9) Chemiluminescence revelation**

- Incubate the blot 5 min with ECL then reveal to the fusion camera reader.
- Analyze image using Gel analyzer software to semi quantify the different bands obtained.

#### 1.9.4 Protocol Luminex R&D systems

For human samples analysis, multiplexing kits were composed by CCL2, VEGF-A, TNF- $\alpha$ , IL-8, IL-6, IL-1 $\beta$  and CCL5/RANTES detection Ab (LXSAHM). For mice plasma analysis multiplexing kits were composed by IL-6 and CCL2 (LXSAMSM).

Luminex<sup>®</sup> Assay Magnetic Multiplexing Kits are designed for use with the Luminex<sup>®</sup> MAGPIX CCD imager. Alternatively, the kits can be used with typical dual-flow sorting and detection platforms: Luminex<sup>®</sup> 200 or Bio Rad<sup>®</sup> Bio-Plex<sup>®</sup>.

The analyte-specific antibodies are pre-coated on color-coded magnetic microparticles. The microparticles, standards, and samples are pipetted into the wells and the immobilized antibodies bind the analytes of interest. After washing out all unbound material, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. After washing to remove any unbound biotinylated antibody, a streptavidin-phycoerythrin (Streptavidin-PE) conjugate, which binds to the biotinylated antibody, is added to each well. A final wash removes the unbound Streptavidin-PE, the microparticles are resuspended in buffer and read using the Luminex<sup>®</sup> MAGPIX analyzer. A magnet in the analyzer captures and holds the super paramagnetic microparticles in a monolayer. Two spectrally distinct light-emitting diodes (LEDs) illuminate the beads. One LED identifies the analyte being detected and the second LED determines the amplitude of the signal derived from PE, which is directly proportional to the amount of analyte bound. Each well is imaged with a CCD camera.

Use the standard concentrations on the certificate of analysis and calculate third party dilutions for the remaining levels. Average duplicate readings for each standard and sample and subtract the median mean fluorescence intensity (MFI). Create a standard curve for each analyte by reducing the data using computer software capable of generating a 5-parameter logistic curve fit (5-PL). If the samples have been diluted, the concentration read on the calibration curve must be multiplied by the dilution factor.

Nevertheless, this technology presents some limitations. Even if, Luminex is more sensitive than ELISA, it is not possible to analyze lysis cell or tissue. Only, plasma, serum and supernatant can be analyzed.

#### 1.9.5 ELISA assay

The concentrations of inflammatory and vascular markers were determined by ELISA assay (enzyme-linked immunosorbent assay) on cells and tissues lysate, according to the

manufacturer's specifications (Abcam). The human inflammatory markers assayed included: IL-6 (ab178013), IL-8 (ab214030) and IL-1 $\beta$  (ab46052) (Abcam). The mouse inflammatory and vascular markers assayed included IL-6 (ab100713), IL-1 $\beta$  (ab100705), VCAM-1 (ab201278) and COX-2 (ab210574) (Abcam). Pay attention to use T-PER as lysis buffer to carry out ELISA assay. Using the microplate reader (TECAN Infinite 200 PRO), record the OD (Optical Density) at 450 nm. Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values. Determine the concentration of the target protein in the sample by interpolating the blank control subtracted absorbance values against the standard curve. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.

#### 1.9.6 *In vitro* protein and RNA extraction

##### **Cell protein extraction**

Lysis buffer: T-PER, for ELISA assay (ThermoFischer Scientific) or RIPA (Sigma) supplemented with supplemented with a cocktail of protease inhibitors (Complete Mini, Roche Diagnostics) and phosphatase inhibitors (25X solution: 125mM NaF, 250mM -glycerophosphate, 25mM Na3VO4). T-PER lysis buffer was used to carried out ELISA assay while RIPA was used to performed Western Blot.

- ⇒ After cell stimulation, wash the cells twice with the appropriate buffer (PBS or HEPES) on the 6 well-plate
- ⇒ Add 200  $\mu$ L of lysis buffer in each well on ice
- ⇒ Use a scraper to lysis and detach all the cells
- ⇒ Recover cells lysate and centrifuge immediately at 10 000g during 10 min at +4°C
- ⇒ Recover the supernatant and stored at -80°C until protein quantification

##### **Cell RNA extraction**

The total RNAs were extracted from cells using Macherey-Nagel™ NucleoSpin™ RNA Plus XS Protocol:

- ⇒ Add 100  $\mu$ L of LB1 and vortex vigorously and use a scraper to lysate all cells. Carry out on ice.
- ⇒ Sample can be stored at -20°C until extraction.
- ⇒ Add 100  $\mu$ L volume of Lysis Buffer LB2 to the lysate and mix

- ⇒ Place a NucleoSpin® gDNA Removal Column XS(yellow ring) in a Collection Tube, transfer the homogenized lysate to the column, and centrifuge for 2 min at 100g followed by 10 s at 10 000g. Discard the column and continue with the flowthrough.
- ⇒ Add 150 µL Binding Solution BSXS to the flowthrough
- ⇒ Transfer the whole lysate to the NucleoSpin®RNA Plus XS Column and centrifuge for 1 min at 300g followed by 10 sec at 10 000g
- ⇒ Add 100 µL Wash Buffer MDB onto the column and centrifuge for 10 s at 10 000g. Discard the flow through
- ⇒ Add 500 µL Buffer WB2 onto the column and centrifuge for 10 s at 10 000g. Discard the flowthrough
- ⇒ Add 200 µL Buffer WB2 onto the column and centrifuge for 2 min at full speed ~20 000g to dry the membrane.
- ⇒ Add 20 µL of RNase-free H<sub>2</sub>O and centrifuge 1 min at 11 000g.
- ⇒ RNA purity and concentration are determined by spectrophotometry using a NanoDrop ND-2000 (Thermo Scientific).
- ⇒ Conserve RNA samples at -80°C until RT-qPCR.

#### 1.9.7 *Ex vivo* protein and RNA extraction

##### **Mouse skin Protein extraction:**

- ⇒ After skin sampling, put immediately samples in liquid nitrogen.
- ⇒ Store samples at -80°C.
- ⇒ The day of the protein extraction, immerge skin samples in Eppendorf containing T-PER (ThermoFischer Scientific) supplemented with supplemented with a cocktail of protease inhibitors (Complete Mini, Roche Diagnostics) and phosphatase inhibitors (25X solution: 125mM NaF, 250mM -glycerophosphate, 25mM Na<sub>3</sub>VO<sub>4</sub>).
- ⇒ Add to the tube two Stainless Steel Beads, 5 mm (Qiagen)
- ⇒ Lyze samples using Tissue Lyzer at 20Hz for 2 minutes. Repeat the operation 4 times
- ⇒ Gently remove the beads
- ⇒ Centrifuge tubes at 10 000g for 10 min at 4°C.
- ⇒ Recover the supernatant. If necessary, repeat the previous step.

⇒ Store samples at -80°C until protein quantification and ELISA assay.

#### **Mouse skin RNA extraction:**

⇒ Immerse skin samples in Eppendorf containing cold RNAlater (Sigma R0901), then stabilizes samples at +4°C for up to 3 weeks or indefinitely at -80 °C.

The total RNAs were extracted from mouse skin using the RNeasy Fibrous Tissue Mini Kit (Qiagen).

- ⇒ Thaw tubes containing RNAlater and skin at +4°C.
- ⇒ Prepare the lysis buffer: RLT Buffer + 1% β-mercapto-ethanol.
- ⇒ Add 300 µl of lysis buffer to the each tubes and two Stainless Steel Beads, 5 mm (Qiagen)
- ⇒ Lyze samples using Tissue Lyzer at 20Hz during 2 minutes. Repeat the operation 4 times
- ⇒ Gently remove the beads
- ⇒ Centrifuge tubes at 10 000g for 10 min at 4°C.
- ⇒ Recover the supernatant. If necessary, repeat the previous step.
- ⇒ Add 590µL of RNase free water into the tube, then 10µL of Proteinase K
- ⇒ Invert tube 2-3 times to homogenize
- ⇒ Incubate 10 min at 55°C
- ⇒ Centrifuge tubes at 10 000g for 4 min at RT
- ⇒ Transfer the supernatant into a new Eppendorf tube. Be careful not to pick up the base containing the debris
- ⇒ Add 450µL of 96-100% ethanol and transfer 700µL to the column
- ⇒ Centrifuge 30sec / 10 000g / RT, empty the bin
- ⇒ Transfer the rest of the sample to the column.
- ⇒ Centrifuge 30sec / 10 000g / RT
- ⇒ Add 350µL of RW1 to the column
- ⇒ Centrifuge 30sec / 10 000g / RT
- ⇒ Mix 10µL of DNase with 70µL of RDD on each column
- ⇒ Incubate at RT for 15min

- ⇒ Add 350µL RW1 to the column, centrifuge 30sec / 10 000g / RT and discard the flow-through
- ⇒ Add 500µL RPE on column, centrifuge for 30sec / 10 000g / RT and discard the flow-through Add 500µL RPE to the column and centrifuge 2min30sec / 10 000g at RT
- ⇒ Place the RNeasy Mini spin column in a new 1.5 ml collection tube (supplied). Add 60 µl RNase-free water directly to the RNeasy Mini spin column membrane. Close the lid gently. To elute the RNA, centrifuge for 1 min at 10 000g at RT.

Evaluate the quality and concentration of the extracted RNA:

- ⇒ RNA purity and concentration are determined by spectrophotometry using a NanoDrop ND-2000 (Thermo Scientific). RNA purity is evaluated by calculating a 260nm/280nm absorbance ratio indicating the absence of proteins.
- ⇒ The ratio 260nm/230nm indicating the absence of contamination by organic molecules. Extraction of RNA from mouse skin with this protocol resulted in concentrations between 300 and 800 ng/µL.
- ⇒ Conserve RNA samples at -80°C until RT-qPCR.

### 1.9.8 RT-qPCR

#### **Protocol RT:**

RNAs are retrotranscribed using the PrimeScript™ RT reagent Kit (Takara refRR037A).

Prepare the reaction mix for each RNA sample:

Reagents	Volume
5X PrimeScript buffer	2 µL
PrimeScript RT Enzyme Mix	0,5 µL
Oligo dT Primer (50 µM)	0,5 µL
Random 6mers (100 µM)	0,5 µL
Total of RNA	500 ng
H <sub>2</sub> O RNase free	QSP 10µL

- ⇒ Program the thermal cycler (Biorad) for retro transcription:

Temperature	Time
37°C	15 min
85°C	5 secondes
4°C	illimited

Immediately use the resulting cDNA for the PCR reaction or store the aliquots at -20°C. The reaction is considered absolute, the cDNA concentration of the resulting solution is then at 50 ng /  $\mu$ L.

### Protocol qPCR:

The reaction of qPCR was performed with the SYBR Premix Ex Taq™ II kit (Takara ref RR820W). The gene expression results presented in this work were designed by Eurogentec (Table 5: Primer list). All primers were standardized by the RPL13A and ACTB for mouse samples and GAPDH and TBP for in vitro sample as housekeeping gene.

⇒ Dilute to 1/10th the volume of cDNA required for each sample.

⇒ Prepare the reaction mix in order to make duplicates:

Reagent	Volume
Takara mix	5 $\mu$ L
Primer Eurogentec	1 $\mu$ L
H <sub>2</sub> O RNase free	2 $\mu$ L
cDNA diluted at 5 ng/mL	2 $\mu$ L

⇒ Place the duplicate samples on a 96-well plate with transparent bottom (Eurogentec), add a negative control (RNase free water).

⇒ Film the plate

⇒ Program the Aria Mix (Agilent) to perform qPCR in real time:

Step	Temperature	Duration	Number of cycles
<b>Denaturation</b>	95°C	30 sec	1
<b>Amplification</b>	95°C 60°C 72°C	5 sec 30 sec 30 sec	40
<b>Melting curve</b>	95 °C 60°C 95°C	1 min 30 sec 30 sec	1

The purity is systematically verified by controlling the unicity of the dissociation peak of the double-stranded products obtained by the melting curve, and by the absence of amplification observed in the “negative well”. The results are expressed in relative quantity. The method used is that of  $2^{-\Delta\Delta Ct}$ , with double normalization by the expression of housekeeping genes. According to the primer list below:

Species	Gene	Gene ID	Primer	Primer sequence
Human	<b>VEGFA</b>	7422	Forward	5' – TGC TGT CTT GGG TGC ATT GG – 3'
			Reverse	5' – GCA TAA TCT GCA TGG TGA TGT TGG – 3'
	<b>COL1A1</b>	1277	Forward	5' – CAG CCG CTT CAC CTA CAG C – 3'
			Reverse	5' – TTT TGT ATT CAA TCA CTG TCT TGC C – 3'
	<b>ELN</b>	2006	Forward	5' – GTT GGT GGC TTA GGA GTG TCT G – 3'
			Reverse	5' – CGG CAC TTT CCC AGG CTT C – 3'
	<b>MMP1</b>	4312	Forward	5' – GCT TTC CTC CAC TGC TGC T – 3'
			Reverse	5' – CTT GCC TCC CAT CAT TCT TC – 3'
	<b>MMP2</b>	4313	Forward	5' – TCT TCC CCT TCA CTT TCC TG – 3'
			Reverse	5' – ACT TGC GGT CGT CAT CGT – 3'
	<b>ICAM1</b>	3383	Forward	5'-AGG CCA CCC CAG AGG ACA AC-3'
			Reverse	5'- CCC ATT ATG ACT GCG GCT GCT A-3'
	<b>VCAM1</b>	7412	Forward	5'-CGT CTT GGT CAG CCC TTC CT-3'
			Reverse	5'-ACA TTC ATA TAC TCC CGC ATC CTT C-3'
	<b>IL6</b>	3569	Forward	5'-GAA CTC CTT CTC CAC AAG CGC CTT-3'
			Reverse	5'-CAA AAG ACC AGT GAT GAT TTT CAC CAG G-3'
	<b>IL8</b>	3576	Forward	5'-TCT GCA GCT CTG TGT GAA GG-3'
			Reverse	5'-ACT TCT CCA CAA CCC TCT GC-3'
	<b>CCL2</b>	6347	Forward	5'-AGC AAG TGT CCC AAA GAA GC-3'
			Reverse	5'-CAT GGA ATC CTG AAC CCA CT-3'
	<b>P50</b>	4790	Forward	5'-GCA GAT GGC CCA TAC CTT CA-3'
			Reverse	5'-CAC CAT GTC CTT GGG TCC AG-3'
	<b>P65</b>	5970	Forward	5'-CCA GAC CAA CAA CAA CCC CT-3'
			Reverse	5'-TCA CTC GGC AGA TCT TGA GC-3'
	<b>GAPDH</b>	2597	Forward	5'-CCG GGA AAC TGT GGC GTG ATG G-3'
			Reverse	5'-AGG TGG AGG AGT GGG TGT CGC TGT T-3'

	<b>TBP</b>	6908	Forward	5'-TGT ATC CAC AGT GAA TCT TGG TTG- 3'
			Reverse	5'-GGT TCG TGG CTC TCT TAT CCT C- 3'
	<b>COX2</b>	5743	Forward	5'-CGG TGA AAC TCT GGC TAG ACA G-3'
			Reverse	5'-GCA AAC CGT AGA TGC TCA GGG A-3'
	<b>TNF</b>	7124	Forward	5'-CTC TTC TGC CTG CTG CAC TTT G-3'
			Reverse	5'-ATG GGC TAC AGG CTT GTC ACT C-3'
	<b>IL-1<math>\beta</math></b>	3553	Forward	5'-CCA CAG ACC TTC CAG GAG AAT G-3'
			Reverse	5'-GTG CAG TTC AGT GAT CGT ACA GG-3'
	<b>CD68</b>	968	Forward	5'-CGA GCA TCA TTC TTT CAC CAG CT-3'
			Reverse	5'-ATG AGA GGC AGC AAG ATG GAC C-3'
	<b>CD64</b>	2209	Forward	5'-ATA CAG GTG CCA GAG AGG TCT C-3'
			Reverse	5'-CCA GCT TAT CCT TCC ACG CAT G-3'
	<b>CD206</b>	4360	Forward	5'-AGC CAA CAC CAG CTC CTC AAG A-3'
			Reverse	5'-CAA AAC GCT CGC GCA TTG TCC A-3'
	<b>TGM2</b>	7052	Forward	5'- TGT GGC ACC AAG TAC CTG CTC A-3'
			Reverse	5'- GCA CCT TGA TGA GGT TGG ACT C-3'
<b>P16</b>	1029	Forward	5'-CCA ACG CAC CGA ATA GTT ACG- 3'	
		Reverse	5'-GCT ACC TGA TTC CAA TTC CCC T- 3'	
<b>P53</b>	7157	Forward	5'-AGT ATT TGG ATG ACA GAA- 3'	
		Reverse	5'-ATG TAG TTG TAG TGG ATG- 3'	
<b>Mouse</b>	<b>TNF</b>	21926	Forward	5'-GGT GCC TAT GTC TCA GCC TCT T-3'
			Reverse	5'-GCC ATA GAA CTG ATG AGA GGG AG-3'
	<b>IL1-<math>\beta</math></b>	16176	Forward	5'-TGG ACC TTC CAG GAT GAG GAC A-3'
			Reverse	5'-GTT CAT CTC GGA GCC TGT AGT G-3'
	<b>RPL13A</b>	22121	Forward	5'-CTG CTC TCA AGG TTG TTC GGC T-3'
			Reverse	5'-CCT TCC GTT TCT CCT CCA GAG T-3'
	<b>ACTB1</b>	11461	Forward	5'-CAT TGC TGA CAG GAT GCA GAA GG-3'
			Reverse	5'-TGC TGG AAG GTG GAC AGT GAG G-3'

### 1.9.9 Immunofluorescence

Immunofluorescence was performed on dermal fibroblast after confluency on glass coverslip to evaluate the extracellular matrix (ECM) formation in inflammatory condition. The following

antibodies were used: Rabbit polyclonal anti-collagen I (Novotec, Bron, France) and Rabbit polyclonal anti-elastin antibody (Abcam, Cambridge, UK) as primary antibody. As secondary antibody, Goat anti-rabbit IgG Alexa Fluor 488 (Life Technologies). Nuclear counterstaining using DAPI was purchased from Sigma-Aldrich.

## **Protocol:**

### **1) Cell fixation**

- ⇒ For elastin network observation, use methanol. Add 2 ml of 100% cold methanol to the box containing the slides for 20 minutes.
- ⇒ For collagen network observation, use PFA (Paraformaldehyde) 4%: Add 2 ml of PFA 4% 10 min at RT.
- ⇒ Rinse twice with PBS 1X.

At the end of this step, keep it in the fridge with PBS 1X (+ 0.4% PFA for PFA fixation).

NB: Using PFA fixation: a permeabilization step must be performed with adding Triton X100 0.1 % for 10 min.

### **2) Site saturation**

- ⇒ Add the cells (Glass slides) in a mixture of PBS 1X - Goat's milk serum 5%.
- ⇒ Incubate for 1h at RT.

### **3) Hybridization with the primary antibody**

- ⇒ Dilute of the primary antibody (Ab) to the desired dilution in PBS 1X buffer - 0.1 % Goat serum.
- ⇒ Add 100 µl primary Ab diluted on each glass coverslip.
- ⇒ Incubate at 4°C overnight (or 2h at RT).
- ⇒ Wash 3 times during 5min with PBS 1X at RT.

### **4) Hybridization with the secondary antibody**

- ⇒ Dilute the secondary antibody to 1/1000 in 0.1% goat serum solution coupled with a fluorescent marker.
- ⇒ Place 100 µl of diluted secondary Ab on coverslip.
- ⇒ Incubate for 1 hour at RT in the dark.
- ⇒ Wash 3 times during 5min with PBS 1X at RT in the dark.

## 5) Cell nucleus staining

- ⇒ Counter-staining with DAPI diluted 500-fold in 0.1% goat serum solution.
- ⇒ Incubate 5 min at room temperature in the dark.
- ⇒ Wash 3 times during 5min with PBS 1X at RT

## 6) Assembly

- ⇒ Place the mounting medium (Permafluor) on a glass slide. Then slide the glass disk over it.
- ⇒ Coverslip are ready to be analysed

Image acquisition was carried out using a Nikon microscope (Nikon TE300, Champigny-sur-Marne, France) with a coolsnap Fx CCD camera (Photometrics, Tucson, AZ, United States) with MetaVue software (Universal Imaging Corporation, West Chester, PA, United States). Image analysis and semi-quantification were performed using ImageJ software. Results were obtained from 4 independent experiments from 3 donors of fibroblasts. Two images were acquired per condition.

### 1.10 Statistical analysis

The data of this thesis work are expressed in mean  $\pm$  SD (Standard Deviation). The statistical analyses are realized using the software GraphPad Prism (version 8.0; GraphPad). A student unpaired t-test is performed to compare 2 unmatched samples. An analysis of variance test (ANOVA) one way or two way is used to compare samples from 3 or more groups with Dunnett or Bonferroni post-test. Differences to the mean are considered significant when  $p < 0.05$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , ns= not significant.

## Experimental results

## 1.11 Determination of cytokine mix concentration

It has been reviewed that TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$  may alter vascular processes and cells behaviors, inducing inflammation stress. A mix of these cytokines induces synergistic effect producing inflammatory responses, which could induce at low concentration expression of chemokine like CCL2 or RANTES implicated in vessels permeability on endothelial cells. Concentration of inflammatory mix was determined by gene expression on HDMEC. We showed that HDMEC stimulated with 10 IU/mL of cytokine mix induced significant upregulation of pro-inflammatory vascular gene respect to unstimulated cells (Control). Indeed, IL-6, IL-8, CCL2 and VEGF-A were 380, 96, 32 and 2-fold upregulated than control (Figure 12). While stimulation with 1 IU/mL induce non-significant upregulation of pro-inflammatory gene except for CCL2 which was induced 5-fold than control (Figure 12C). In this way, we decided to use for next study 10 IU/mL as cytokine concentration to induce inflammatory conditioned medium from HDMEC.

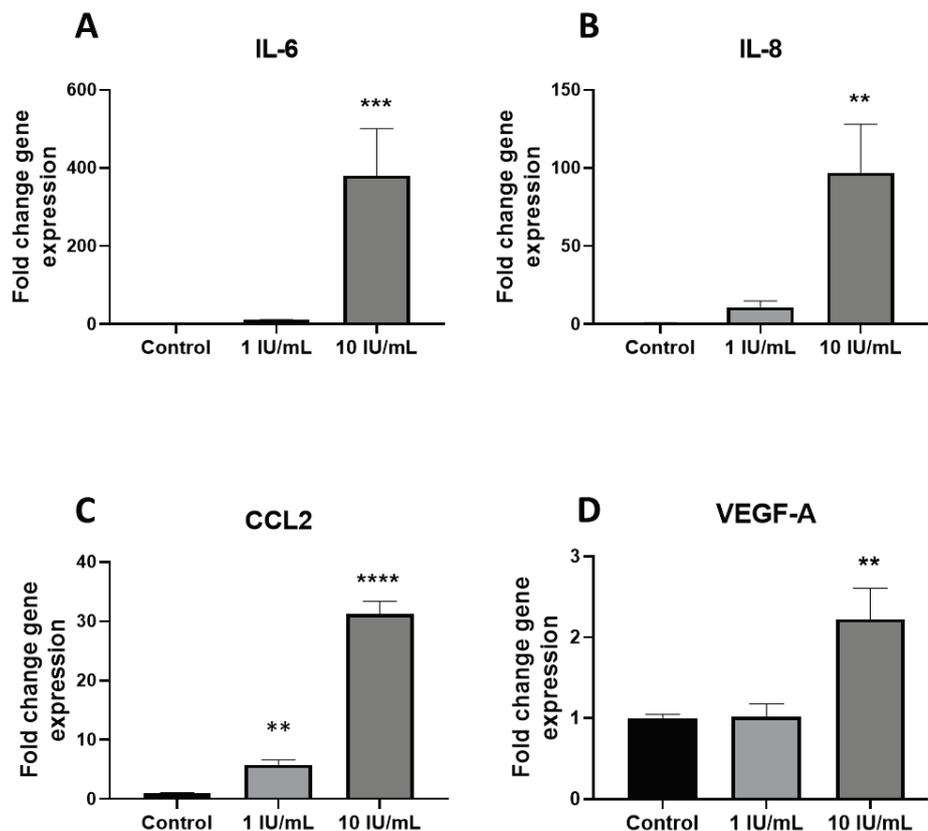


Figure 12: Inflammatory dose response on HDMEC

Effect of low-grade inflammatory stimulation on HDMEC gene expression of (A) IL-6, (B) IL-8, (C) CCL2 and (D) VEGF-A. HDMEC were stimulated with 1 IU/mL or 10 IU/mL of mix of cytokines composed by TNF- $\alpha$ , IL-1 $\beta$  and IFN-g for 24h and compared to unstimulated cells (Control). Gene expression was normalized with *GAPDH* and *TBP*. Data are representative of at least three independent experiments. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  vs control (Unstimulated HDMEC).

## 1.12 Low grade inflammation on HDMEC, impact extracellular matrix formation

In this study entitled " Impact of Human Dermal Microvascular Endothelial Cells on Primary Dermal Fibroblasts in Response to Inflammatory Stress" we determined the effect of a low-grade inflammatory stimulation on HDMEC and the effect of the conditioned medium produced by these cells on dermal fibroblasts behaviors. This study, published in Frontiers, Cell and Developmental Biology, is presented hereafter (Front. Cell Dev. Biol., 03 April 2019 | <https://doi.org/10.3389/fcell.2019.00044>).



# Impact of Human Dermal Microvascular Endothelial Cells on Primary Dermal Fibroblasts in Response to Inflammatory Stress

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The aim of the present study was to evaluate the impact of the microenvironment produced by dermal microvascular endothelial cells, secondary to a pro-inflammatory challenge, on 2D culture models using dermal fibroblasts and in 3D reconstructed skin model using dermal fibroblasts and keratinocytes from healthy donors. We hypothesized that specific microvascular endothelial low grade inflammation could change fibroblasts phenotype and be involved in extracellular matrix (ECM) modification and skin alteration. Following IFN $\gamma$ , TNF $\alpha$ , IL-1 $\beta$  pro-inflammatory stress on Human Dermal Endothelial Cells (HDMEC) we observed the increased release of Chemokine ligand 2 (CCL2), IL-6 and IL-8 but not VEGF-A in the conditioned medium (CM). The subsequent addition of this endothelial pro-inflammatory CM in dermal fibroblasts revealed an upregulation of *IL6*, *IL8* and *CCL2* but no *NF- $\kappa$ B* gene expression. The resulting ECM formation was impaired with a reduction of the collagen 1 network and a decrease in *COL1A1* gene expression in 2D and 3D models. Collagen 1 and pro-LOX protein expression were significantly reduced confirming an impairment of the collagen network related to endothelial inflammation secretion. To conclude, this work showed that, without any immune cells, the endothelial secretion in response to a pro-inflammatory stress is able to activate the fibroblasts that will maintain the pro-inflammatory environment and exacerbate ECM degradation.

**Keywords:** fibroblasts, endothelial cells, inflammation, extracellular matrix, collagen, elastin

## INTRODUCTION

Skin microcirculation is located deep in the dermis layer up to the reticular dermis close to the epidermis. Skin microcirculation plays a significant role in regulating skin homeostasis, thermoregulation, blood pressure and inflammatory response (Charkoudian, 2003; Gutterman et al., 2016). It also allows nutrients and systemic factors to reach the whole skin tissue

**Abbreviations:** CCL2, chemokine ligand 2; CM, conditioned medium; COL1A1, collagen 1 alpha 1; ECM, extracellular matrix; ELN, elastin; HDMEC, Human Dermal Microvascular Endothelial Cell; ICAM-1, InterCellular Adhesion Molecule 1; IFN- $\gamma$ , Interferon gamma; IL-1 $\beta$ , Interleukin 1 Beta; IL-6, Interleukin 6; IL-8, Interleukin 8; LOX, Lysyl Oxydase; MMP, Matrix metalloproteinase; pro-LOX, pro-Lysyl Oxydase; TNF- $\alpha$ , Tumor Necrosis Factor alpha; VCAM-1, Vascular cell adhesion protein 1; VEGF-A, Vascular endothelial growth factor A.

throughout the skin microvascular network from arteries to small capillaries close to the epidermis. Microvascular endothelial cells are the major components of dermal blood vessels and are clearly involved in skin inflammatory process. Endothelial cells participate to synthesize and secrete chemokine and cytokine and are implicated in recruiting immune cells in response to dysfunctions due to different factors (e.g., UV, heat, pathological and physiological processes) (Swerlick and Lawley, 1993).

Specific inflammatory mediators are described as endothelium activators like IL-1 $\beta$  or TNF- $\alpha$ , permitting vessels hyperpermeability allowing leucocyte infiltration. These vascular modulations are involved in skin diseases linked to a chronic inflammation (McGee et al., 1995; Huggenberger and Detmar, 2011). *In vitro*, TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$  may alter vascular processes and cells behaviors, inducing inflammation stress. A mix of these cytokines induces synergistic effect producing inflammatory responses, which could induce at low concentration expression of chemokine like CCL2 or RANTES implicated in vessels permeability on endothelial cells (Goebeler et al., 1997).

Thus, inflammation is a common condition that affects the vasculature, but is also a skin feature that occurs in long-term pathologies, as well as in short-term conditions at high or low-grade level (Huggenberger and Detmar, 2011). Dermal fibroblasts are the key cells to synthesize the ECM composed by many components including collagen and ELN fibers. Fibroblasts are also known to stimulate vascular inflammation through cytokines and chemokines production (Singer and Clark, 1999), leading to endothelial activation and leukocytes attraction (Buckley et al., 2001; Enzerink and Vaheri, 2011).

Interestingly, the impact of microvascular endothelial cells microenvironment on skin integrity in a low-grade inflammatory context is poorly understood. The aim of the present study was to evaluate the impact of the microenvironment produced by dermal microvascular endothelial cells, secondary to a pro-inflammatory challenge, on 2D culture models using dermal fibroblast from healthy donors. We hypothesized that specific endothelial low grade inflammation could change fibroblasts phenotype and be involved in ECM modification and skin alteration. We validated this hypothesis in 2D culture model and then confirmed these results in a 3D full-thickness reconstructed human skin.

## MATERIALS AND METHODS

### Cell Culture

Human dermal microvascular endothelial cells (HDMEC, Promocell Heidelberg, Germany) were cultured in Endothelial cell medium from Promocell composed by DMEM containing heparin 90  $\mu$ g/ml, hydrocortisone 1  $\mu$ g/ml supplemented with 2% Fetal Bovine Serum (FBS), 0.4% Endothelial Cell Growth Supplement (Promocell). Complete medium was supplemented with 1% penicillin/streptomycin (Gibco, Life Technologies, Carlsbad, CA, United States) at 37°C and 5% CO<sub>2</sub>. Human dermal fibroblasts were kindly provided by the Cell and Tissue Bank (Hôpital Edouard-Herriot, Lyon, France). Normal abdominal

human skin tissue explants were obtained from the surgical discard of anonymous healthy patients with informed consent of adult donors in accordance with ethical guidelines (French Bioethics law of 2004) and declared to the French research ministry (Declaration no. DC-2008-162 delivered to the Cell and Tissue Bank of Hospices Civils de Lyon). Exclusion criteria were related to positive test result for hepatitis B or C, or HIV as well as obesity history. Human dermal fibroblasts were cultured in a medium consisted in a ratio 1 :1 of Dulbecco's Modified Eagle's Medium with Glutamax and F-12 medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% FBS (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and 1% penicillin/streptomycin (Gibco Life Technologies). HDMEC were used below 10 population doubling level and dermal fibroblasts below 20.

### Antibodies and Reagents

The following antibodies were used: Rabbit polyclonal anti-collagen I (Novotec, Bron, France), Rabbit polyclonal anti-elastin antibody (Abcam, Cambridge, Royaume-Uni), Rabbit monoclonal anti-pro-LOX antibody (D8F2K, Cell Signaling), Rabbit polyclonal anti- $\beta$ -actin antibody (ab8227, Abcam Cambridge, Royaume-Uni). As secondary antibody, Goat anti-rabbit IgG Alexa Fluor 488 (Life Technologies) was used for immunofluorescence and Goat anti-rabbit IgG, HRP-linked antibody for western blot (Cell signaling). Nuclear counterstaining using DAPI was purchased from Sigma-Aldrich. rhIFN- $\gamma$  (c-60724), rhTNF- $\alpha$  (c-63719), and IL-1 $\beta$  (c-61120) were purchased from Promokine (Heidelberg, Germany).

### Inflammatory Conditioned Medium From HDMEC

To produce a pro-inflammatory CM from HDMEC, cells were seeded in a 75 cm<sup>2</sup> flask (4  $\times$  10<sup>5</sup> cells/flask) 24 h before stimulation. HDMEC were stimulated with 0 IU/mL as control, 10 IU/mL or 100 IU/mL (for 3D skin models experiments) of a mix of cytokines composed by rhIFN- $\gamma$ , rhTNF- $\alpha$ , and rhIL-1 $\beta$  during 24 h at 37°C 5% CO<sub>2</sub> (CM $\emptyset$ , control; CM10, conditioned medium produce with 10 IU/mL cytokines mix; CM100, conditioned medium produce with 100 IU/mL cytokines mix). Conditioned media were then collected and centrifuged at 10000  $\times$  g for 10 min at 4°C. Supernatant of each stimulation experiment was then collected and stored at -80°C until used.

### Effect of the Conditioned Medium on Human Dermal Fibroblasts

Human dermal fibroblasts extracted from abdominal skin biopsies from adult donors were seeded in 6 well plates (200 000 cells/well). 24 h after cell adhesion, fibroblasts medium was changed by endothelial medium during 24 h. Dermal fibroblasts were then washed twice with PBS and stimulated with the different CM obtained (CM $\emptyset$  or CM10) during 48 h at 37°C 5% CO<sub>2</sub>. Results were obtained from 3 independent experiments from 3 donors of fibroblasts.

## Effect of the Conditioned Medium on 3D Human Full Thickness Skin

Full-thickness human 3D skin models (from Lab Skin Creations, Lyon, France) were cultured in 6 well plates using an insert membrane of 0.4  $\mu\text{m}$  of pore size (Millicell, Burlington, United States). Reconstructed skins were cultured in skin medium provided by Lab Skin Creations. 24 h before stimulation, skins were cultured in endothelial cells medium and then stimulated with the different CMs (CM $\emptyset$  or CM100). From preliminary tests it has been established on human biopsies that CM100 and 5 days of stimulation were necessary to observe modifications on skin integrity in presence of CM. 3D Skin models were then stimulated with CM100 during 5 days at 37°C under 5% of CO<sub>2</sub>. Results were obtained from 7 independent experiments using 3D skin models.

## RNA Isolation and Real-Time Quantitative PCR

Total RNA was isolated using RNeasy Kit for 2D *in vitro* models and RNeasy Fibrous Tissue Mini Kit for 3D skin models (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. 500 ng of total RNA was reverse-transcribed into cDNA using PrimeScript™ RT reagent kit (Takara, Shiga, Japan) and analyzed by Real-Time QPCR using SYBR® Premix Ex Taq II (Takara) on an AriaMx Realtime PCR system (Agilent Genomics, Santa Clara, CA, United States). All primer (Supplementary Table S1) were provided by Eurogentec. Results were normalized to the *GAPDH* expression level for HDMEC, and dermal fibroblast samples.

Results were obtained from 3 independent experiments and 3 donors of fibroblasts have been used for 2D experiments. Relative quantification was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  quantification method. The primers are listed in the Supplementary Table S1.

## Western Blotting

Dermal fibroblasts were lysed in RIPA buffer from Sigma and supplemented with protease and phosphatase inhibitor at 4°C. Cells lysate was centrifuged 10 min at 10000 *g* and 4°C. Supernatant were collected. Proteins were denatured 5 min at 95°C, separated on 10% polyacrylamide gel according to protein size and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Ref. IPVH00010). Membranes were blocked with 1 × tris-buffered saline (TBS) 0.1% Tween and 5% non-fat milk for 1 h and blotted at 4°C overnight with primary antibodies diluted as recommended by the manufacturers in 1 × TBS, 0.1% Tween and 5% non-fat milk. Membranes were incubated for 1 h at room temperature with secondary antibodies diluted as recommended by the manufacturers in 1 × TBS, 0.1% Tween. Then membranes were incubated 5 min with ECL (Thermo Fisher, Ref. 34080) and were revealed by chemiluminescence. Western blot results were analyzed using GelAnalyzer software.

## Immunofluorescence Microscopy

For immunofluorescence, conditioned media (CM $\emptyset$  or CM10) were added when fibroblasts were at confluence on coverslip.

Eight-day post confluence, cells were fixed in cold methanol (−20°C) for 20 min to investigate ELN network or were fixed in PBS supplemented with 4% paraformaldehyde at room temperature for 10 min to study collagen I network. Cells were incubated overnight at 4°C with a rabbit polyclonal anti-collagen I (Novotec, Bron, France) antibody diluted at 1:400 in PBS – goat serum 0.1% or a rabbit polyclonal anti-elasticin antibody (Abcam, Cambridge, Royaume-Uni) diluted at 1:250 in PBS – goat serum 0.1%. This was followed by a second incubation with a goat anti-rabbit IgG Alexa Fluor 488 (Life Technologies) diluted in 1:1000 PBS – goat serum 0.1% (Life Technologies) at room temperature for 1 h. Nuclear counterstaining using DAPI (Sigma) was carried out with a dilution 1:500 in PBS – goat serum 0.1%. Negative controls were performed by omitting the primary antibody. Image acquisition was carried out using a Nikon microscope (Nikon TE300, Champigny-sur-Marne, France) with a coolsnap fx CCD camera (Photometrics, Tucson, AZ, United States) with MetaVue software (Universal Imaging Corporation, West Chester, PA, United States). Image analysis and semi-quantification were performed using ImageJ software. Results were obtained from 4 independent experiments from 3 donors of fibroblasts. Two images were acquired per condition.

## Luminex Assay

The determination of the amount of multiple cytokines in CM $\emptyset$  and CM10 was carried out using Human Magnetic Luminex Assay from R&D Systems (Minneapolis, MN, United States) according to the manufacturer's instructions. Levels of CCL2 (MCP-1), IL-6, IL-8 (CXCL8), VEGF-A, IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$  were analyzed using Bio-Plex MAGPIX Multiplex Reader and *Bio-Plex* Manager software from Bio-Rad (Bio-Rad Laboratory, Hercules, CA, United States).

## Statistical Analysis

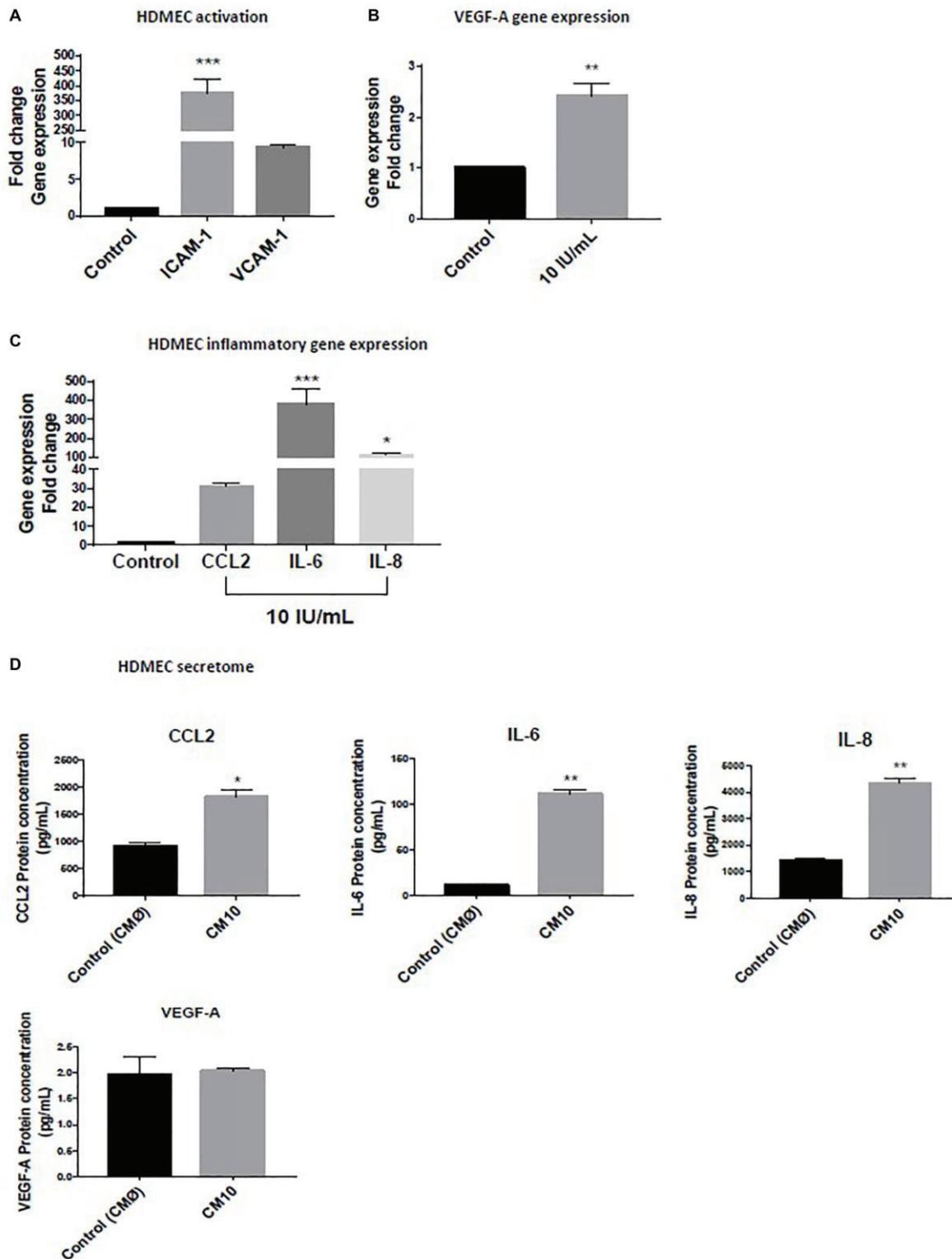
All statistical analyses were performed using Prism (version 7.0, GraphPad Software Inc.). All results are expressed as mean  $\pm$  SD. Statistically significant differences were calculated by one-way analysis of variance (followed by Dunnett's test post-test) or unpaired Student's *t*-test.  $p < 0.05$  was considered statistically significant. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

## RESULTS

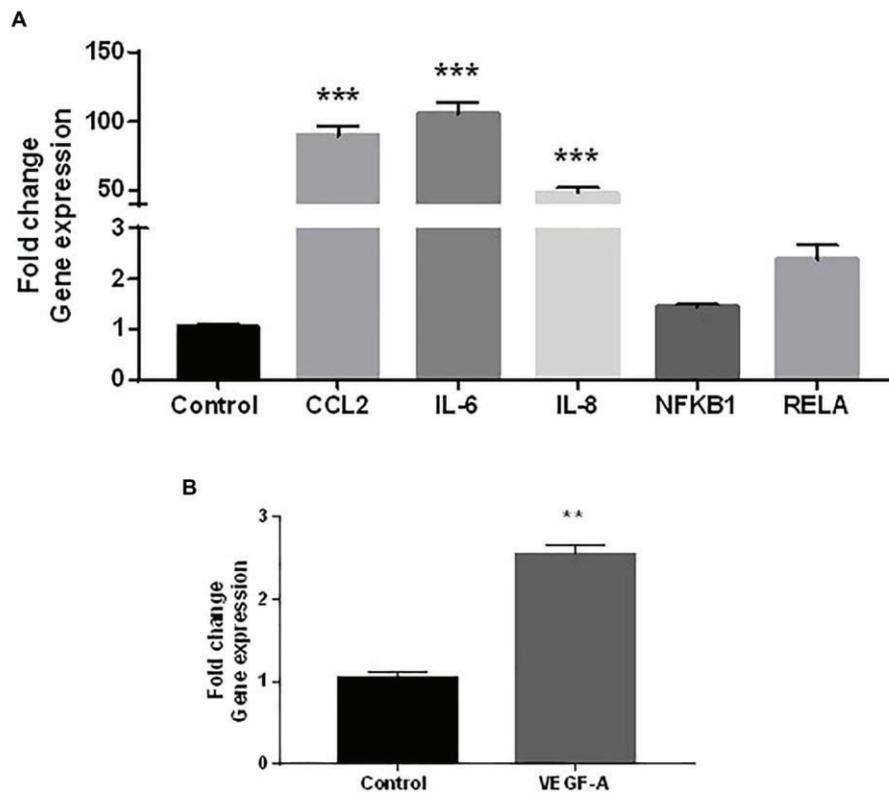
### HDMEC Activation and Secretion Under Low-Grade Inflammatory Stimulation

Human Dermal Endothelial Cells were treated with low-grade inflammatory mix (rhIFN- $\gamma$ , rhTNF- $\alpha$ , and rhIL-1 $\beta$ ) and we observed an upregulation of *ICAM-1* and *VCAM-1* (Figure 1A) demonstrating that the low-grade inflammatory mix activated the dermal microvascular endothelial cells, as confirmed by 2.5-fold increase in *VEGF-A* gene expression (Figure 1B).

We observed a significant HDMEC transcriptional activation of the pro-inflammatory gene expression markers, *CCL2*, *IL-6*, and *IL-8* (Figure 1C) in response to the initial inflammatory cocktail that resulted of an increased in HDMEC protein



**FIGURE 1** | Effect of low grade inflammatory stimulation on HDMEC Gene expression of **(A)** markers of vascular activation of **(B)** *VEGF-A* **(C)** pro-inflammatory cytokines. **(D)** Expression of proteins secreted in the conditioned medium. Luminex assay for CCL2, IL-6, and IL-8 levels and VEGF-A protein expression. Gene expression was normalized with *GAPDH*. Data are representative of at least three independent experiments. Analysis of cytokine secretion was performed in duplicate for each sample using *t*-test. For vascular inflammation results were compared and analyzed to control condition using ANOVA Dunnett's test. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.



**FIGURE 2 |** Effect of CM10 on fibroblast gene expression. RT-qPCR expression analysis of genes coding **(A)** *CCL2*, *IL-6*, and *IL-8*, *NFKB1*, *RELA* and **(B)** *VEGF-A* following CM10 stimulation versus control CMØ. Gene expression was normalized with *GAPDH*. **(A)** Results were compared and analyzed to control conditions using ANOVA Dunnett's test, except for *VEGF-A* where gene expression data were analyzed using a *t*-test. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

secretion of CCL2, IL-6, and IL-8 in the CM10 compared to the control condition CMØ (**Figure 1D**). The protein fold increases were 1.5, 12, and 2.8, respectively (**Figure 1D**). In contrast, protein expression of TNF- $\alpha$ , IL-1 $\beta$ , and IFN $\gamma$  were not detectable in CM10, revealing absence or non-significant levels of these cytokines in HDMEC secretome. VEGF-A protein expression was not significantly different between the two groups (**Figure 1D**).

### Pro-inflammatory Endothelial Conditioned Medium Stimulates Further Fibroblast Inflammatory Gene Expression

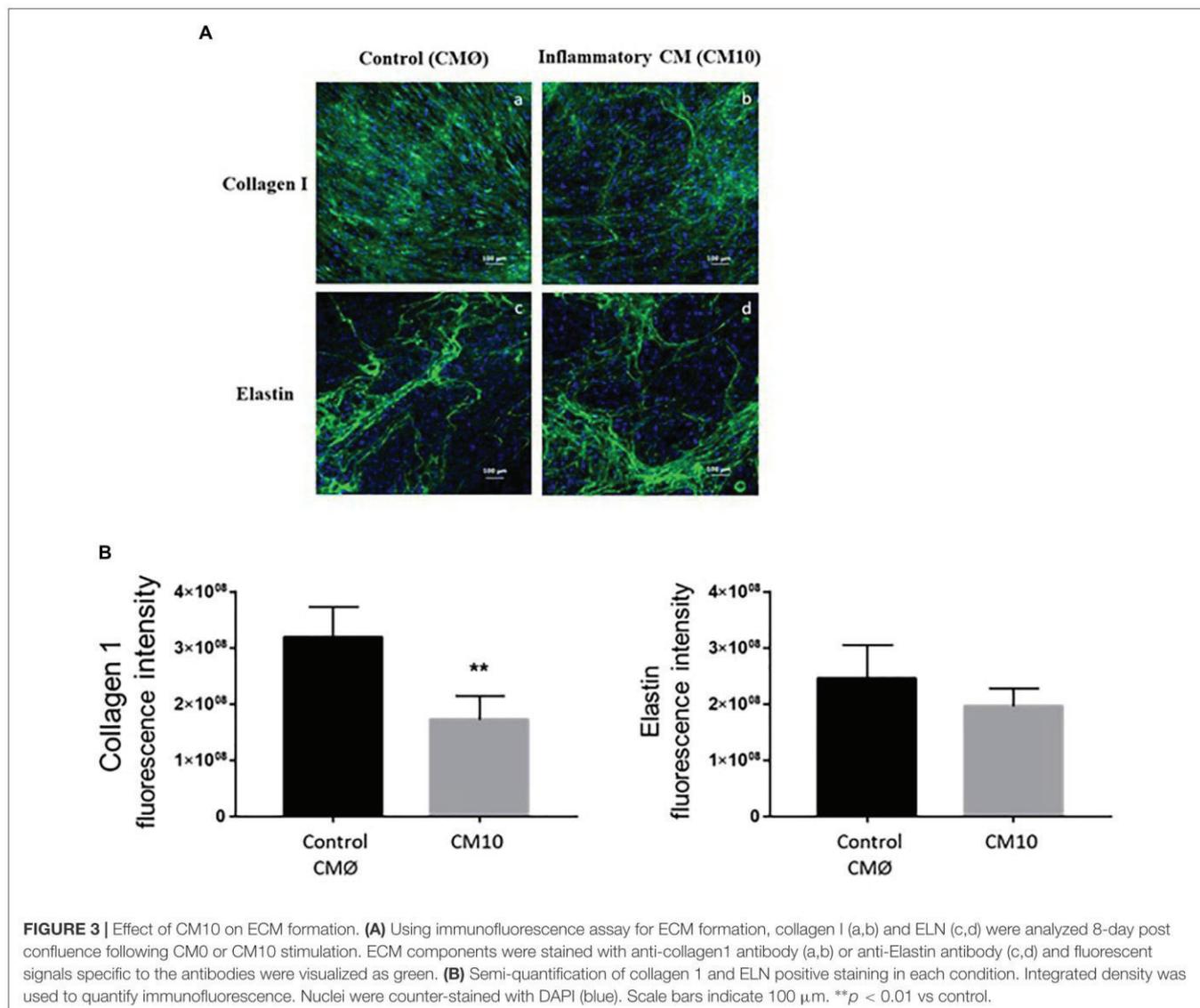
The secretion of increased inflammatory cytokines released from HDMEC under low-grade inflammation could influence dermal fibroblasts function. To investigate this possibility in a cell culture system, dermal fibroblasts were stimulated with CMØ or CM10 for 48 h. It appeared that *CCL2*, *IL-6*, and *IL-8* gene expression were significantly up-regulated in dermal fibroblasts in presence of CM10 compared to control condition CMØ (**Figure 2A**). In contrast, no significant differences were observed in pro-inflammatory NF- $\kappa$ B pathway, NF- $\kappa$ B sub-unit *NFKB1* and *RELA* gene expression in fibroblasts challenged by CM10 compared to CMØ.

*VEGF-A* gene expression was also 2.5-fold increased by dermal fibroblasts under CM10 compared to CMØ (**Figure 2B**).

### Pro-inflammatory Endothelial Conditioned Medium Altered ECM

In order to investigate collagen type I and ELN ECM formation in contact with CMØ or CM10, we used immunofluorescence to analyze their production within the ECM. We confirmed that dermal fibroblasts treated with CM10, 8-day post confluence, synthesized less collagen I than cells treated with CMØ (**Figure 3A**). Collagen I network appeared to be less organized in presence of CM10 compared to CMØ. Semi-quantification of collagen I labeling was significantly decreased in presence of CM10 compared to CMØ while ELN labeling semi-quantification tended to be reduced but did not reach significant differences (**Figure 3B**).

We then analyzed the expression of genes encoding proteins involved in ECM rearrangement in fibroblasts treated with CM10 or CMØ (**Figure 4A**). *MMP1* and *MMP2* gene expression were significantly increased by CM10 while *ELN* gene expression was unchanged and *COL1A1* gene expression was significantly reduced. In addition, collagen I protein expression was reduced in presence of CM10 compared to CMØ (**Figure 4B**) confirming the reduction of the collagen I network as observed by



immunofluorescence (Figure 3A). Pro-LOX protein expression, the pro-enzyme form of LOX responsible for collagen and/or ELN cross-link, was also reduced by CM10 compared to CMØ (Figure 4B).

### Effect of CM100 on 3D Human Full Thickness Skin Gene Expression

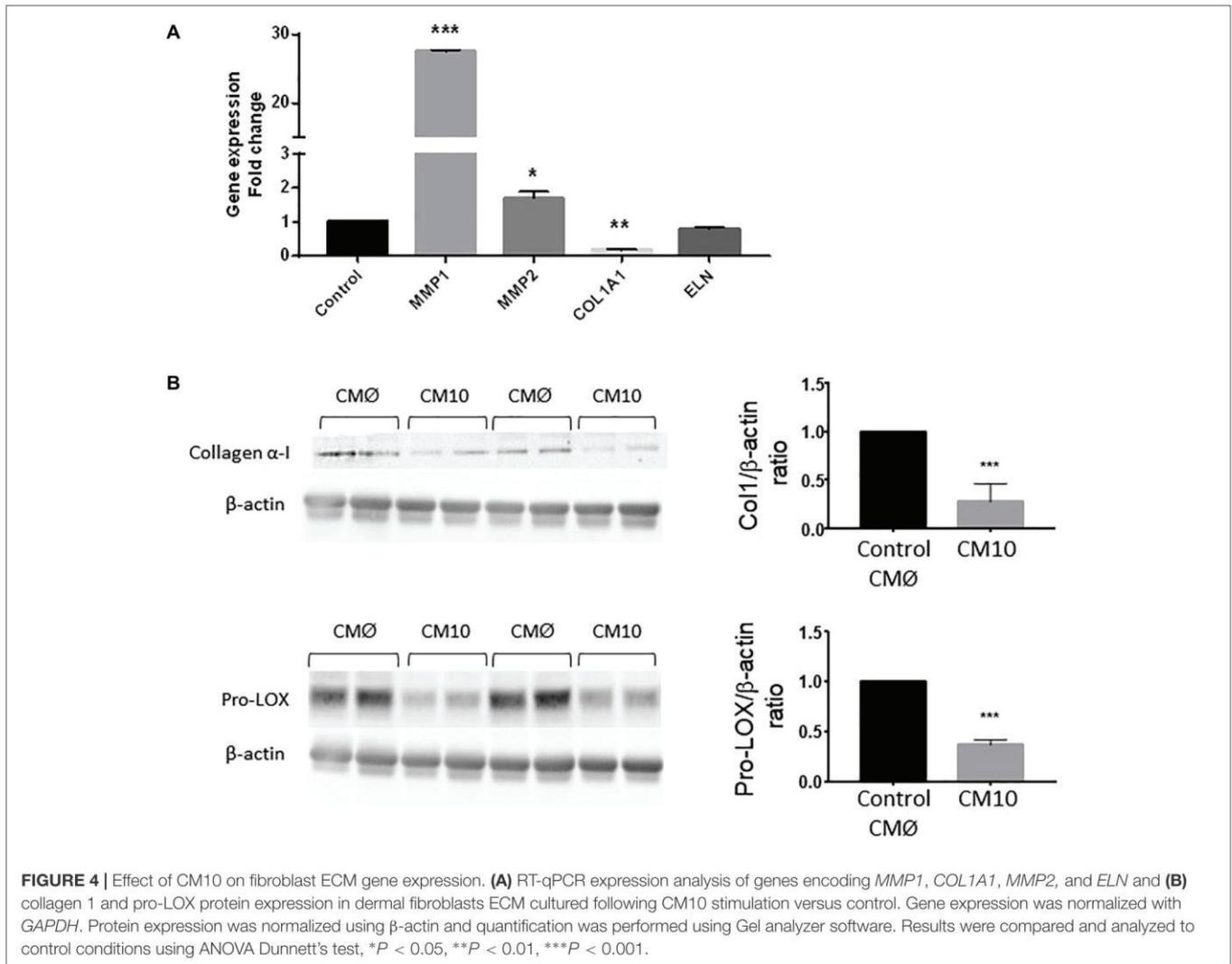
To go further into the comprehension of the effect of vascular pro-inflammatory CM on ECM, we used 3D reconstructed skin including keratinocytes and fibroblasts that were challenged with CMØ or CM100 for 5 days. Gene expression of cellular senescence marker *p16* and the stress marker *p53* were both not different between CMØ and CM100 (Figure 5A).

In 3D skin model, *COL1A1* and *ELN* gene expression were significantly downregulated in presence of CM100 (Figure 5B). Nevertheless, in this model there was no upregulation of *MMPs* in presence of CM100 compared to CMØ.

## DISCUSSION

In the present study, we showed that the activated endothelial cell secretome released following pro-inflammatory stress promoted a pro-inflammatory fibroblast phenotype that could be self-sustained without any immune cells. Indeed, *CCL2*, *IL-6*, and *IL-8* released in the endothelial secretome following  $\text{IFN}\gamma$ ,  $\text{TNF}\alpha$ , and  $\text{IL-1}\beta$  stress, are responsible for increasing *CCL2*, *IL-6*, and *IL-8* fibroblast gene expression generating a low-grade inflammatory fibroblast environment. The pro-inflammatory cocktail could thus maintain a perpetual fibroblast inflammatory phenotype that could be responsible for an exacerbation in ECM degradation with an increase in *MMP1* gene expression and unstable collagen 1 network.

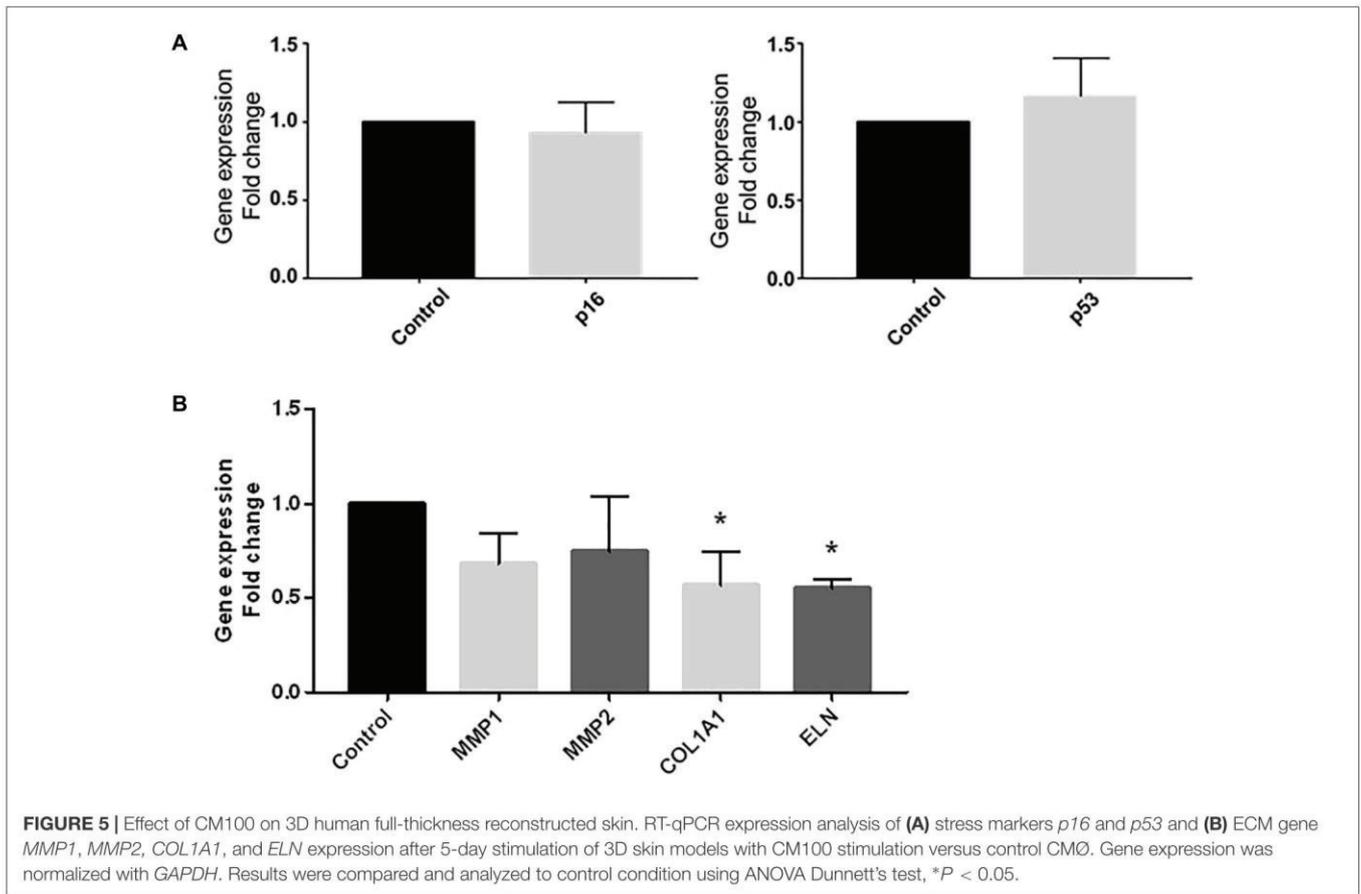
Dermal microcirculation is the conduit in which the stressors will affect the surrounding environment. In homeostasis, fibroblasts and endothelial cells are both involved in the angiogenic process and in ECM formation (Slany et al., 2016).



In a pro-inflammatory microenvironment, dermal microvascular endothelial cells play an important role in homeostasis and in inflammatory responses. HDMECs synthesize and secrete chemokine and cytokines which are implicated in recruiting immune cells in response to inflammatory disorders (Qazi et al., 2011). This inside-out inflammatory response has been supplemented with an outside-in signaling involving activated pro-inflammatory fibroblasts interacting with immune cells to direct endothelial activation (Enzerink and Vaheri, 2011). In this context, fibroblasts play a critical role in modulating immune cells such as leukocytes and could be responsible for the establishment of chronic inflammation (Buckley et al., 2001).

In the present study, we focused on activated endothelial cell secretion without any immune cell interaction that could impact dermal cell behaviors such as dermal fibroblast and the ECM formation. To play their roles, endothelial cells have to be activated in order to interact with their environment. In an inflammatory context, endothelial cells release different inflammatory factors like IL-1 $\beta$  and TNF- $\alpha$  or by an upregulation of leucocyte adhesion molecule as *ICAM-1* and *VCAM-1*, which

are two markers used to confirm the endothelial cells activation (Detmar et al., 1992; Videm and Albrigtsen, 2008). Endothelial cell activation permits the interaction with the environment, playing a key role in inflammatory development (Swerlick et al., 1992). In our models, we showed that HDMEC were activated in presence of low-grade inflammation with an upregulation of *ICAM-1* and *VCAM-1*. These activation are responsible for the loss of vascular integrity allowing secretion of cytokines and chemokines that could interact with tissues like skin structure (Adams and Shaw, 1994). HDMECs are described as critical elements for releasing proteins like VEGF-A or other growth factors. Moreover, HDMECs are also implicated in the cutaneous inflammation, secreting cytokines and chemokines that could directly interact with components of the dermis (Swerlick and Lawley, 1993). It appeared that low-grade inflammatory mix (CM10) composed of 10 IU/mL of IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  induced upregulation of cytokines and chemokines on dermal microvascular endothelial cells as CCL2, IL-6, and IL-8 which are known to be chemoattractant for T cells, dendritic cells and monocytes. The addition of the activated endothelial secretome



onto dermal fibroblasts induced a switch from normal to pro-inflammatory phenotype with an upregulation of *CCL2*, *IL-6*, and *IL-8* gene expression perpetuating fibroblasts pro-inflammatory environment.

The crosstalk between endothelial cells and fibroblasts has been studied through fibroblasts influencing endothelial cells, since fibroblasts provide ECM support as well as the production of several molecules like VEGF for angiogenesis (Villaschi and Nicosia, 1994; Martin et al., 2001; Berthod et al., 2006; Stunova and Vistejnova, 2018). In addition, (Villaschi and Nicosia, 1994) suggested that endothelial cells might also affect fibroblasts since cytokines, chemokines and other molecules present in the microenvironment could affect skin integrity altering ECM components (Van Linthout et al., 2014). In skin aging, it has been shown that *IL-8* and *IL-6* are clearly linked to MMPs modulation and *COL1A1* gene and protein expression (Dasu et al., 2003; Kolar et al., 2012; Quan and Fisher, 2015). We showed that the low-grade inflammatory microenvironment produced by activated endothelial cells induced a strong up-regulation of *MMP1* in dermal fibroblasts suggesting collagen I degradation associated to a reduction in collagen I synthesis due to *COL1A1* gene downregulation. In addition, the significant decrease in Pro-LOX protein expression strengthens the reduction of collagen cross-linking. Indeed, the primary function of the LOX family is the covalent cross-linking of collagens and/or ELN into a stable, insoluble ECM, which is important for the maintenance of tensile

strength and structural integrity of most tissues (Noblesse et al., 2004). The collagenous matrix deposited during LOX reduction should be more easily degradable (Trackman, 2005). In contrast, *ELN* gene expression was not modulated by the vascular pro-inflammatory microenvironment. Only a few studies have evaluated skin ELN production in an inflammatory context apart from skin aging (Ganceviciene et al., 2012) and wound healing (Almine et al., 2013) studies. We confirmed the 2D results with a downregulation of *COL1A1* and the 3D skin model revealed a significant reduction in *ELN* gene expression, suggesting that specific inflammatory vascular environment could alter skin integrity, inducing modification of gene implicated in ECM components. These results show the significant role of skin microvessels on skin integrity, which may vary depending on inflammatory conditions.

Indeed, the loss of vascular integrity is involved in the development of skin or autoimmune diseases, inducing inflammatory processes as chronic inflammatory conditions like psoriasis or lupus (Heidenreich et al., 2009; Achtman and Werth, 2015). Endothelial cells play a significant role in inflammatory responses and link to pro angiogenic and pro-inflammatory protein secretion as described. Among these pro-inflammatory proteins, VEGF-A is described as a predominant protein implicated in immune response. In inflammatory conditions, it appeared that VEGF is not only secreted by endothelial cells but also by macrophages, T cells and fibroblasts, which are

involved in angiogenesis and inflammatory processes (Mor et al., 2004; Enzerink and Vaheri, 2011). In our study, it appeared that HDMEC up-regulated *VEGF-A* gene expression, but no differences were observed in VEGF-A protein secretion in the CM, after 24 h of stimulation with the low-grade inflammatory cytokines mix. This result is probably linked to the short biological half-life, and the rapid metabolic degradation of VEGF-A in the medium (Lee et al., 2000).

To conclude, this study showed that the secretory micro-environment related to endothelial inflammation could trigger a perpetual fibroblast inflammatory phenotype that could be responsible for an exacerbation in ECM degradation with an increase in MMP1 and unstable collagen 1 network. In contact with endothelial inflammatory secretion, and no immune cells, it appears that fibroblasts switch from normal to pro-inflammatory phenotype and are less involved in ECM synthesis.

## AUTHOR CONTRIBUTIONS

LL, GA, JL, GL, and DS-R conceived and designed the study. BS and JD acquired, analyzed, and interpreted the data. BS, LL, JD, GA, JL, GL, and DS-R critically revised the manuscript for important intellectual content. DS-R obtained funding. BS, LL, JD, GA, JL, GL, and DS-R provided the administrative, technical,

or material support. DS-R is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2019.00044/full#supplementary-material>

**TABLE S1** | Details of specific primers and targets used in real-time qPCR experiments.

## REFERENCES

- Achtman, J. C., and Werth, V. P. (2015). Pathophysiology of cutaneous lupus erythematosus. *Arthritis Res. Ther.* 17:182. doi: 10.1186/s13075-015-0706-2
- Adams, D. H., and Shaw, S. (1994). Leucocyte-endothelial interactions and regulation of leucocyte migration. *Lancet* 343, 831–836. doi: 10.1016/S0140-6736(94)92029-X
- Almine, J. F., Wise, S. G., Hiob, M., Singh, N. K., Tiwari, K. K., Vali, S., et al. (2013). Elastin sequences trigger transient proinflammatory responses by human dermal fibroblasts. *FASEB J.* 27, 3455–3465. doi: 10.1096/fj.13-231787
- Berthod, F., Germain, L., Tremblay, N., and Auger, F. A. (2006). Extracellular matrix deposition by fibroblasts is necessary to promote capillary-like tube formation in vitro. *J. Cell Physiol.* 207, 491–498. doi: 10.1002/jcp.20584
- Buckley, C. D., Pilling, D., Lord, J. M., Akbar, A. N., Scheel-Toellner, D., and Salmon, M. (2001). Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol.* 22, 199–204. doi: 10.1016/S1471-4906(01)01863-4
- Charkoudian, N. (2003). Skin blood flow in adult human thermoregulation: how it works, when it does not, and why. *Mayo Clin. Proc.* 78, 603–612. doi: 10.4065/78.5.603
- Dasu, M. R., Barrow, R. E., Spies, M., and Herndon, D. N. (2003). Matrix metalloproteinase expression in cytokine stimulated human dermal fibroblasts. *Burns* 29, 527–531. doi: 10.1016/S0305-4179(03)00154-2
- Detmar, M., Tenorio, S., Hettmannsperger, U., Ruszczak, Z., and Orfanos, C. E. (1992). Cytokine regulation of proliferation and ICAM-1 expression of human dermal microvascular endothelial cells in vitro. *J. Invest. Dermatol.* 98, 147–153. doi: 10.1111/1523-1747.ep12555746
- Enzerink, A., and Vaheri, A. (2011). Fibroblast activation in vascular inflammation. *J. Thromb Haemost.* 9, 619–626. doi: 10.1111/j.1538-7836.2011.04209.x
- Ganceviciene, R., Liakou, A. I., Theodoridis, A., Makrantonaki, E., and Zouboulis, C. C. (2012). Skin anti-aging strategies. *Dermatoendocrinology* 4, 308–319. doi: 10.4161/derm.22804
- Goebeler, M., Yoshimura, T., Toksoy, A., Ritter, U., Bocker, E. B., and Gillitzer, R. (1997). The chemokine repertoire of human dermal microvascular endothelial cells and its regulation by inflammatory cytokines. *J. Invest. Dermatol.* 108, 445–451. doi: 10.1111/1523-1747.ep12289711
- Gutterman, D. D., Chabowski, D. S., Kadlec, A. O., Durand, M. J., Freed, J. K., Ait-Aissa, K., et al. (2016). The human microcirculation: regulation of flow and beyond. *Circ. Res.* 118, 157–172. doi: 10.1161/CIRCRESAHA.115.305364
- Heidenreich, R., Rocken, M., and Ghoreschi, K. (2009). Angiogenesis drives psoriasis pathogenesis. *Int. J. Exp. Pathol.* 90, 232–248. doi: 10.1111/j.1365-2613.2009.00669.x
- Huggenberger, R., and Detmar, M. (2011). The cutaneous vascular system in chronic skin inflammation. *J. Invest. Dermatol. Symp. Proc.* 15, 24–32. doi: 10.1038/jidsymp.2011.5
- Kolar, M., Szabo, P., Dvorankova, B., Lacina, L., Gabius, H. J., Strnad, H., et al. (2012). Upregulation of IL-6, IL-8 and CXCL-1 production in dermal fibroblasts by normal/malignant epithelial cells in vitro: immunohistochemical and transcriptomic analyses. *Biol. Cell* 104, 738–751. doi: 10.1111/boc.20120018
- Lee, R. J., Springer, M. L., Blanco-Boise, W. E., Shaw, R., Ursell, P. C., and Blau, H. M. (2000). VEGF gene delivery to myocardium: deleterious effects of unregulated expression. *Circulation* 102, 898–901. doi: 10.1161/01.CIR.102.8.898
- Martin, T. A., Harding, K., and Jiang, W. G. (2001). Matrix-bound fibroblasts regulate angiogenesis by modulation of VE-cadherin. *Eur. J. Clin. Invest.* 31, 931–938. doi: 10.1046/j.1365-2362.2001.00914.x
- McGee, D. W., Bamberg, T., Vitkus, S. J., and McGhee, J. R. (1995). A synergistic relationship between TNF-alpha, IL-1 beta, and TGF-beta 1 on IL-6 secretion by the IEC-6 intestinal epithelial cell line. *Immunology* 86, 6–11.
- Mor, F., Quintana, F. J., and Cohen, I. R. (2004). Angiogenesis-inflammation cross-talk: vascular endothelial growth factor is secreted by activated T cells and induces Th1 polarization. *J. Immunol.* 172, 4618–4623. doi: 10.4049/jimmunol.172.7.4618
- Noblesse, E., Cenizo, V., Bouez, C., Borel, A., Gleyzal, C., Peyrol, S., et al. (2004). Lysyl oxidase-like and lysyl oxidase are present in the dermis and epidermis of a skin equivalent and in human skin and are associated to elastic fibers. *J. Invest. Dermatol.* 122, 621–630. doi: 10.1111/j.0022-202X.2004.22330.x
- Qazi, B. S., Tang, K., and Qazi, A. (2011). Recent advances in underlying pathologies provide insight into interleukin-8 expression-mediated

- inflammation and angiogenesis. *Int. J. Inflam.* 2011:908468. doi: 10.4061/2011/908468
- Quan, T., and Fisher, G. J. (2015). Role of age-associated alterations of the dermal extracellular matrix microenvironment in human skin aging: a mini-review. *Gerontology* 61, 427–434. doi: 10.1159/000371708
- Singer, A. J., and Clark, R. A. (1999). Cutaneous wound healing. *N. Engl. J. Med.* 341, 738–746. doi: 10.1056/NEJM199909023411006
- Slany, A., Bileck, A., Kreutz, D., Mayer, R. L., Muqaku, B., and Gerner, C. (2016). Contribution of human fibroblasts and endothelial cells to the hallmarks of inflammation as determined by proteome profiling. *Mol. Cell Proteomics* 15, 1982–1997. doi: 10.1074/mcp.M116.058099
- Stunova, A., and Vistejnova, L. (2018). Dermal fibroblasts-A heterogeneous population with regulatory function in wound healing. *Cytokine Growth Factor Rev.* 39, 137–150. doi: 10.1016/j.cytogfr.2018.01.003
- Swerlick, R. A., and Lawley, T. J. (1993). Role of microvascular endothelial cells in inflammation. *J. Invest. Dermatol.* 100, 111S–115S. doi: 10.1038/jid.1993.33
- Swerlick, R. A., Lee, K. H., Li, L. J., Sepp, N. T., Caughman, S. W., and Lawley, T. J. (1992). Regulation of vascular cell adhesion molecule 1 on human dermal microvascular endothelial cells. *J. Immunol.* 149, 698–705.
- Trackman, P. C. (2005). Diverse biological functions of extracellular collagen processing enzymes. *J. Cell Biochem.* 96, 927–937. doi: 10.1002/jcb.20605
- Van Linthout, S., Miteva, K., and Tschöpe, C. (2014). Crosstalk between fibroblasts and inflammatory cells. *Cardiovasc. Res.* 102, 258–269. doi: 10.1093/cvr/cvu062
- Videm, V., and Albrigtsen, M. (2008). Soluble ICAM-1 and VCAM-1 as markers of endothelial activation. *Scand. J. Immunol.* 67, 523–531. doi: 10.1111/j.1365-3083.2008.02029.x
- Villaschi, S., and Nicosia, R. F. (1994). Paracrine interactions between fibroblasts and endothelial cells in a serum-free coculture model. Modulation of angiogenesis and collagen gel contraction. *Lab. Invest.* 71, 291–299.

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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### 1.13 Derma fibroblasts responses with low-grade inflammatory stimulation at 8 days

In the previous study, we showed that inflammatory microenvironment produced by HDMEC induce ECM alteration after 24h stimulation on dermal fibroblasts. And more precisely, a collagen I network altered correlated with a decrease in LOX expression. To evaluate ECM formation in long time stimulation, ECM gene expression, collagen I and LOX protein expression on dermal fibroblasts were analyzed after 8 days in contact with vascular inflammatory conditioned medium. We showed that after 8 days of stimulation with CM10, ECM gene expression of COL1A1, ELN and LOX were significantly downregulated respect to CM $\emptyset$  (Figure 13A). And confirmed by a decrease in protein expression on collagen I and prolysin oxidase (Pro-Lox) in presence of CM10 (Figure 13B).

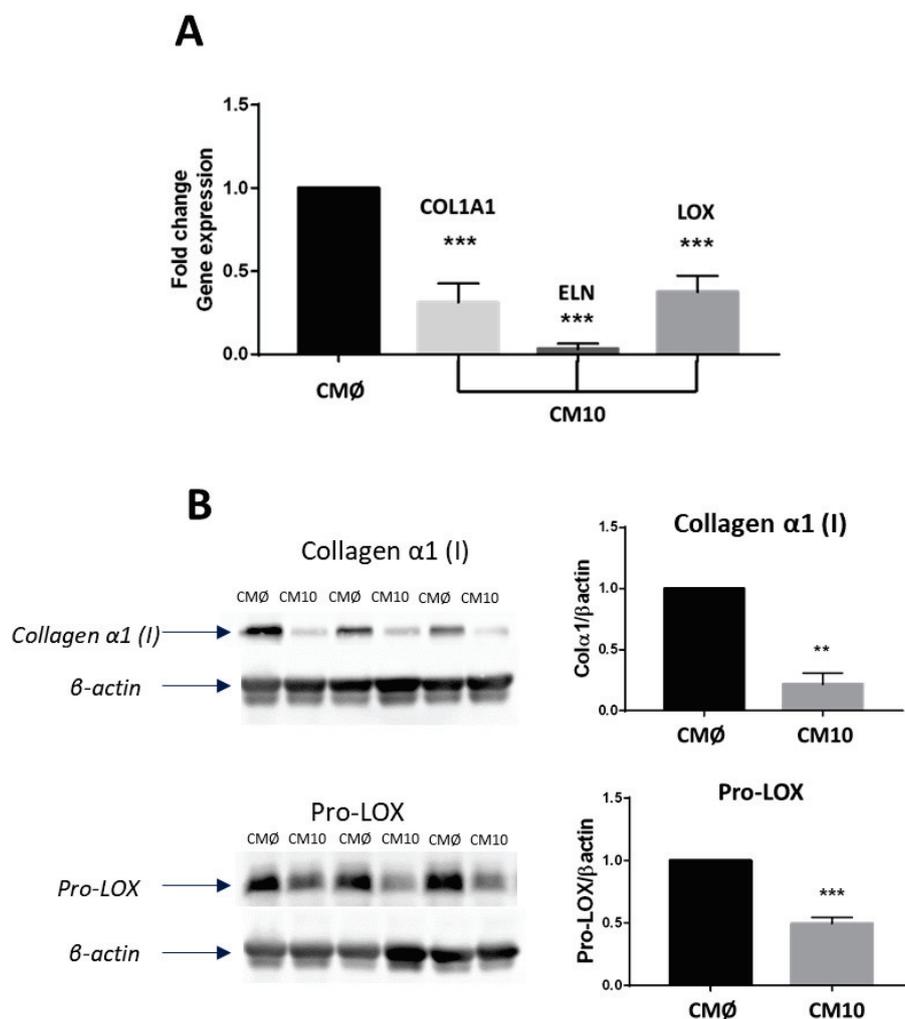


Figure 13: Effect of long-time CM10 stimulation on dermal fibroblasts

Effect of long time CM10 stimulation on fibroblast. (A) RT-qPCR expression analysis of genes encoding *COL1A1*, *ELN* and *LOX* and (B) collagen 1 and pro-LOX protein expression in dermal fibroblasts ECM cultured following CM10 stimulation versus control. Gene expression was normalized with *GAPDH*. Protein expression was normalized using  $\beta$ -actin and quantification was performed using Gel analyzer software. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs CM $\emptyset$ .

**To conclude, we showed that low level of inflammation using a mix of cytokine can induce strong inflammatory responses by microvascular endothelial cells. Inflammatory vascular secretum impacted dermal fibroblasts behavior without any immune cell implication, leading to ECM alteration at short time and longtime stimulation. Inflammation is the hallmark of the innate immune response to tissue damage. Controlled inflammation mediates the activation, expansion and recruitment to the tissues of cells and mainly by immune cells such as macrophages and dendritic cells (41) but also stromal cells (185). Thus, inflammation is a common condition that affects the vasculature, but is also a skin feature that occurs in long-term pathologies, as well as in short-term conditions at high or low-grade level (66). Inflammatory disorders can induce on endothelial cells, secretion of chemokine and cytokine leading to immune cells recruitment (186). This inside-out inflammatory response has been supplemented with an outside-in signaling involving activated pro-inflammatory fibroblasts interacting with immune cells to direct endothelial activation (187). Nevertheless, immune cells can also directly produce inflammatory microenvironment, modulating microvascular network and also dermal fibroblast behaviors in response to extern and intern stimuli. In this way, according to the main objectives with AbbVie (Allergan Legacy) collaboration, we evaluated the potential inflammatory modulation of crosslinked HA and their degradation products on inflammatory dermal cell and immune cells.**

## 1.14 HA characterization and endotoxin quantification

Late adverse effect can be observed in the presence of crosslinked HA few weeks or month after intradermal injection, suggesting a possible implication of degradation products in nodule and granuloma development. As described in Introduction “Chapter II: Hyaluronic acid to medical devices”, HA can be degraded by different way. According to physiological process, we focused on human hyaluronidase to degrade two different crosslinked HA. In partnership with AbbVie (Legacy Allergan), we evaluated the different degradation products of Volbella (VYC-15L) from VYCROSS® technology and UltraPlus XS (HYC-24L+) from HYLACROSS® technology after different duration of degradation. The objective was to produce different range sizes of low molecular weight fragments (LMW) of VYC-15L and HYC-24L+ to study their possible vascular and inflammatory effects *in vitro* and *in vivo*.

### 1.14.1 Low molecular weight of crosslinked HA characterization

The two crosslinked HA were degraded with human recombinant hyaluronidase for 3 different duration of degradation: 30 minutes, 2 hours, and 6 hours. We obtained different profile between each filler and each kinetic. Indeed, we observed that the molar mass distribution was different between the hyaluronidase-induced degradation products from VYC-15L and HYC-24L+. VYC-15L after 30 minutes, 2 hours and 6 hours of degradation induced lower fragments compared to HYC-24L+ degraded at the same degradation time. After 30 minutes, 2 hours and 6 hours of degradation, more of 33%, 55% and 75% of the total hyaluronidase-induced degradation products from VYC-15L were smaller than 40kDa respectively (Figure 14), while 10%, 27% and 55% of total HYC-24L+ fragments were smaller than 40kDa respectively. Furthermore, degradation products from HYC-24L+ contained more HMW fragments with a molecular weight > 500 kDa than those from VYC-15L. The results indicate that HYC-24L+ is less degraded than VYC-15L at the same time of degradation.

These differences have been confirmed with the characterization of Mw and the polydispersity coefficient between VYC-15L and HYC-24L+ fragments (Figure 15). Indeed, for VYC-15L, degradation for 30 minutes, 2 hours and 6 hours induced an average of fragment range sizes of ~100 kDa, ~90 kDa and 30 kDa, respectively. While with the same kinetics of degradation, HYC-24L+ was degraded into fragments with an average of fragments range sizes at ~200 kDa with 30 minutes of degradation, ~180 kDa with 2 hours and ~80 kDa with 6 hours of degradation (Figure 15A).

The different polydispersity coefficients between VYC-15L and HYC-24L+ showed that HYC-24L+ degradation products have equivalent heterogeneous range sizes than VYC-15L products, except after 6 hours of degradation (Figure 15B). We showed that LMW-VYC15L obtained at 30 min, 2h and 6h had a polydispersity at 2.2, 3.2 and 2.4, respectively, while LMW-HYC-24L+ had coefficient of polydispersity at 2.3 for 30 min, 3.3 for 2h and 3.3 for 6h degradation. The closer the polydispersity coefficient is to 1, the more homogeneous the samples are. All these results are summarized in Table 4. We obtained different profile of range sizes of HA fragments, with an average of fragments sizes from 200 kDa to 30 kDa, confirming the main production of low molecular weight of HA fragments as described in literature (93).

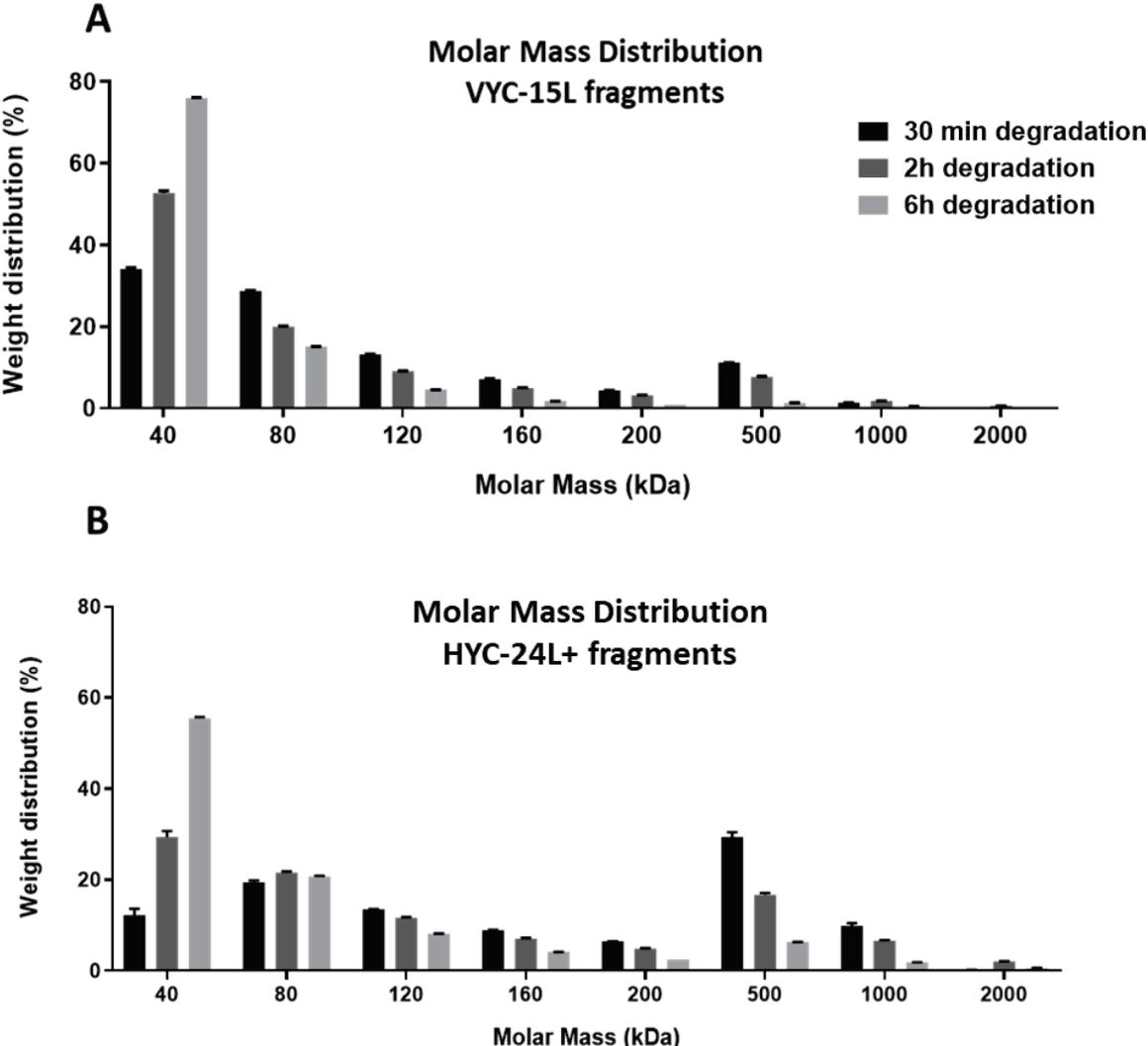


Figure 14: Molar mass distribution of VYC-15L and HYC-24L+ fragments

Molar mass distribution results are obtained by SEC-MALS. A and B corresponds to molar mass distribution of VYC-15L and HYC-24L+ fragments obtained after 30 minutes, 2h and 6h of degradation with human recombinant hyaluronidase. Short time degradation (30min) is represented in black column, medium time degradation (2h) in dark gray and longtime degradation in light grey. Percentage (%) of weight distribution correspond to free HA repartition in different range of molar mass. Molar masses are represented in kDa.

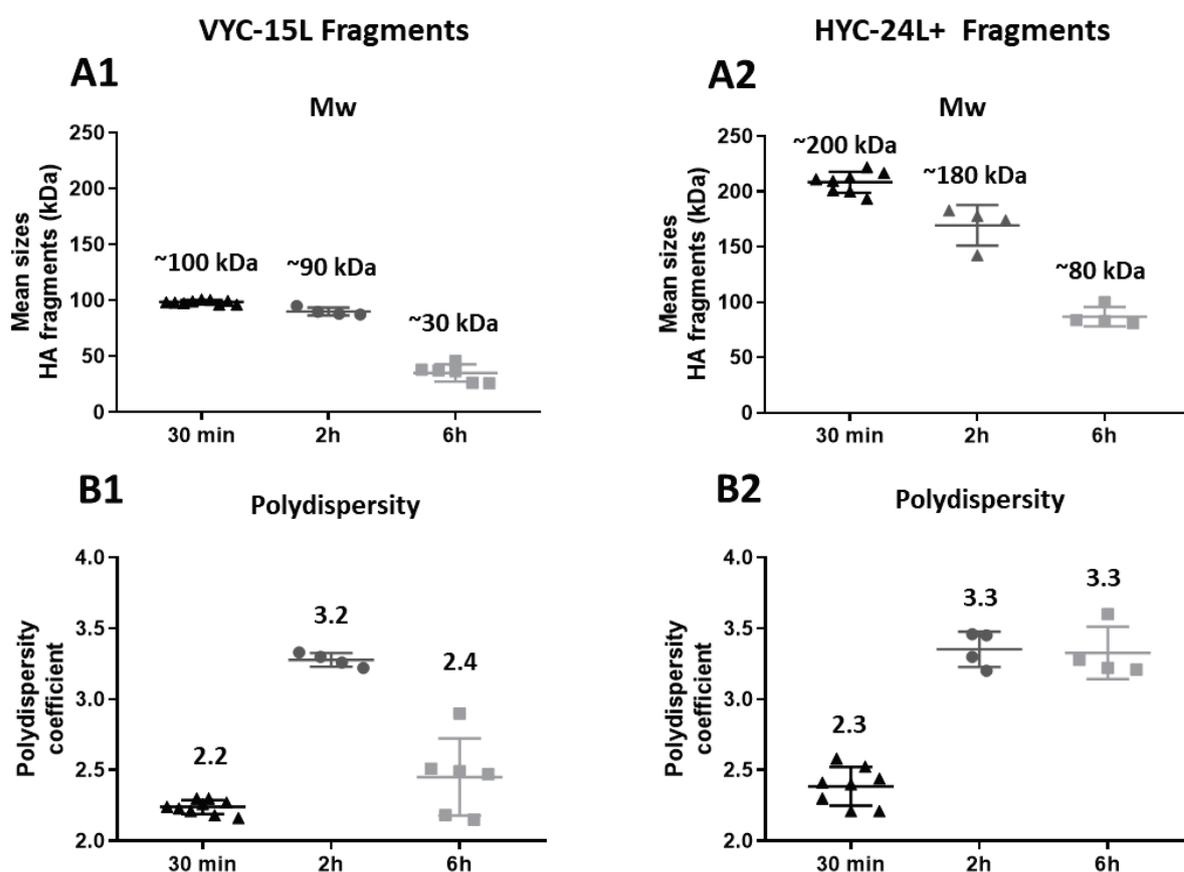


Figure 15: HA characterization: Mw and polydispersity

Molecular weights (Mz), and polydispersity coefficients have been determined by SEC-MALS. A1 and A2 correspond to the average of Mw of LMW of HA in kDa. B1 and B2 correspond to polydispersity coefficient. Data are representative of at least three independent experiments.

Degradation time		Short (30min)	Medium (2h)	Long (6h)
VYC-15L	Mw	~ 100 kDa	~ 90 kDa	~ 30 kDa
	Polydispersity	2,2	3,2	2,4
HYC-24L+	Mw	~ 200 kDa	~ 180 kDa	~ 80 kDa
	Polydispersity	2,3	3,3	3,3

*Table 4: HA fragments characterization*

## 1.15 Skin cell and tissue responses to cross-linked HA in low-grade inflammatory conditions

In this study entitled "Skin cell and tissue responses to cross-linked HA in low-grade inflammatory conditions" we determined the effect of HA degraded and non-degraded under a low-grade inflammation on dermal skin cells, immune cells and in vivo model. This study, will be submitted in Frontiers, Cell and Developmental Biology.

# Skin cell and tissue responses to cross-linked HA in low-grade inflammatory conditions

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## 1. Introduction

Hyaluronic acid (HA), a major glycosaminoglycan of the extracellular matrix, plays an important role in a number of biological processes such as wound healing, cancer or embryonic development (93). The size of HA fragments generated during degradation can also play a role in migration, apoptosis or immune regulation by activating the immune cells, such as macrophages and dendritic cell (99). The high molecular weight of HA (HMW-HA) (>500kDa) are commonly described to have an anti-inflammatory effect, while low molecular weight of HA fragments (LMW-HA) (10 kDa to 500 kDa) and oligomers of HA (0.1 to 10 kDa) have been described as involved in inflammatory responses, functioning as an alarm signal to immune system, particularly for monocytes/macrophages (93, 188). Indeed, some studies showed that pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , are released in the presence of LMW-HA (93). Moreover, the accumulation of LMW-HA fragments seems to be strongly related to the severity of inflammatory pathologies and chronic diseases such as rheumatoid arthritis, liver injury, asthma and lung injuries, suggesting dysregulation in HA metabolism (88, 189). It has been reviewed that LMW-HA accumulation was correlated to an increase in HA catabolism with turnover of the matrix in these inflammatory diseases. In normal condition, LMW-HA were commonly described as pro-inflammatory. However, controversial studies have shown that the inflammatory responses observed on *in vitro* and *ex vivo* murine immune cells were due to an endotoxin contamination on samples rather than the size of the fragments (103, 190, 191); depending on the source of HA and hyaluronidase used (103).

Cross-linked HA is used in a variety of medical applications, including as a dermal filler in aesthetics applications. Although typically regarded as an inert, biocompatible material due to its similarity with native HA, there have been rare instances of adverse effects which can occur from 4 weeks to > 1-year post-treatment (169, 192). Although the etiology of these events is unknown, a number of hypotheses have been proposed, including biofilm-related response, a result of mechanical irritation with the product, or the presence of bacteria

transferred into the skin during injection (171, 193). They have also been proposed to be related to LMW-HA in the filler products, either as a component of the product or following its degradation (192, 193).

An additional factor that has been proposed in these events is an activation of the systemic immune response, like which occurs with flu, cold, or seasonal allergies (192, 193). Moreover, filler treatments are not recommended in case of active auto-immune diseases such as systemic lupus erythematosus, rheumatoid arthritis, or mixed connective tissue disease (169) that all share a low-grade inflammatory microenvironment (194). This low level inflammation may impact cell-cell interactions between immune cells, microvascular endothelial cells, and dermal fibroblasts leading to extracellular matrix alteration (195). A chronic low-level inflammation could worsen cells alteration in time, and auto-immune diseases leading to chronic diseases (194, 196, 197).

Although the effects of HA on the inflammatory response have been examined, the effect of cross-linked hyaluronic acid and its degradation products on the skin, under low-level inflammatory conditions, remains unexplored. In the present study we used two different cross-linking technologies, VYCROSS™ (VYC-15L) and HYLACROSS™ (HYC-24L+), and their hyaluronidase-induced degradation products (165, 166). We hypothesized that, in a low-grade inflammatory microenvironment, the LMW degradation fragments could induce immune responses that could affect cells found in the dermis. Macrophages M1 and mature dendritic cells, both derived from monocytes THP-1 lineages (as immune cells models), inflammatory dermal fibroblasts and inflammatory human dermal microvascular endothelial cell (HDMEC) were used *in vitro* to study the effects of HA products and their LMW degradation fragments in the low-grade inflammatory environment. To further study *in vivo* the vascular and inflammatory responses, we used HA and their LMW degradation fragments in a low-grade inflammatory murine model.

## **2. Material and methods**

### **2.1 Hyaluronic acid degradation and characterization**

Hyaluronic acid VYC-15L and HYC-24L+ (Allergan Aesthetics, Pringy, France) were degraded using the human recombinant hyaluronidase PH20 HYLENEX® (Halozyme Therapeutics, San

Diego, California), for 6 hours at 150 UI/ml at 37°C. The enzyme was inactivated using silicon-bath at 100°C for 10 min. A vehicle control solution was comprised of inactivated hyaluronidase. VYC-15L fragments and HYC-24L+ fragments were analyzed by HPLC and ASTRA software (Wyatt technology). Each analysis was carried out 3 times coming from different preparation of same lot.

## **2.2 Endotoxin quantification**

The vehicle control, VYC-15L fragments and HYC-24L+ fragments were quantified by Endosafe PTS (Charles River). The Endosafe®- PTS (Portable Test System) is a rapid, point-of-use test system which is comprised of a test cartridge along with a hand-held spectrophotometer. For this test we used cartridge with 0.01 EU/mL sensitivity (PTS2001F). All experiments were carried out according to the manufacturer's instructions.

## **2.3 Macrophages M1 polarization**

THP-1 monocytes were incubated for 24h at 5 % CO<sub>2</sub> 37°C with PMA (Phorbol 12-myristate 13-acetate from Sigma) to induce the differentiation into macrophages M0. Then macrophages M0 were polarized into pro-inflammatory M1 for 66 hours in the presence of 100 ng/ml of LPS, 100ng/ml of PMA and 20 ng/ml of IFN $\gamma$  (Peprotech, France). Polarization of macrophages M1 were confirmed via cytokine profile (Supplementary Table 1 and Supplementary Figure 1) and were then put at rest for 24h, as previously reported (174).

## **2.4 Mature Dendritic cell (mDC) differentiation**

THP-1 monocytes were harvested by centrifugation, resuspended in serum-free culture medium (without 10% heat-inactivated FBS) at a concentration of 2\*10<sup>5</sup>cells/ml, into 6 well plate with 200 ng/mL of IL-4, 100 ng/mL of GM-CSF, 200 ng/mL of ionomycin (Sigma) and 20 ng/mL of TNF- $\alpha$  (Peprotech) and incubated for 4 days at 5 % CO<sub>2</sub> 37°C. Medium exchange was performed every 2 days with fresh cytokine-supplemented medium. After cell differentiation confirmed via cytokine profile (Supplementary Figure 2), cells were cultured with complete medium (175).

## **2.5 Human Cell culture**

Macrophages M1 from THP-1 were cultured at 37°C 5% CO<sub>2</sub> in RPMI1640 (Gibco Life Technologies) and supplemented with 10% heat-inactivated FBS, 4.5 g/L glucose (Sigma-

Aldrich, Saint-Quentin-Fallavier), 1% penicillin/streptomycin 10 mM of HEPES, 1 mM sodium pyruvate and 0.05 mM of 2-mercaptoethanol (Gibco Life Technologies). Mature Dendritic cell (mDC) from THP-1 were cultured in RPMI1640 (Gibco Life Technologies) and supplemented with 10% heat-inactivated FBS, 4.5 g/L glucose (Sigma-Aldrich, Saint-Quentin-Fallavier), 1% penicillin/streptomycin 10 mM of HEPES and 1 mM sodium pyruvate in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Human dermal fibroblasts were kindly provided by the Cell and Tissue Bank (Hôpital Edouard-Herriot, Lyon, France). Normal breast human skin tissue explants were obtained from the surgical discard of anonymous healthy patients with informed consent of adult donors in accordance with ethical guidelines (French Bioethics law of 2004) and declared to the French research ministry (Declaration no. DC-2008-162 delivered to the Cell and Tissue Bank of Hospices Civils de Lyon). Exclusion criteria were related to positive test result for hepatitis B or C, or HIV as well as obesity history. Human dermal fibroblasts were cultured in a medium consisted in a ratio 1 :1 of Dulbecco's Modified Eagle's Medium with Glutamax and F-12 medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% FBS (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and 1% penicillin/streptomycin (Gibco Life Technologies). Human dermal microvascular endothelial cells (HDMEC) from adult donors (Promocell Heidelberg, Germany) were cultured at 37°C 5% CO<sub>2</sub> in the endothelial cell medium from Promocell composed by Dulbecco's Modified Eagle's Medium (DMEM) containing heparin 90 µg/ml, hydrocortisone 1 µg/ml supplemented with 2% Fetal Bovine Serum (FBS), 0.4% Endothelial Cell Growth Supplement (Promocell) and Epidermal Growth Factor (recombinant human) at 10ng/ml. Complete medium was supplemented with 1% penicillin/streptomycin (Gibco, Life Technologies, Carlsbad, CA, United States) at 37°C and 5% CO<sub>2</sub>. THP-1 cells, dermal fibroblasts and HDMEC were used below 20, 20 and 10 population doubling level, respectively.

## **2.6 Effect of HA and HA degradation products on immune cell response**

Macrophages M1 and mDC were seeded at 200 000 cells/ml and stimulated with the vehicle control, VYC-15L fragments, VYC-15L, HYC-24L+ fragments and HYC-24L+ at 100 µg/ml for 24h. LPS at 1µg/mL was included as a positive control. Cell supernatants were then collected and centrifuged at 10000 ×g for 10 min at 4°C. The amount of cytokines in macrophages M1 and mature DC (mDC) supernatant was determined using Human Magnetic Luminex Assay from R&D Systems (Minneapolis, MN, United States) and the levels of IL-8 (CXCL8), TNF-α, and IL-1β were analyzed using Bio-Plex MAGPIX Multiplex Reader and *Bio-Plex* Manager software

from Bio-Rad (Bio-Rad Laboratory, Hercules, CA, United States). Results were obtained from 3 independent experiments.

## **2.7 Effect of HA and HA degradation products on preconditioned dermal skin cells**

HDMEC and dermal fibroblasts were seeded at 50 000 cells/ml for 24h. After cell adhesion, cells were preconditioned using inflammatory medium produced by macrophages M1 at 1/100 dilution factor for 48h to induce a low-grade inflammatory phenotype as previously described (195). The preconditioned cells were then washed twice with PBS or HEPES, depending on cell type, and stimulated with the Vehicle Control, VYC-15L fragments, VYC-15L, HYC-24L+ fragments, or HYC-24L+ at 100 µg/ml for 72h. LPS at 1µg/mL was included as a positive control. Results were obtained from 3 independent experiments from 3 donors for HDMEC and dermal fibroblasts. Cell supernatants were collected and centrifuged at 10000 ×g for 10 min at 4°C. IL-8, IL-6 and IL-1β were quantified in Dermal fibroblasts and HDMEC supernatant using ELISA assay on from Abcam. Preconditioned cells were compared to unstimulated cells (∅).

## **2.8 Induction of low-grade inflammation in healthy mice**

Experiments were carried out in healthy male C57BL/6 mice (20g body weight) from Charles River (Le Genest-St-Isle, France). Mice were housed with ad libitum access to food and water, in a regulated environment with a constant temperature of 24°C and a day/night cycle of 12h/12h. All experiments were conducted in accordance with European Union recommendations for animal experimentation and approved by Rhone-Alpes ethics committee and then by the Ministry of Research - Project Authorization Service (agreement #18529). Special effort was made to minimize the number as well as the stress and suffering of mice used in this study. Before experimentation, animals were acclimatized for 1 week. To induce a systemic low-grade inflammatory phenotype, 100 µl of super low dose of LPS (O26:B6) at 5 ng/kg or PBS (control) was injected by intraperitoneal injection (IP) 3 times per week for 6 weeks as previously described (176, 177). In order to characterize the low-grade inflammation phenotype, blood was collected after sacrifice by intracardiac puncture and centrifuged at 3000 rpm during 10 min at 4°C to study CCL2 and IL-6 in the plasma using Luminex assay (R&D system). Cutaneous biopsy (punch 8mm ∅ at the back) was also performed for histological analysis and to study in the skin TNF-α and IL-1 β gene expression

using RT-qPCR and COX-2, IL-6 and VCAM-1 protein quantification using ELISA assay. These parameters collected from low-grade inflammatory mice were compared with those from healthy mice. Acute intradermal injection (20 $\mu$ l) of LPS at 5 $\mu$ g/ml was used as a positive control to induce strong inflammation in healthy mice.

## **2.9 Assessment of acetylcholine (ACh)-mediated vasodilation**

Two days prior to the microvascular experiments, the back was depilated with a depilatory lotion in mice under isoflurane to provide hairless areas for skin blood flow measurements and iontophoretic delivery of acetylcholine (ACh 2% Sigma) performed in low-grade inflammatory mice.

One day prior to the microvascular experiments, low-grade inflammatory mice received an acute intradermal injection (20 $\mu$ l) of the Vehicle Control, VYC-15L fragments (5.5 mg/ml), VYC-15L (15 mg/ml), HYC-24L+ fragments (8.8 mg/ml) or HYC-24L+ (24 mg/ml).

For the microvascular experiments, all mice were anaesthetized with thiopental (65 mg.kg<sup>-1</sup>, i.p.) and placed in an incubator (MMS, Chelles, France) to maintain a stable skin temperature (35.0 $\pm$ 0.5 $^{\circ}$ C). The cutaneous blood flow was measured using a laser Doppler probe (PF 4001 Master, Periflux, Perimed, Sweden) in response to iontophoretic delivery of ACh with an anodal current application (100  $\mu$ A for 10 s) at the site of intradermal injection. The laser Doppler signal expressed in arbitrary units (a.u.) was digitized with a 200 Hz sampling frequency using a computerized acquisition system (MP 100, Biopac System, CA). Local iontophoretic drug delivery was chosen to assess the *in vivo* cutaneous microvascular function while avoiding any systemic effects. Data collection started with a 1-minute control period before the ACh delivery and was continued for 20 minutes.

## **2.10 Mouse skin analysis**

For histological analysis, mouse skin was taken from the lower back 24h after an acute intradermal injection (20 $\mu$ l) of the Vehicle Control, VYC-15L fragments, VYC-15L, HYC-24L+ fragments, or HYC-24L+. Skin biopsy was fixed in AFA solution (alcohol, formalin, acetic acid). Samples were embedded in paraffin and sectioned at a thickness of 5  $\mu$ m. After dewaxing, the cuts were successively stained with Harris hematoxylin, eosin G and saffron. The matrix elements were colored in pinkish-yellow, the cytoplasm in pink, the nuclei in purple and HA in dark violet.

Skin tissue lysate was analyzed by ELISA assay on IL-6 from Abcam (Cambridge, UK). Skin tissue lysate was prepared by homogenization in T-PER lysis buffer and supplemented with protease and phosphatase inhibitor (ThermoFisher Scientific) in Tissue Lyzer using magnetic beads. Skin tissue lysates were centrifuged 10 min at 10000g and 4°C. Supernatant were then collected and quantified.

### **2.11 Mouse RNA Isolation and Real-Time Quantitative PCR**

Total RNA of skin samples was isolated using RNeasy Fibrous Tissue Mini Kit for skin biopsies (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. 500 ng of total RNA was reverse-transcribed into cDNA using PrimeScript™ RT reagent kit (Takara, Shiga, Japan) and analyzed by Real-Time QPCR using SYBR® Premix Ex Taq II (Takara) on an AriaMx Realtime PCR system (Agilent Genomics, Santa Clara, CA, United States). All primers were provided by Eurogentec. RPL13A forward GATGGTGGTTCCTGCTGCCC and reverse GGCTTTCTCTTCCTCCTCCTCC; ACTB forward CATTGCTGACAGGATGCAGAAGG and reverse TGCTGGAAGGTGGACAGTGAGG; TNF- $\alpha$  forward GGTGCCTATGTCTCAGCCTCTT and reverse GCCATAGAACTGATGAGAGGGAG and IL-1B forward TGGACCTTCCAGGATGAGGACA and reverse GTTCATCTCGGAGCCTGTAGTG. Results were normalized to RPL13A and ACTB. Relative quantification was calculated using the  $2^{-\Delta\Delta C_t}$  quantification method.

### **2.12 Statistical analyses**

Statistical analyses were performed using Prism (version 8.0, GraphPad Software Inc.). The results are expressed as mean $\pm$ SD. ACh-mediated vasodilation was expressed as the percentage changes of the cutaneous blood flow over the basal values, calculated as the average over the 1-min control period before the iontophoretic delivery of ACh. An unpaired t-test or one-way analysis of variance followed by Dunnett's multiple comparison test was used to compare two or more independent groups, respectively. A p-value less than 0.05 was regarded as statistically significant.

### 3. Results

#### 3.1 HA degradation products: characterization and endotoxin quantification

After a 6-hour incubation with hyaluronidase, VYC-15L and HYC-24L+ were degraded into fragments with a mean molecular weight of 30 and 80 kDa and a polydispersity of 2.4 and 3.3, respectively (Table 1).

VYC-15L fragments, HYC-24L+ fragments, and the Vehicle Control contained less than 0.02 EU/ml of LPS (Figure 1), showing that the degradation process did not introduce endotoxin and can be considered endotoxin free in comparison to the positive LPS control (500 EU/ml).

#### 3.2 Cytokines release by M1 and mDC in response to HA and HA degradation products

In the condition medium of macrophages M1 at the 24h time point (Figure 2A), TNF- $\alpha$  protein was overexpressed in the presence of VYC-15L fragments (233 $\pm$ 13 pg/ml), VYC-15L (407 $\pm$ 10 pg/ml), HYC-24L+ fragments (227 $\pm$ 8 pg/ml) and HYC-24L+ (384 $\pm$ 10 pg/ml) compared to the vehicle control (187 $\pm$ 5 pg/ml). IL-1 $\beta$  protein was only overexpressed in the presence of VYC-15L (296 $\pm$ 9 pg/ml) and HYC-24L+ (255 $\pm$ 5 pg/ml) compared to the vehicle control (94 $\pm$ 3 pg/ml). In contrast, IL-8 protein was not modified in any HA treatment compared to the vehicle control (21475 $\pm$ 390 pg/ml). However, the overexpression of cytokines by M1 mentioned above was much lower compared to the positive LPS control (Supplementary Figure 3A).

In the condition medium of mDC at the 24h time point (Figure 2B), no difference of TNF- $\alpha$  protein level was observed between conditions. Level of IL-1 $\beta$  was too low to be detectable by Luminex assay except for LPS condition as positive control (Supplementary Figure 3B). IL-8 protein was only over expressed in the presence of VYC-15L fragments (4842 $\pm$ 325 pg/ml) compared to the vehicle control (2943 $\pm$ 242 pg/ml), although this overexpression was much lower compared to the positive LPS control (29697 $\pm$ 500 pg/ml) (Supplementary Figure 3B).

No differences were observed for any cytokines and any HA treatment group at the 48h and 72h time points (Supplementary Figure 4), suggesting a transient effect of HA products in contrast to the positive LPS stimulation that lasted up to 72h (data not shown).

### **3.3 Cytokines released by preconditioned fibroblasts (low-grade inflammatory phenotype) in response to HA and HA degradation products at 72h**

Compared to non-inflammatory dermal fibroblasts, IL-8, IL-1 $\beta$  and IL-6 were overexpressed in fibroblasts preconditioned with M1 macrophages, but to a lesser extent than the positive LPS stimulation (Supplementary Figure 5), showing a low-grade inflammation in 72h post-preconditioned fibroblasts.

In preconditioned dermal fibroblasts, VYC-15L fragments, VYC-15L, HYC-24L+ fragments and HYC-24L+ did not induce overexpression of cytokines (IL-8, IL-1 $\beta$  and IL-6) compared to the vehicle control at 72h (138 $\pm$ 17pg/ml for IL-8; 105 $\pm$ 5pg/ml for IL-1 $\beta$  and 68 $\pm$ 1.7pg/ml for IL-6) (Figure 3), except a slight down-regulation of IL-1 $\beta$  (1.2-fold) in response to VYC-15L, HYC-24L+ fragments and HYC-24L+. In contrast, the positive LPS stimulation induced a strong overexpression for IL-8 (407 $\pm$ 9 pg/ml), IL-1 $\beta$  (140 $\pm$ 5 pg/ml) and IL-6 (503 $\pm$ 7 pg/ml) in preconditioned dermal fibroblasts (Supplementary Figure 5).

### **3.4 Cytokines released by preconditioned HDMEC (low-grade inflammatory phenotype) in response to HA and HA degradation products at 72h**

Compared to non-inflammatory HDMEC, IL-8, IL-1 $\beta$  and IL-6 were overexpressed in preconditioned HDMEC, but to a lesser extent than LPS stimulation (Supplementary Figure 6), showing a low-grade inflammation in 72h post- preconditioned HDMEC.

In preconditioned HDMEC, VYC-15L fragments, VYC-15L, HYC-24L+ fragments, and HYC-24L+ did not induce overexpression of cytokines (IL-8 and IL-1 $\beta$ ) compared to the vehicle control at 72h (756 $\pm$ 6.9 pg/ml for IL-8 and 44 $\pm$ 3.9 pg/mL for IL-1 $\beta$ ) (Figure 4). However, IL-6 expression (Figure 4A) decreased in the presence of VYC-15L fragments (25 $\pm$ 2 pg/ml) and VYC-15L (53 $\pm$ 6 pg/ml) compared to the vehicle control (76 $\pm$ 3 pg/ml). In contrast, the positive LPS stimulation induced a strong overexpression for IL-8 (794 $\pm$ 13 pg/ml), IL-1 $\beta$  (76 $\pm$ 4 pg/ml) and IL-6 (792 $\pm$ 15 pg/ml) in preconditioned HDMEC (Supplementary Figure 6).

### 3.5 *In vivo* mouse model of low-grade inflammation

The plasma level of IL-6 was higher in low-grade inflammation mice ( $19\pm 4$  pg/ml) than in control mice ( $8\pm 1$  pg/ml), representing an increase of 225%. Similarly, CCL2 plasma level was higher in low-grade inflammation mice ( $239\pm 50$  pg/ml) than in control mice ( $148\pm 43$  pg/ml), representing an increase of 161%.

In the skin, the upregulations of TNF- $\alpha$  (2.8-fold) and IL-1 $\beta$  (1.3-fold) gene expression in the skin of low-grade inflammation mice were not significant compared to control mice (Supplementary Figure 7A). Similarly, the increase of +130% in COX-2 protein concentration in the skin of low-grade inflammation mice ( $2410\pm 385$  pg/ml) was not significant compared to control mice ( $1868\pm 312$  pg/ml), such as positive control ( $1764\pm 256$  pg/ml) (Supplementary Figure 7B). No increase of IL-6 protein concentration in the skin of low-grade inflammation mice ( $103.9\pm 22$  pg/ml) was observed compared to control mice ( $102\pm 16$  pg/ml) (Supplementary Figure 7B). Again, the increase of +123% in VCAM-1 protein concentration in the skin of low-grade inflammation mice ( $8493\pm 1418$  pg/ml) was not significant compared to control mice ( $6903\pm 1695$  pg/ml) (Supplementary Figure 7B). These moderate increases of cytokine expression validated our mouse model of low-grade inflammation in the skin, in comparison to the strong inflammation obtained in the positive control mice (Supplementary Figure 7). Interestingly, low-grade inflammation increased intragroup heterogeneity as shown by the increased coefficient of variation (CV) in low-grade inflammation mice compared to control mice: 21% vs 7% for IL-6 plasma level, 29% vs 20% for CCL2 plasma level, 47% vs 39% for TNF- $\alpha$  gene expression, and 103% vs 71% for IL-1 $\beta$  gene expression and correlated with standard deviation observed.

In addition, no differences were observed between control mice and low-grade inflammation mice on skin structure by histological analysis. No higher immune cell infiltration and differences in thickness were observed in comparison to the infiltration observed in the positive control mice (Supplementary Figure 7C).

On the vascular aspect, basal skin blood flow was not different between control mice ( $45\pm 13$  arbitrary units (a.u.)), low-grade inflammation mice ( $49\pm 11$  a.u.) and positive control mice ( $49\pm 9$  a.u.). Similarly, the ACh-mediated vasodilation was not different between low-grade inflammation mice ( $32\pm 15\%$ ) and control mice ( $43\pm 12\%$ ), in contrast to the augmented ACh-

mediated microvascular reactivity in the positive control mice ( $69\pm 32\%$ ) (Supplementary Figure 7D).

### **3.6 Effects of HA and HA degradation products on skin structure in mice**

To study the effects of HA and HA degradation products in a low-grade inflammation environment, the histological analyses were performed 24h after the intradermal injections of vehicle, VYC-15L fragments, VYC-15L, HYC-24L+ fragments, and HYC-24L+ in low-grade inflammation mice (Figure 5).

Vehicle induced a very limited inflammation (Figures 5A). The injection of VYC-15L fragments and VYC-15L induced a moderate inflammation around the products (Figures 5B and 5C) with immune cell recruitment. While, in the presence of HYC-24L+ products (Figures 5D and 5E) immune cells seemed to be less infiltrated than in the presence of VYC-15L products.

### **3.7 Skin IL-6 in response to HA and HA degradation products in control and low-grade inflammation mice**

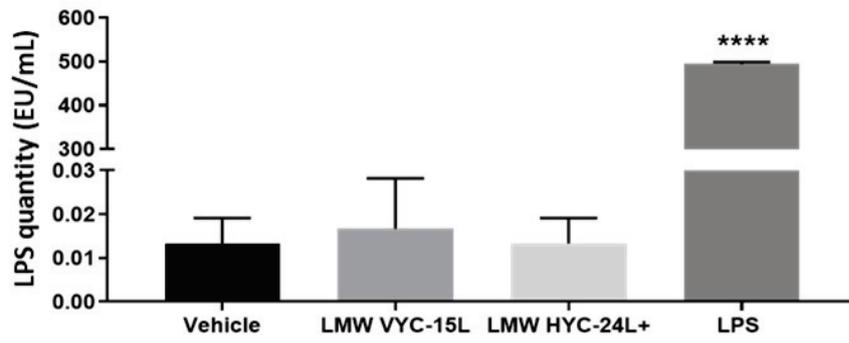
In low-grade inflammation mice, IL-6 protein was only overexpressed in the presence of VYC-15L ( $238\pm 52$  pg/ml) compared to the vehicle control mice ( $102\pm 20$  pg/ml) (Figure 6), associated with an enhanced intragroup heterogeneity.

### **3.8 Effects of HA and HA degradation products on skin microcirculation in mice**

In low-grade inflammation mice, basal skin blood flow was only increased by the injection of VYC-15L ( $67\pm 18$  a.u.) compared to the vehicle control ( $49\pm 11$  a.u.) (Figure 7A). But, ACh-mediated vasodilation was increased by the injection of VYC-15L ( $87\pm 33\%$ ) and VYC-15L fragments ( $72\pm 40\%$ ) compared to the vehicle control ( $32\pm 15\%$ ) (Figure 7B). This enhanced ACh-mediated vasodilation was associated with an elevated heterogeneity as shown by an increased standard deviation.

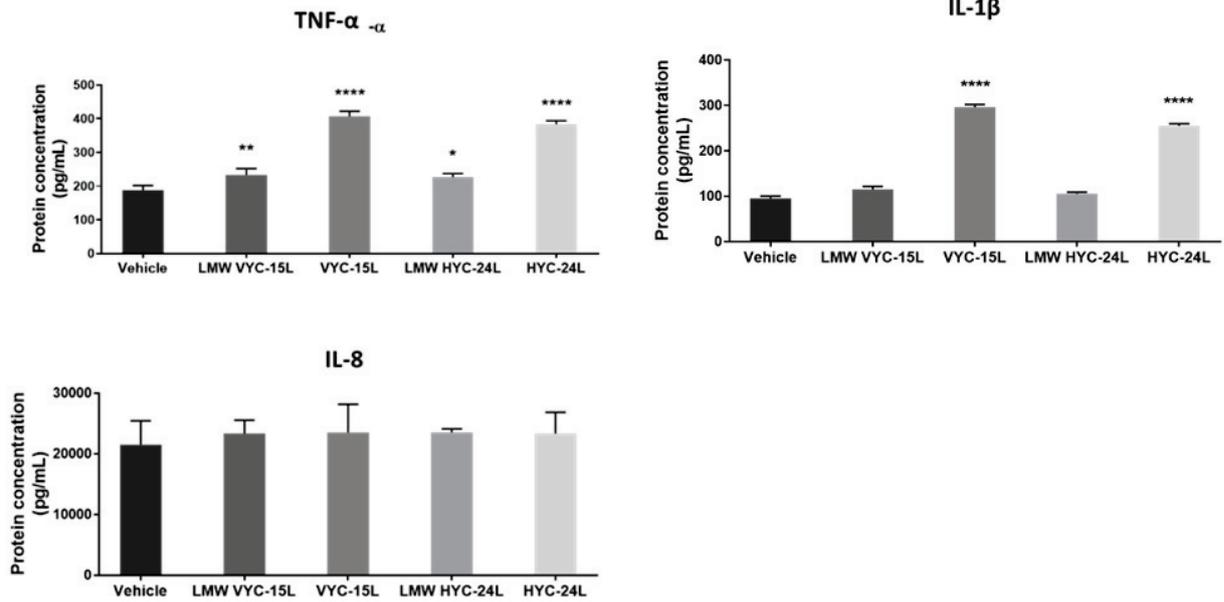
Degraded (6h)	Mean Mw (kDa)	Polydispersity
VYC-15L fragments	30 ± 4,6	2,4
HYC-24L+ fragments	80 ± 1,2	3,3

**Table 1.** characterization (HPLC) of VYC-15L fragments and HYC-24L+ fragments after 6 hours of degradation. Mean (n=3) value ± SD for molecular weight (MW) and polydispersity of samples.

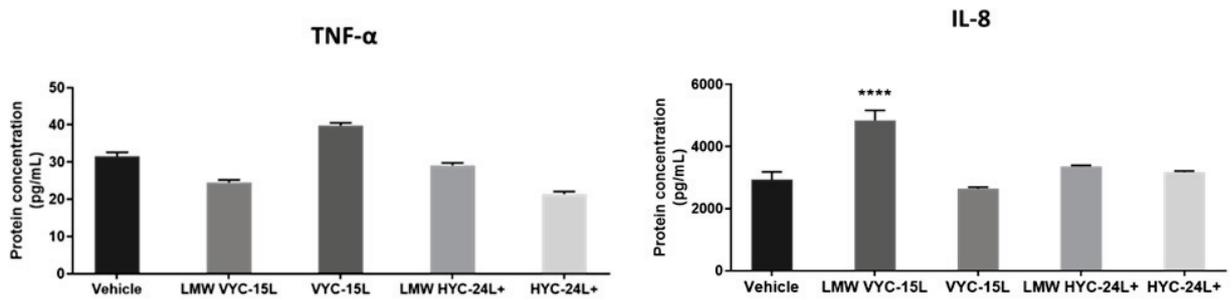


**Figure 1.** Endotoxin quantification. Mean $\pm$ SD of LPS level (EU/ml) in the vehicle control (n=2), LMW VYC-15L (n=2), LMW HYC-24L+ (n=2) and LPS (positive control, n=2) samples. \*\*\*\*P < 0.0001 vs vehicle.

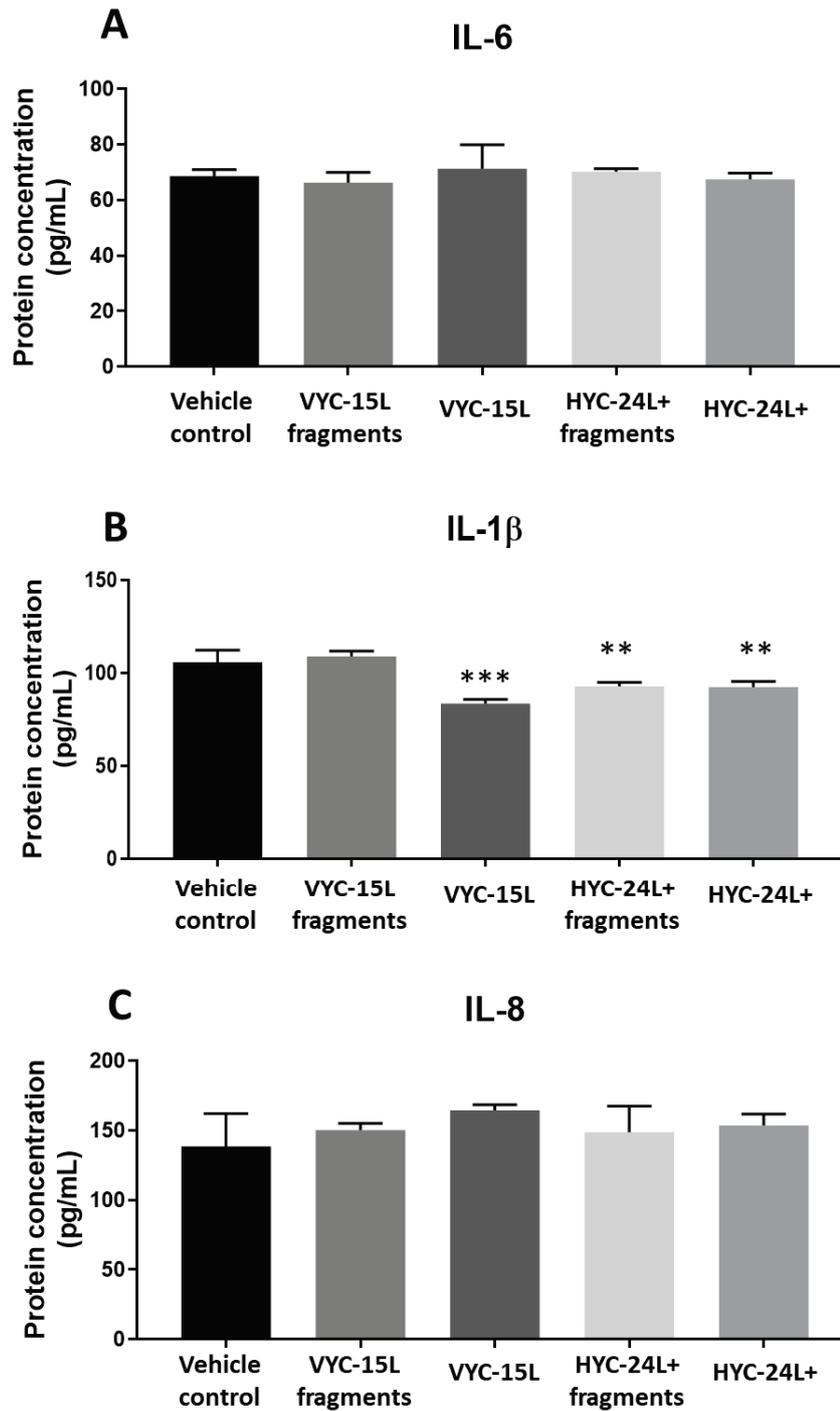
## A Macrophages M1 cytokines secretion



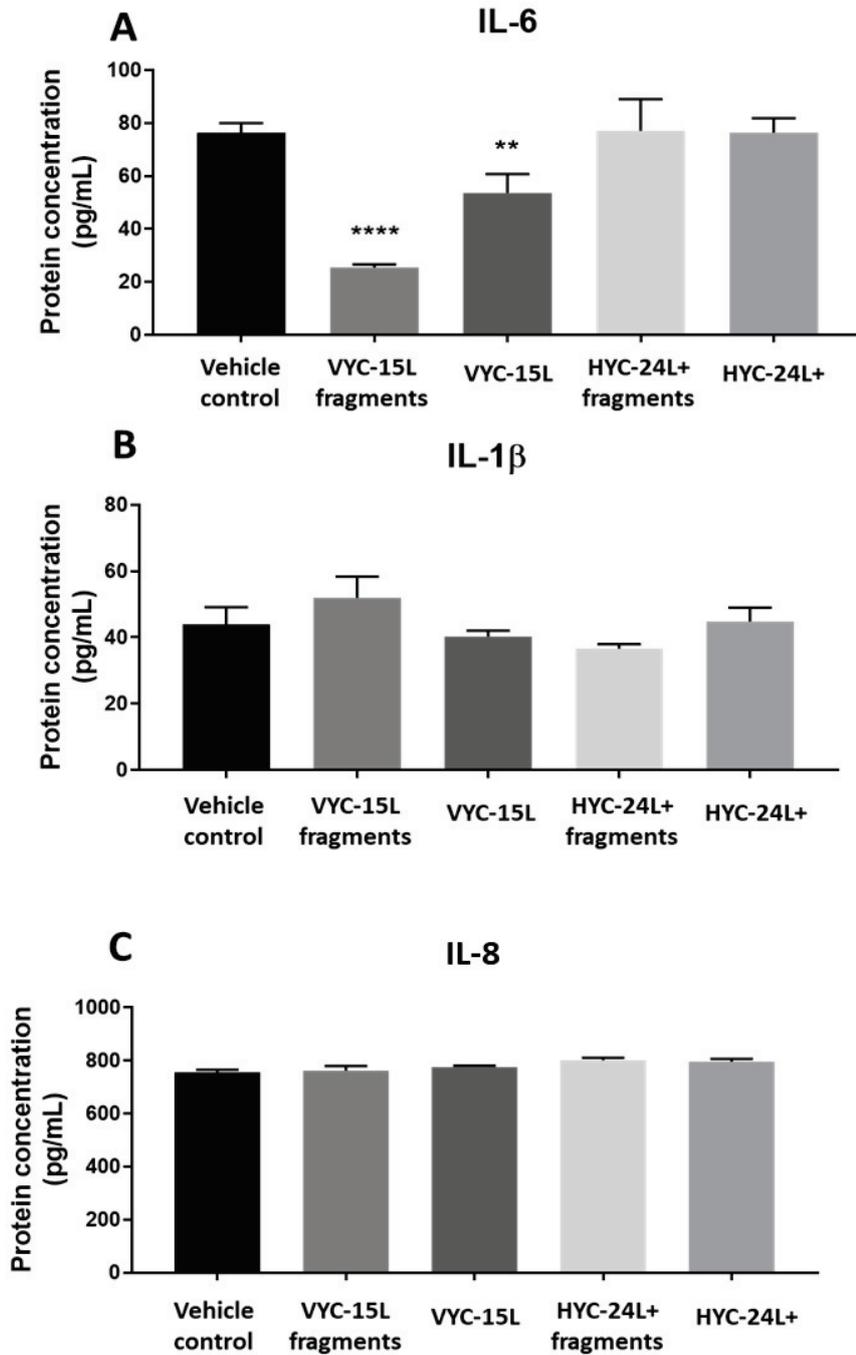
## B mDC cytokines secretion



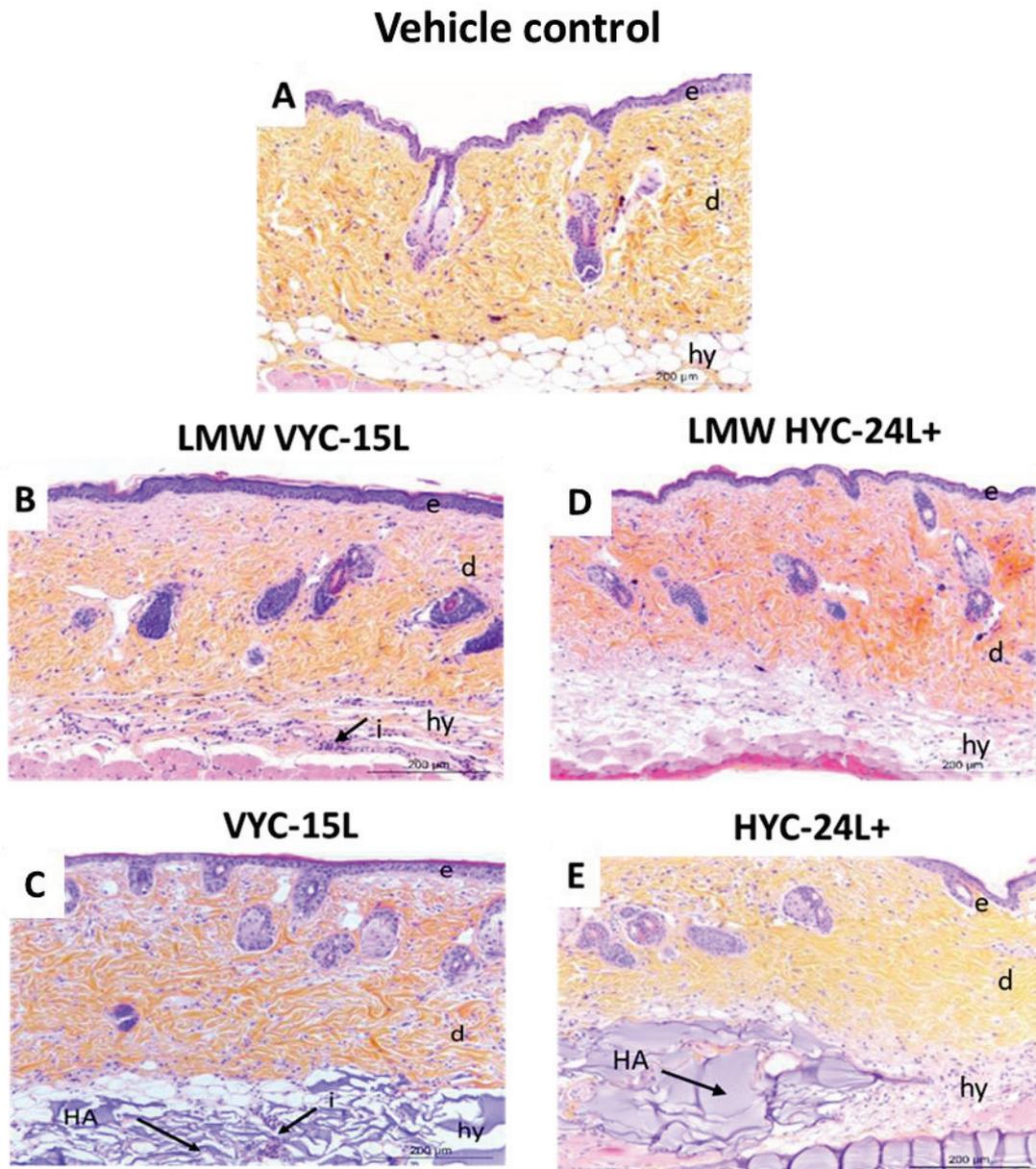
**Figure 2:** Effects of HA and HA degradation products on Cytokines release by M1 and mDC at the 24h time point. Mean $\pm$ SD of protein level (pg/ml) of TNF- $\alpha$ , IL-8 and IL-1 $\beta$  (except for mDC), in the condition medium of M1 after stimulation with vehicle, LMW VYC-15L, VYC-15L, LMW HYC-24L+ and HYC-24L+. \*P<0.01, \*\*P<0.001, \*\*\*\*P<0.0001. vs vehicle.



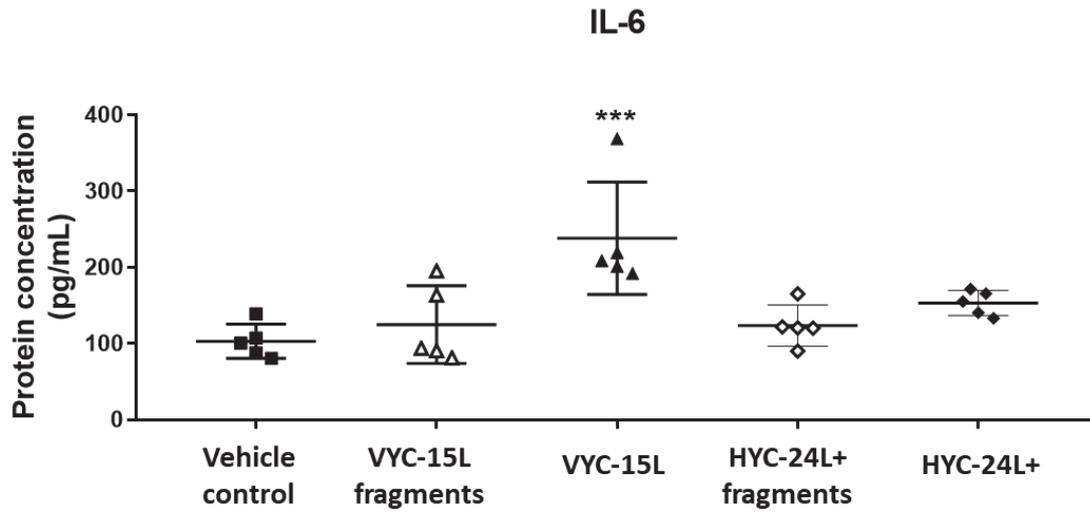
**Figure 3:** Effects of HA and HA degradation products on cytokines release by preconditioned dermal fibroblasts at the 72h time point. Mean $\pm$ SD of protein level (pg/ml) of IL-6, IL-1 $\beta$  and IL-8 in preconditioned dermal fibroblasts stimulated with vehicle, VYC-15L fragments, VYC-15L, HYC-24L+ fragments and HYC-24L+.



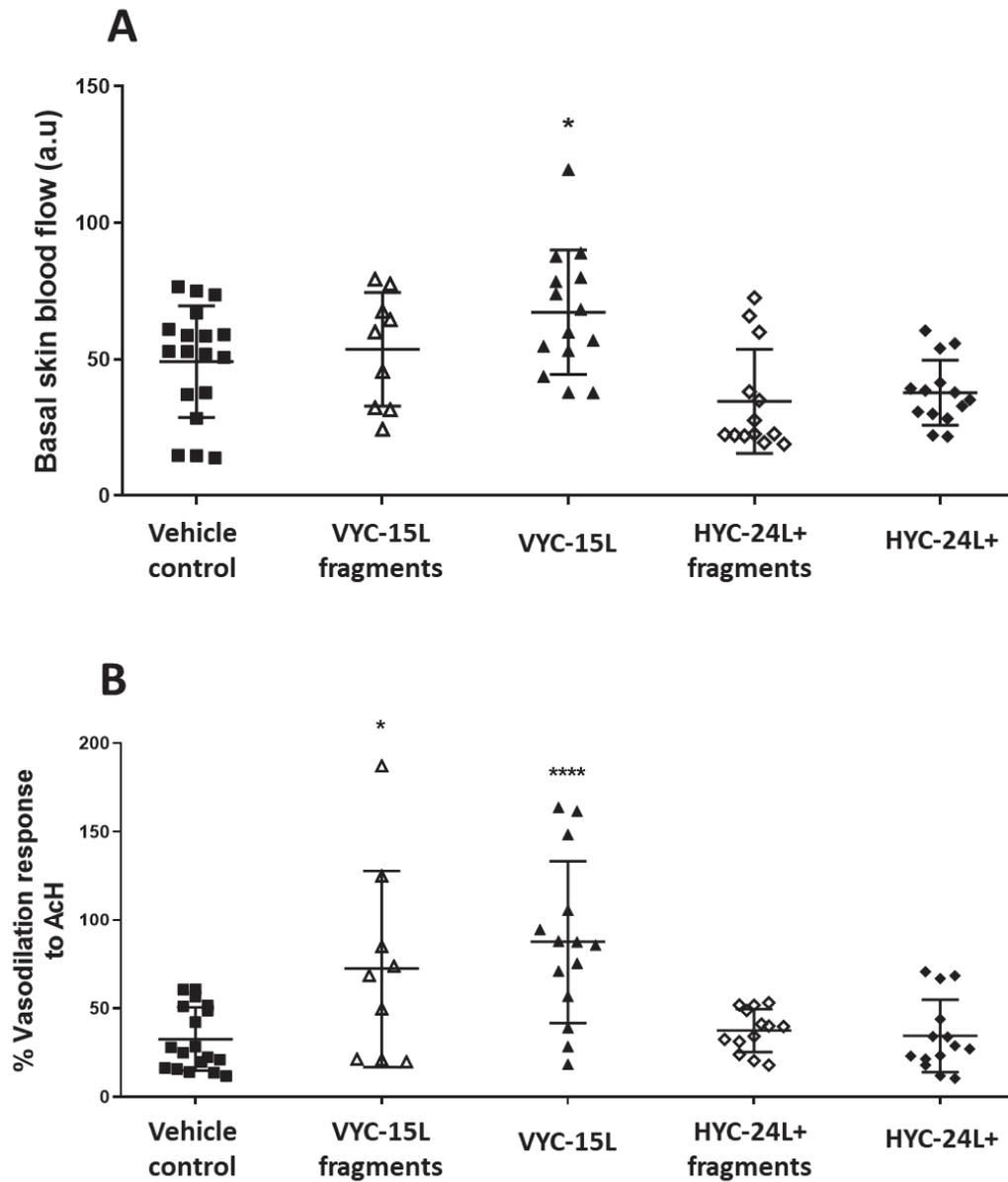
**Figure 4:** Effect of HA and HA degradation products at 72 h on cytokine release by preconditioned HDMEC. Mean $\pm$ SD of Protein level (pg/ml) of IL-6, IL-1 $\beta$  and IL-8 in preconditioned HDMEC stimulated with vehicle, VYC-15L fragments, VYC-15L, HYC-24L+ fragments and HYC-24L+.. \*\*P<0.001, \*\*\*\* P0.0001 vs the vehicle control.



**Figure 5:** Representative HES coloration skin (X20) in low-grade inflammation mice 24h after an acute intradermal injection (20µl) of the vehicle control (A), LMW VYC-15L (B), VYC-15L (C), LMW HYC-24L+ (D) and HYC-24L+ (E). e=epidermis, d=dermis, hy=hypodermis, i=inflammation and HA=hyaluronic acid. Hyaluronic acid is colored in dark violet, extracellular elements in yellow and nuclei of cells in purple.



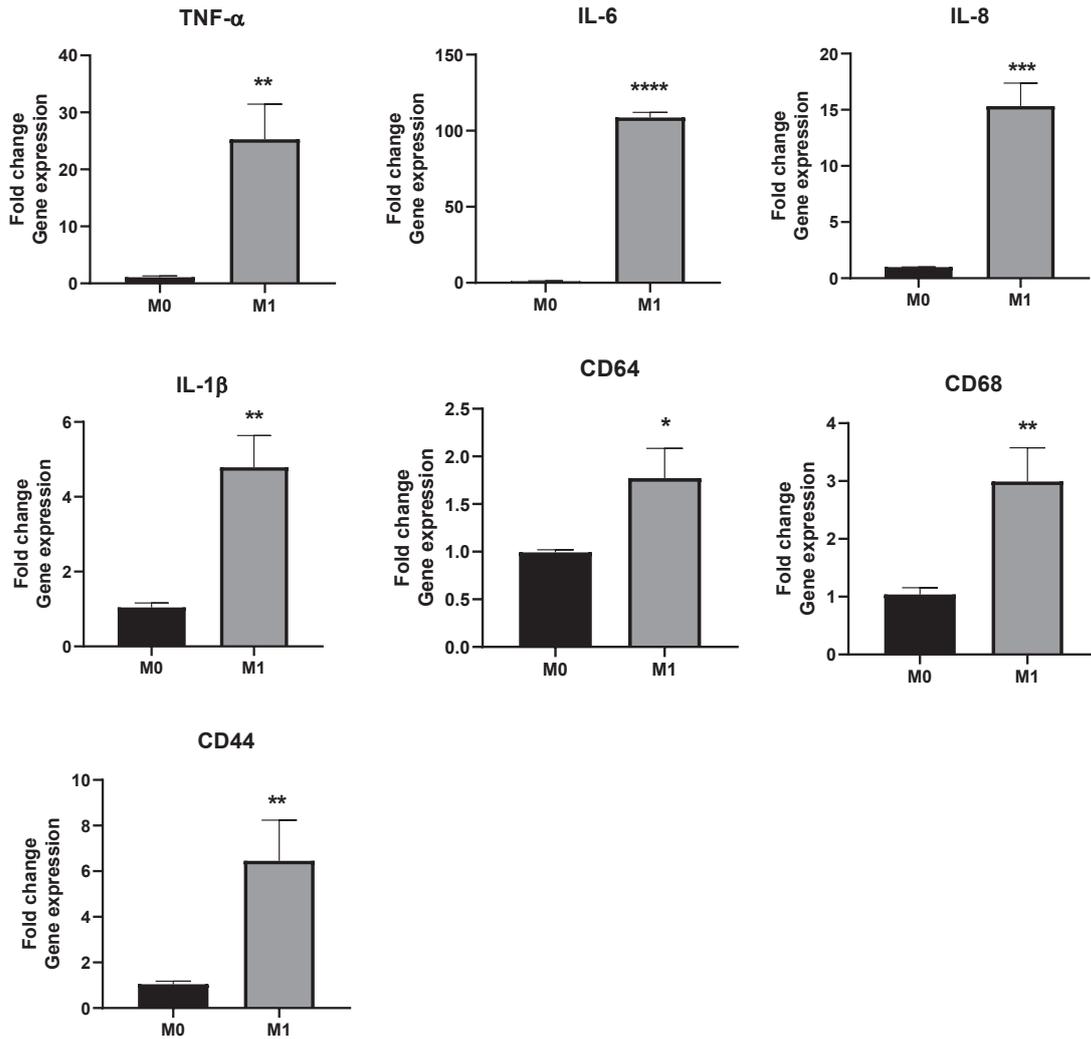
**Figure 6:** IL-6 protein concentration in skin in low-grade inflammatory mice (n=5), 24h after an acute intradermal injection (20 $\mu$ l) of the Vehicle Control, VYC-15L fragments, VYC-15L, HYC-24L+ fragments, and HYC-24L+. All samples were normalized to 1000 $\mu$ g of total protein. \*\*\*P<0.001 vs the Vehicle Control.



**Figure 7:** Basal skin blood flow (A) and ACh-mediated vasodilation (B) in low-grade inflammatory (n=5) mice 24h after intradermal injection of the Vehicle Control, VYC-15L fragments, VYC-15L, HYC-24L+ fragments, and HYC-24L+. \*P<0.05, \*\*\*\*P<0.0001 vs the Vehicle Control. Each point represents one measurement of microvascular experiments.

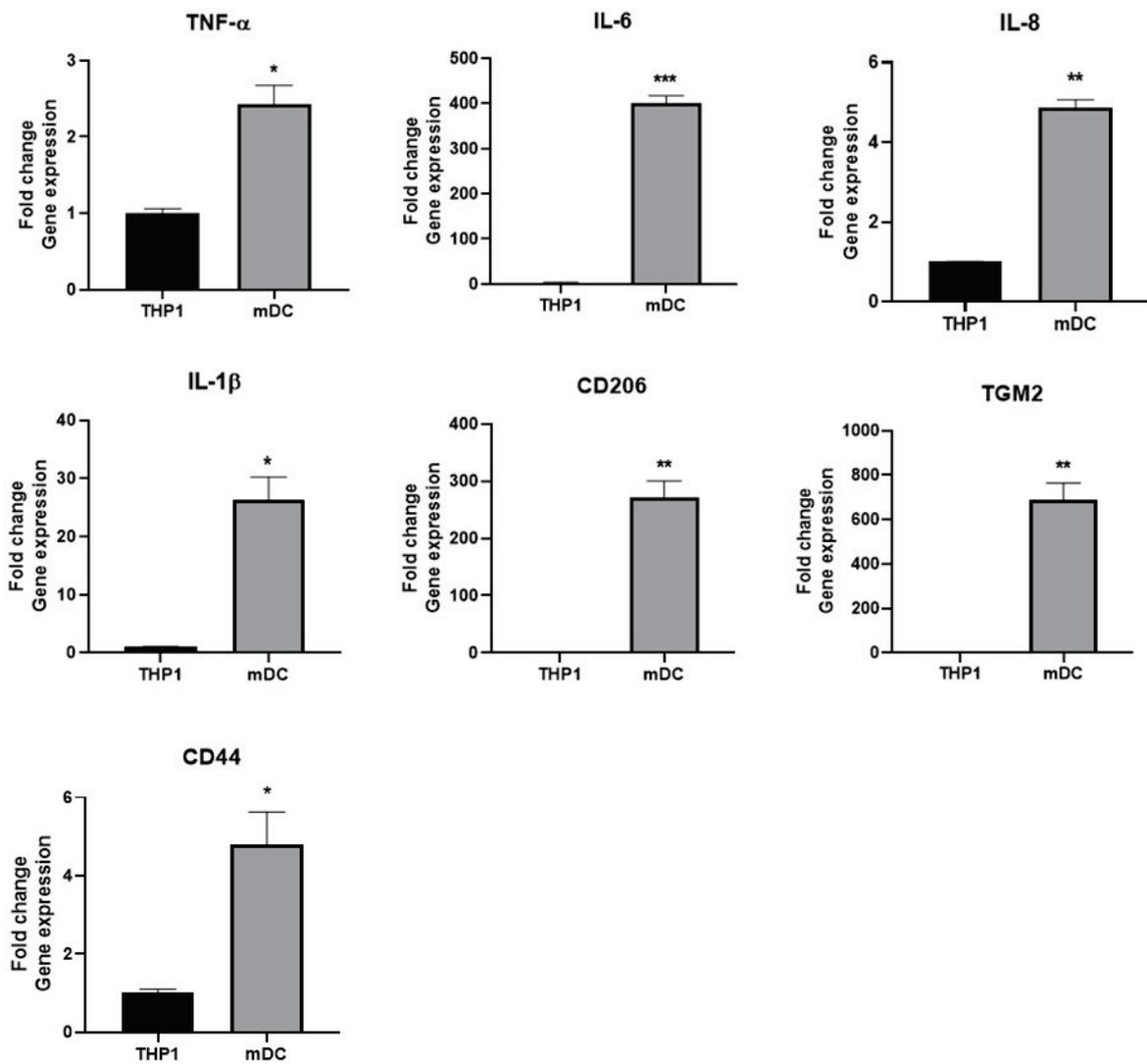
Cytokine	M0 (Mean pg/mL ± SD)	M1 (Mean pg/mL ± SD)	P-value * <0.05
TNF- $\alpha$	119 $\pm$ 2	187 $\pm$ 2	<b>0,0211</b>
IL-8	4151 $\pm$ 51	21475 $\pm$ 39	<b>&lt;0,0001</b>
CCL2	198 $\pm$ 14	213 $\pm$ 11	ns
IL-1 $\beta$	255 $\pm$ 15	94 $\pm$ 2	<b>&lt;0,0001</b>
IFN-gamma	88 $\pm$ 2	181 $\pm$ 7	<b>0,0014</b>
CCL5/RANTES	202 $\pm$ 7	410 $\pm$ 8	<b>&lt;0,0001</b>
GMC-SF	120 $\pm$ 10	308 $\pm$ 5	<b>&lt;0,0001</b>
VEGF-A	72 $\pm$ 2	147 $\pm$ 2	<b>&lt;0,0001</b>

**Supplementary Table 1: Characterization of the pro-inflammatory M1 culture medium**



### Supplementary figure 1: Macrophages M1 phenotype

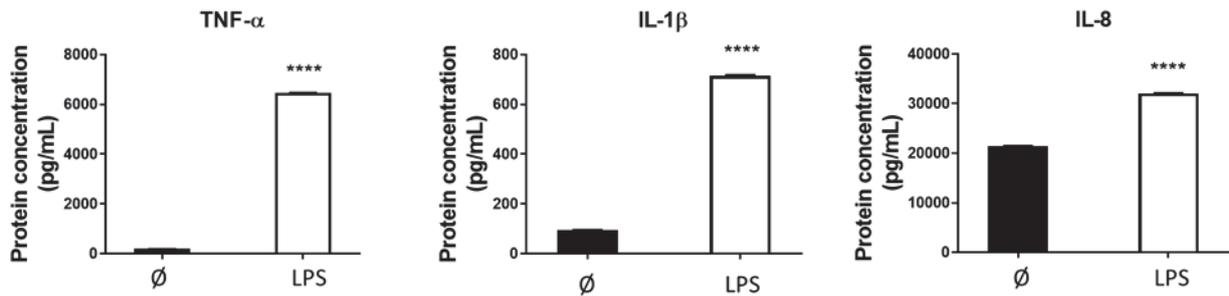
Pro-inflammatory Macrophages M1 phenotypes. M1 markers gene expression of TNF- $\alpha$ , IL-6, IL-8, IL1 $\beta$ , CD44, CD64 and CD68. Genes expressions are normalized with GAPDH and TBP and data are represented in fold change. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 vs. Macrophages M0. Data are representative of at least three independent experiments.



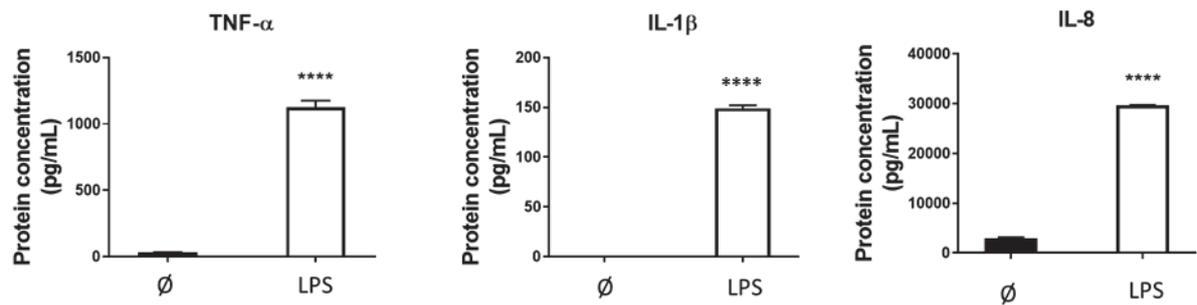
### Supplementary figure 2: Mature Dendritic cell phenotype

Pro-inflammatory mDC phenotypes. mDC markers gene expression of TNF- $\alpha$ , IL-6, IL-8, IL1 $\beta$ , CD44, CD64 and CD68. Genes expressions are normalized with GAPDH and TBP and data are represented in fold change. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. vs. THP1. Data are representative of at least three independent experiments.

### A Macrophages M1 LPS stimulated



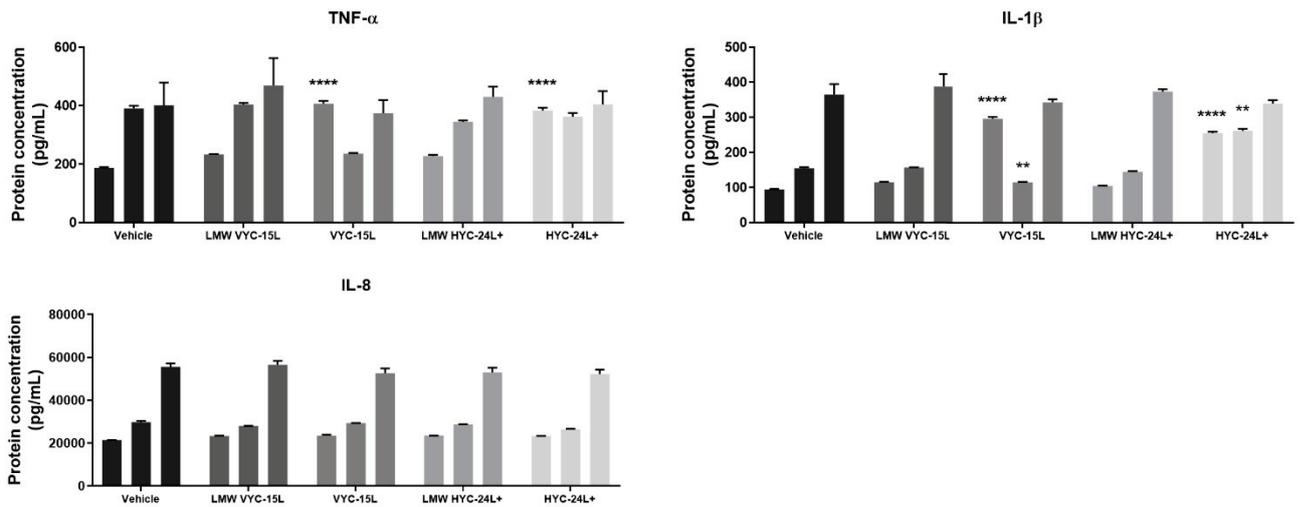
### B mDC LPS stimulated



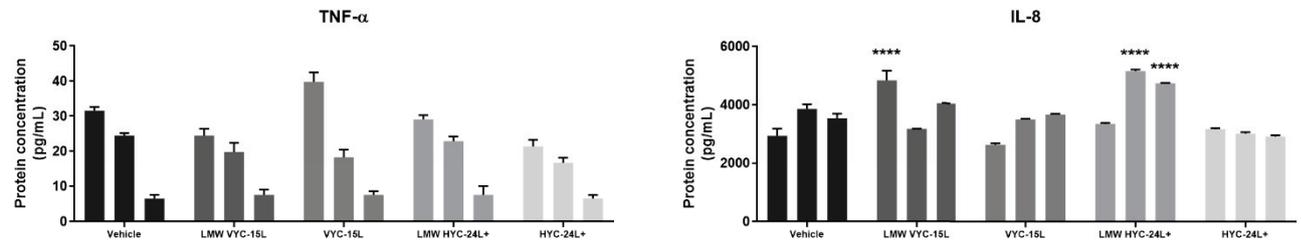
### Supplementary figure 3: LPS effect on M1 and mDC cytokine production at 24h

Supernatant produced by immune cells. (A) macrophages M1 secretion and (B) mDC secretion after 24h stimulation of LPS at 1 $\mu$ g/mL. TNF- $\alpha$ , IL-8 and IL-1 $\beta$  were quantified. Data are representative of at least three independent experiments. \*\*P<0.01, \*\*\*\*P<0.0001. vs. immune cells unstimulated.

### A Macrophage M1 cytokine secretion

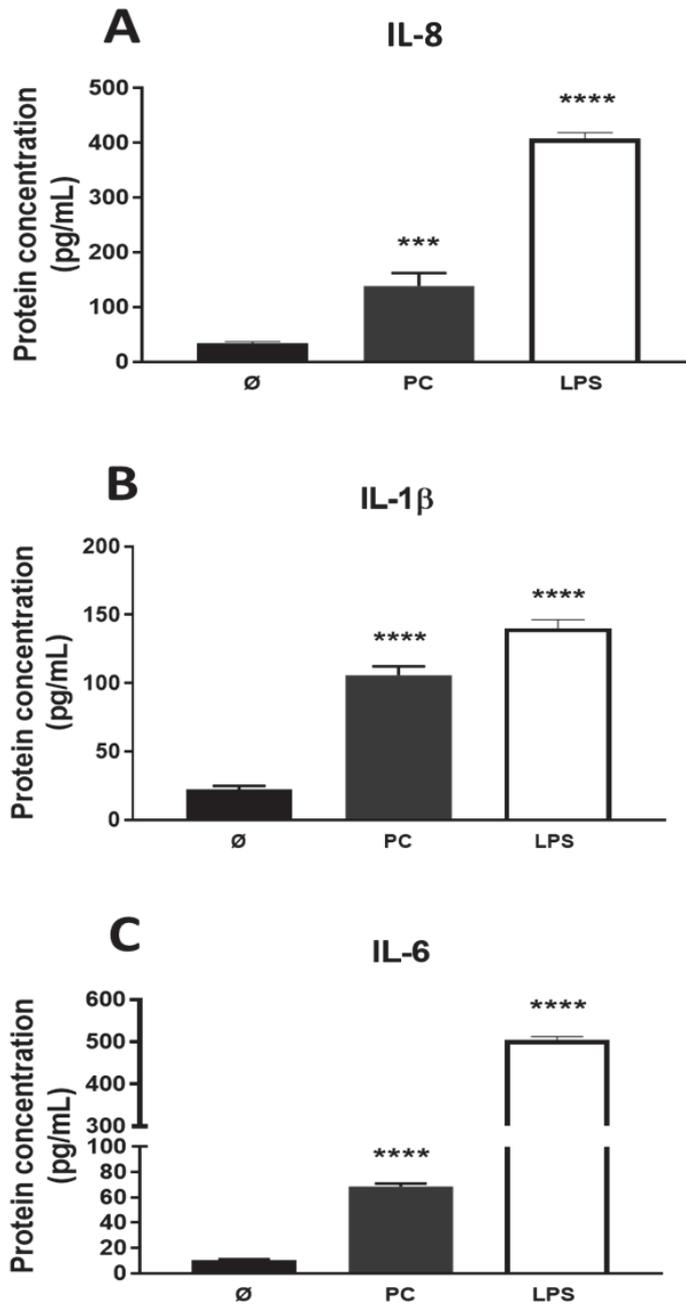


### B mDC cytokine secretion



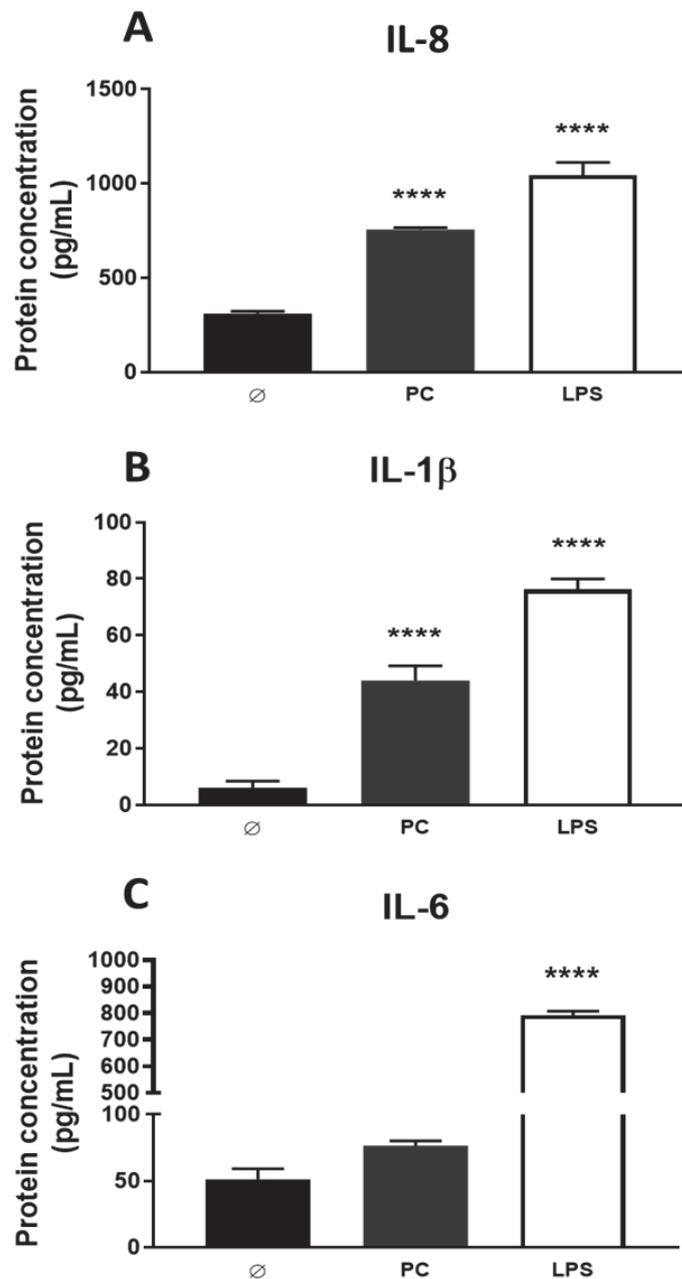
## Supplementary figure 4: long-term exposure 48h and 72h with HA and HA degradation products

Supernatant produced by immune cells. (A) macrophages M1 secretion and (B) mDC secretion after 24h, 48h and 72h stimulation. TNF-α, IL-8 and IL-1β (except for mDC) were quantified in the presence of vehicle, LMW of VYC-15L, VYC-15L, LMW of HYC-24L+ and HYC-24L+. Each column of each condition corresponds to a different kinetics of stimulation, left column corresponds to 24h, middle column corresponds to 48h and right column corresponds to 72h stimulation. Data are representative of at least three independent experiments. \*\*P<0.01, \*\*\*\*P<0.0001. vs. vehicle



**Supplementary figure 5: low-grade inflammation phenotype for preconditioned dermal fibroblasts**

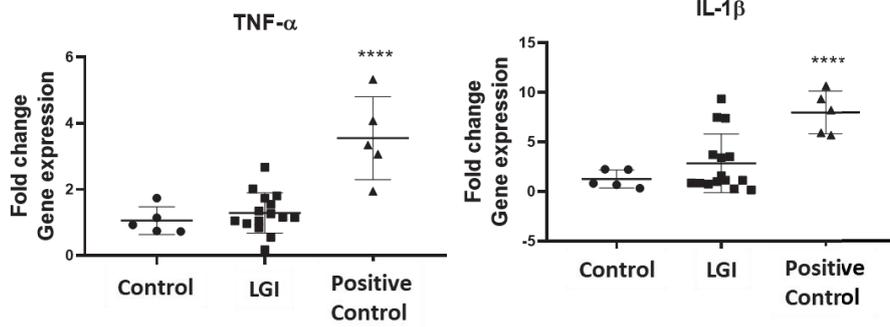
Cytokine release in unstimulated ( $\emptyset$ ), preconditioned (PC) and LPS-stimulated dermal fibroblasts. Protein level (pg/ml) of IL-8, IL-1 $\beta$  and IL-6 72h post preconditioning. LPS is considered as the positive control for inflammation. \*\*\*P<0.001, \*\*\*\*P<0.0001. vs non-preconditioned (non-inflammatory) Fibroblast Unstimulated ( $\emptyset$ ).



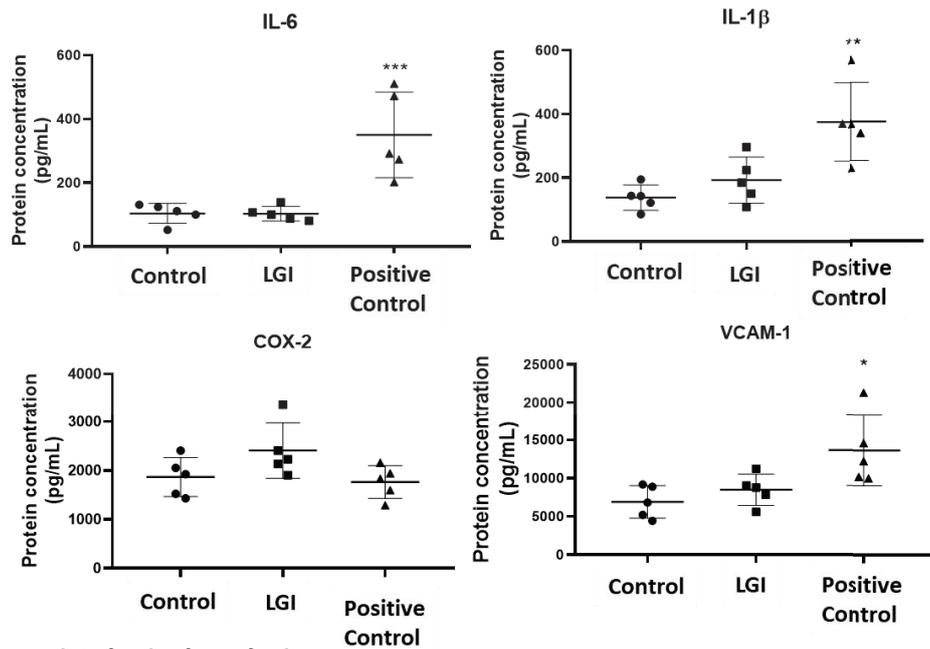
**Supplementary Figure 6: low-grade inflammation phenotype for preconditioned HDMEC**

Cytokine release in unstimulated ( $\emptyset$ ), preconditioned (PC) and LPS-stimulated dermal endothelial cells (HDMEC). Protein level (pg/ml) of IL-8, IL-1 $\beta$  and IL-6 72h-post preconditioning. LPS is considered as positive control for inflammation. \*\*\*\*P<0.00001. vs non-preconditioned (non-inflammatory) HDMEC ( $\emptyset$ ).

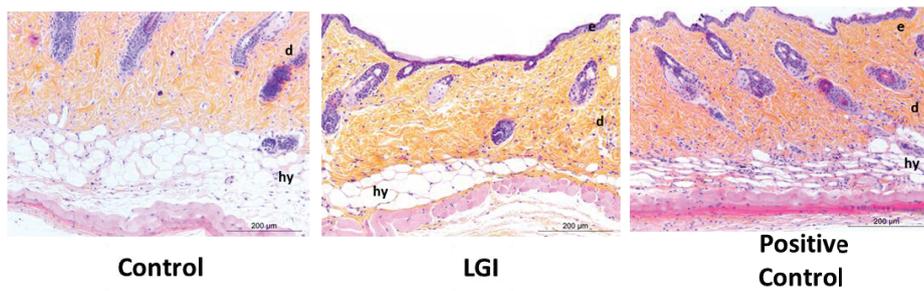
### A skin mRNA expression



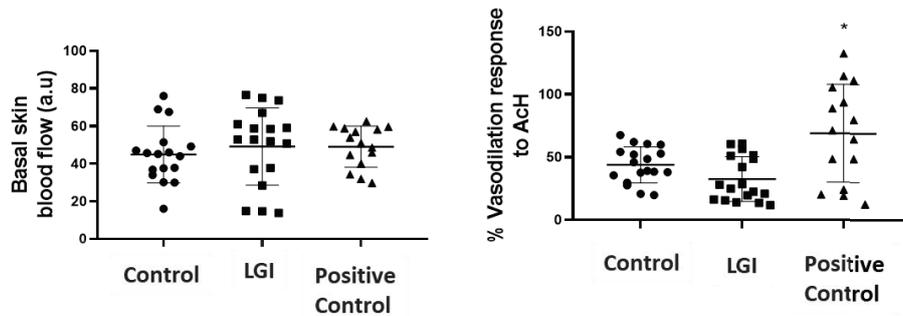
### B skin protein quantification



### C Histological analysis



### D Vascular properties



Supplementary figure 7: low-grade inflammation phenotype in mice

Scatter dot plot showing A, fold change gene expression of TNF- $\alpha$  and IL-1 $\beta$  in skin biopsy from control mice (n=5), low-grade inflammation mice (LGI) induced by a chronic treatment of low dose of LPS (n=15) and positive control mice treated with an acute intradermal injection of LPS (n=5); B, Protein level of IL-6, IL-1 $\beta$ , VCAM-1 and COX-2 in control mice (n=5), low-grade inflammation mice (n=5) and positive LPS control mice (n=5); C, Representative histological analysis with HES coloration skin in one control mouse, one low-grade inflammation mouse and one positive LPS control mice; D, vascular properties with basal skin blood flow and acetylcholine (ach)-mediated microvascular reactivity in control mice (n=17), low-grade inflammation mice (n=18) and positive control mice (n=15). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 vs Control (PBS).

## 1.16 Complementary Results

### 1.16.1 Effect of range sizes of HA on immune cells

To assess the potential inflammatory effect of different range sizes of HA, cytokines released by macrophages M1 and mDC were quantified by Luminex assay. Immune cells were stimulated for 24h, 48h and 72h with the negative vehicle control, the 3 range sizes of HA fragments degraded from VYC-15L and HYC-24L+ and the two non-degraded HA.

On M1, we showed that IL-8, TNF- $\alpha$  and IL-1 $\beta$  were not overexpressed in presence of the 3 different range sizes of each crosslinked HA at 24h, 48h and 72h (Figure 16). As described above, only HA non-degraded induced cytokine overexpression of TNF- $\alpha$  and IL-1 $\beta$  at 24h. However, the overexpression of cytokines by M1 mentioned above was much lower compared to the positive LPS control.

In the condition medium of mDC, no difference of TNF- $\alpha$  protein level has been observed between conditions at 24h, 48h or 72h (Figure 17). IL-8 protein was overexpressed in the presence of VYC-15L fragments compared to the vehicle control. Indeed, at the 24h time point, VYC-15L fragments of 100kD, 90kDa and 30kDa induced  $5935 \pm 222$ ,  $4047 \pm 153$  and  $4842 \pm 320$  pg/mL of IL-8, respectively, while vehicle induced  $2990 \pm 242$  pg/mL of IL-8. Interestingly, we showed that HYC-24L+ fragments induced a decrease in IL-8 secretion. IL-1 $\beta$  was too low to be detectable by Luminex assay but was observed in presence of LPS as positive control.

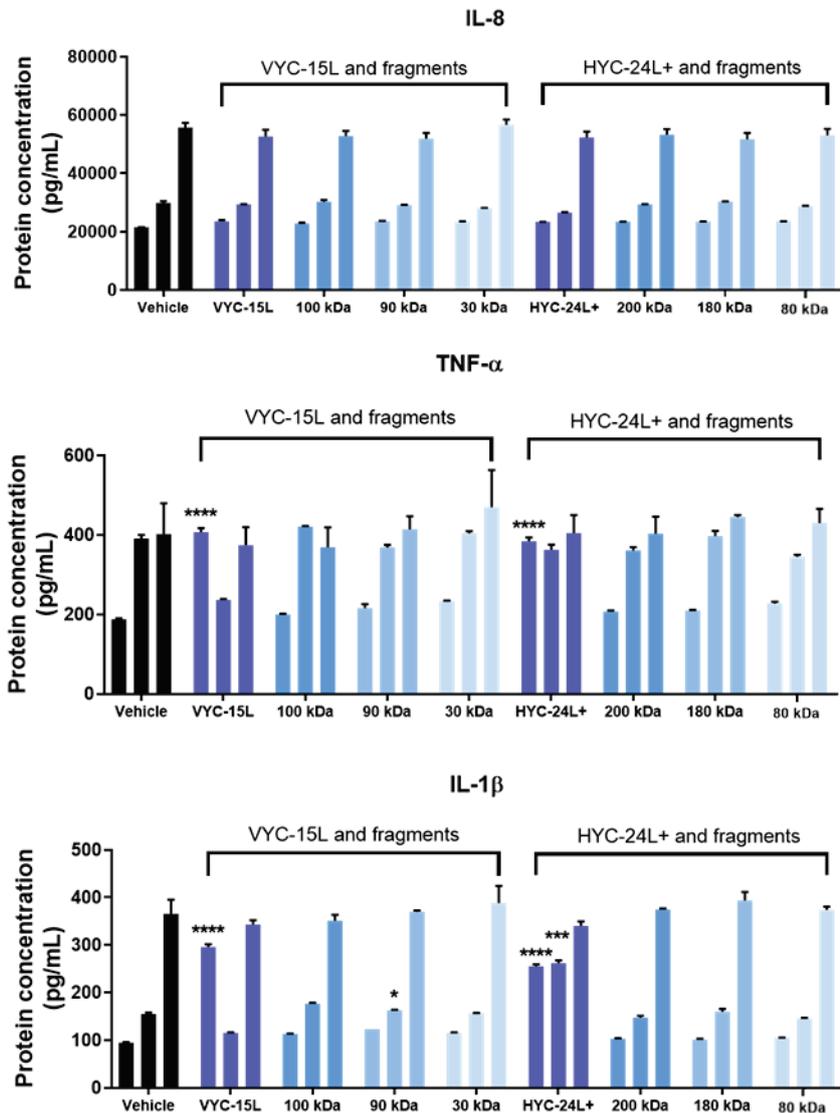


Figure 16: Cytokine secretion of M1 macrophage in the presence of HA

Supernatant produced by macrophages M1 was quantified by Luminex assay. IL-8, TNF- $\alpha$ , IL-1 $\beta$  and CCL5/RANTES were analyzed after 24h, 48h and 72h in the presence of vehicle, LMW of VYC-15L (at 100kDa, 90kDa and 30kDa), VYC-15L, LMW of HYC-24L+ (at 200kDa, 180kDa and 80kDa) and HYC-24L+. Each column of each condition corresponds to a different kinetics of stimulation, left column corresponds to 24h, middle column corresponds to 48h and right column corresponds to 72h stimulation. Data are representative of at least three independent experiments. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$  vs vehicle condition.

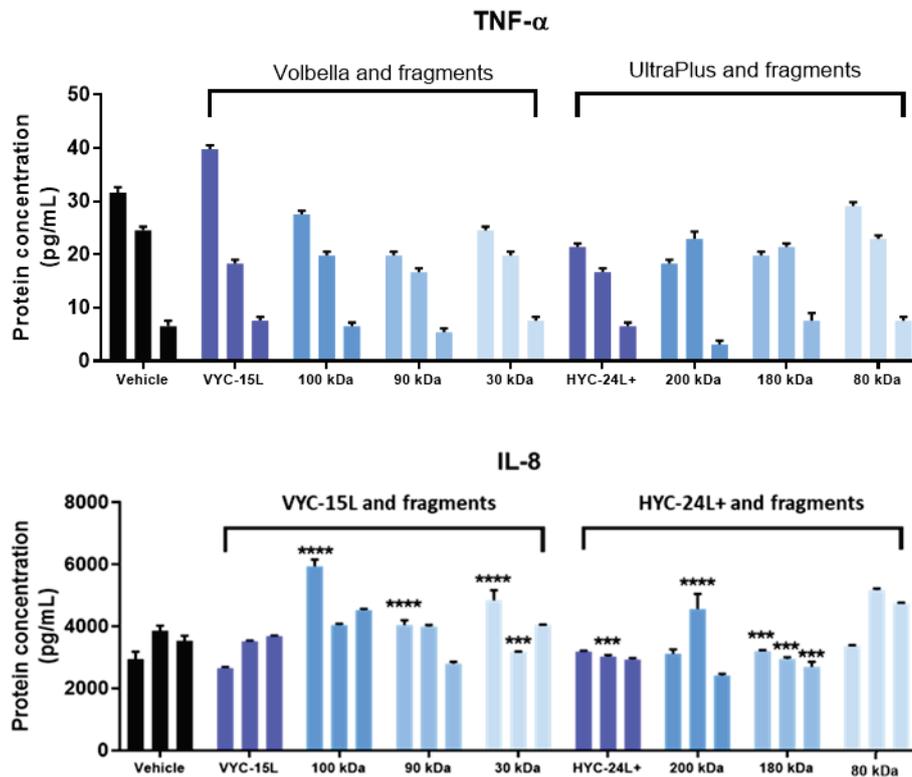


Figure 17: Cytokine secretion of mDC in the presence of HA

Supernatant produced by mDC was quantified by Luminex assay. IL-8, TNF- $\alpha$  and CCL5/RANTES were analyzed after 24h, 48h and 72h in the presence of vehicle, LMW of VYC-15L (at 100kDa, 90kDa and 30kDa), VYC-15L, LMW of HYC-24L+ (at 200kDa, 180kDa and 80kDa) and HYC-24L+. Each column of each condition corresponds to a different kinetics of stimulation, left column corresponds to 24h, middle column corresponds to 48h and right column corresponds to 72h stimulation. Data are representative of at least three independent experiments. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$  vs vehicle condition.

#### 1.16.2 CD44 receptor overexpression is not correlated with inflammatory responses

The main receptor of HA and transmitter of the HA bioactivity and reviewed as involved in inflammatory process has been quantified on the two immune cells model. We wanted to evaluate if these inflammatory modulations could be correlated with a CD44 overexpression on immune cells. CD44 expression was not modified by any HA treatment compared to the vehicle control on M1 and mDC (Figure 18A and B) at the 24h time point, excluding correlation with cytokine modulation.

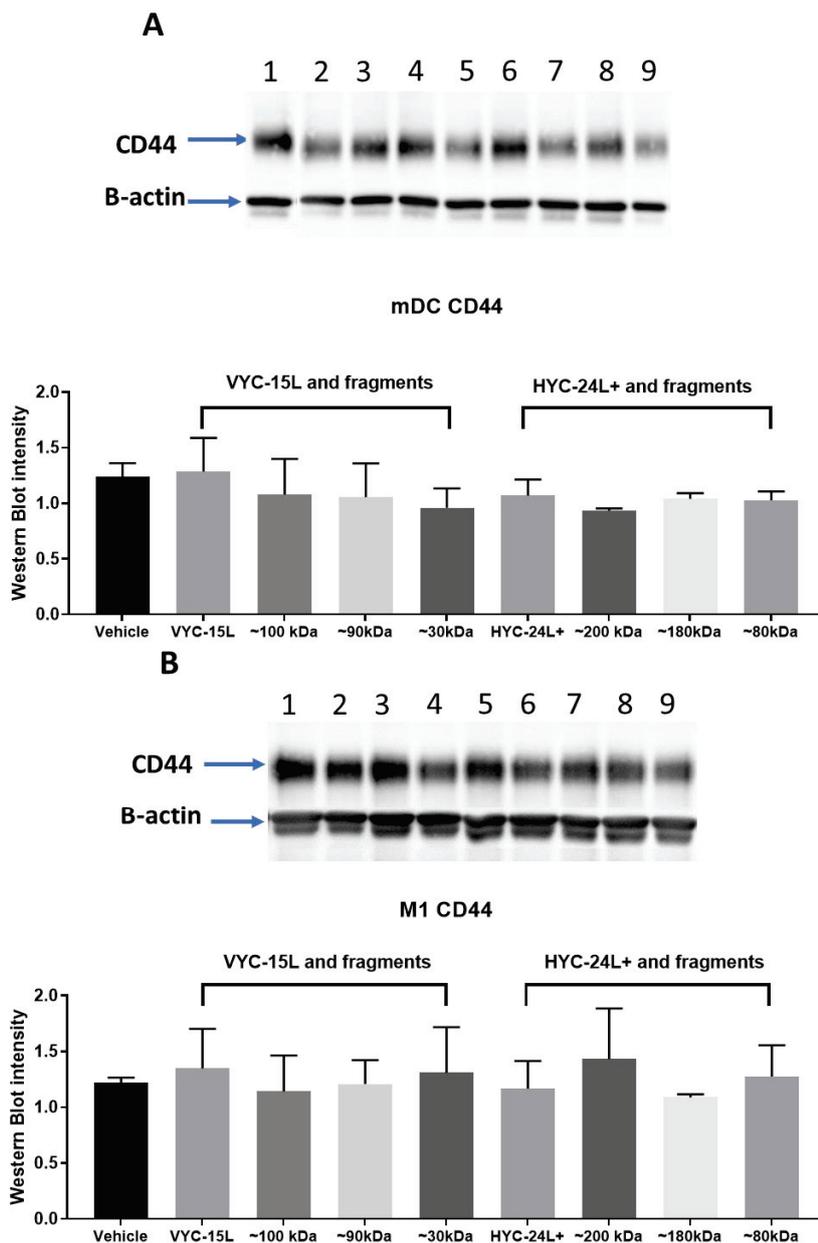


Figure 18: CD44 protein expression on M1 and mDC in the presence of HA

CD44 protein expression in M1 and mDC cells in the presence of HA products. A corresponds to CD44 expression on M1 by Western Blot. B corresponds to CD44 expression on mDC by Western Blot. Immune cells, were lyzated after 24h stimulation in the presence of vehicle (1), VYC-15L (2), LMW VYC-15L 100kDa (3), LMW VYC-15L 90kDa (4), LMW VYC-15L 30kDa (5), HYC-24L+ (6), LMW HYC-24L+ 200kDa (7), LMW HYC-24L+ 180kDa (8) and LMW HYC-24L+ 80kDa (9). Protein expression was normalized using  $\beta$ -actin and quantification was performed using Gel analyzer software. Data are representative of at least two independent experiments.

### 1.16.3 Differences between control mice and low-grade inflammatory mice responses

#### 1.16.3.1 Skin histology in control group

Skin from the control group with vehicle are subnormal and no lesions are observed with rare circulating macrophages and polynuclear cells (Figure 19A).

HA fragments were not detected by HES staining 24h after the injection of HA fragments, but some rare macrophages/mast cells/polynuclear are detected mainly in the hypodermis (Figure 19B and C).

With VYC-15L and HYC-24L+ injection, it has been observed a long and wide amorphous patch of purple color with the HES staining. Small or larger HA were present above the panniculus carnosus in the hypodermis, but some are detected under the panniculus carnosus. These HA localizations induces a limited macrophagic and polynuclear inflammatory reaction (Figure 19D and E).

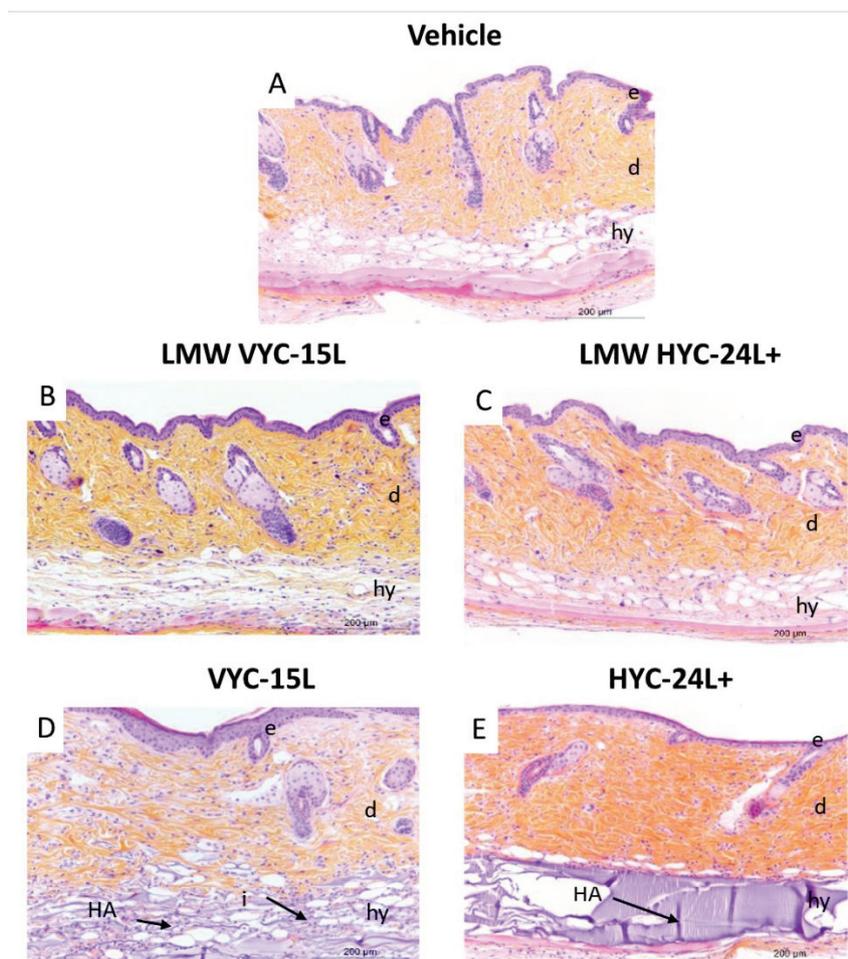


Figure 19: Skin histology of control group

*Histological skin analysis of control group. A corresponds to vehicle condition, B to LMW VYC-15L conditions, C to LMW HYC-24L+, D to VYC-15L condition and E corresponds to HYC-24L+. e = epidermis, d= dermis, hy = hypodermis, i= inflammation and HA= hyaluronic acid. Hyaluronic acid is colored in dark violet, extracellular elements in yellow and nuclei of cells in purple. Representative images from n= 5 mice per conditions. All staining and observations were carried out by NOVOTEC.*

#### *1.16.3.2 No cytokine overexpression in control group mice*

Without reaching significance, VYC-15L also tended to increase the production of IL-6 ( $177\pm 68$  pg/mL) in the control group compared to vehicle ( $103\pm 31$  pg/mL). In contrast, IL-6 was not increased in any other conditions in control (Figure 20A).

No significant differences were observed for IL-1 $\beta$  protein quantification between all condition. Nevertheless, it has been observed higher heterogeneity in IL-1 $\beta$  inflammatory responses in LGI group. Indeed, for LGI group the coefficient of variation for vehicle, LMW VYC-15L, VYC-15L, LMW HYC-24L+ and HYC-24L+ were respectively 38%, 45%, 28%, 24% and 35%, while in the control group the coefficients of variation were respectively 29%, 20%, 8%, 23% and 23% (Figure 20B).

The positive LPS stimulation induced by an intradermal injection of LPS at 5 $\mu$ g/mL induced significant overexpression of IL-6 in control group ( $350\pm 130$  pg/mL) and LGI group ( $350\pm 147$  pg/mL) compared to vehicle condition ( $102\pm 20$  pg/mL). Similarly, the positive LPS stimulation induced a significant overexpression of IL-1 $\beta$  in control group ( $374\pm 122$  pg/mL) and in LGI group ( $419\pm 95$  pg/mL) compared to vehicle condition in control group ( $137\pm 39$  pg/mL).

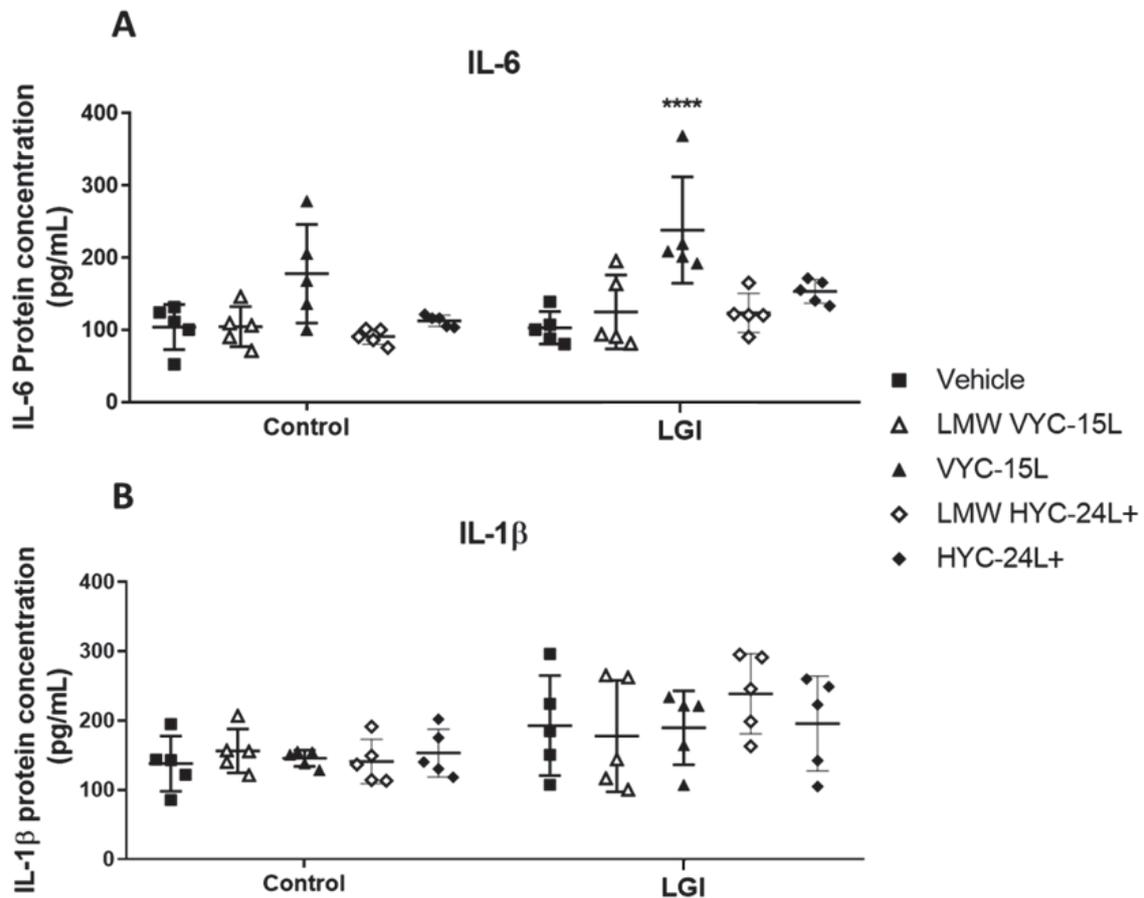


Figure 20: Inflammatory modulation in low-grade phenotypes in the presence of HA

Inflammatory modulation in low-grade phenotypes. Skin IL-6 and IL-1 $\beta$  in control mice (n=5) and low-grade inflammation mice (n=5) in response to vehicle, LMW VYC-15L, VYC-15L, LMW HYC-24L+ and HYC-24L+ after 24h intradermal injection. IL-6 and IL-1 $\beta$  were quantified by ELISA assay and samples were normalized to 1000 $\mu$ g of total protein per conditions. \*P < 0.05, \*\*\*\*P<0.0001 vs respective vehicle.

#### 1.16.3.3 Control group did not induce higher ACh-mediated vasodilation

ACh-mediated vasodilation was not different between control and LGI groups except in the presence of VYC-15L and its fragment in LGI group (Figure 21B) as described previously (Table 5). These differences, confirmed that low-grade inflammatory model reacted differently in presence of VYC-15L (Figure 21B). LGI microenvironment modulated vasodilation pathway in the presence of crosslinked HA and their degradation products.

Furthermore, we showed that an acute intradermal injection of LPS (positive control) strongly increased basal skin blood flow in LGI group, but not in control group (Figure 21). In these conditions (dilated vessels), ACh could not induce vasodilation in the presence of LPS in LGI

group. In contrast, ACh-mediated vasodilation was increased by LPS (69±32%) compared to vehicle (43±12) in the control group as described previously (Table 6)

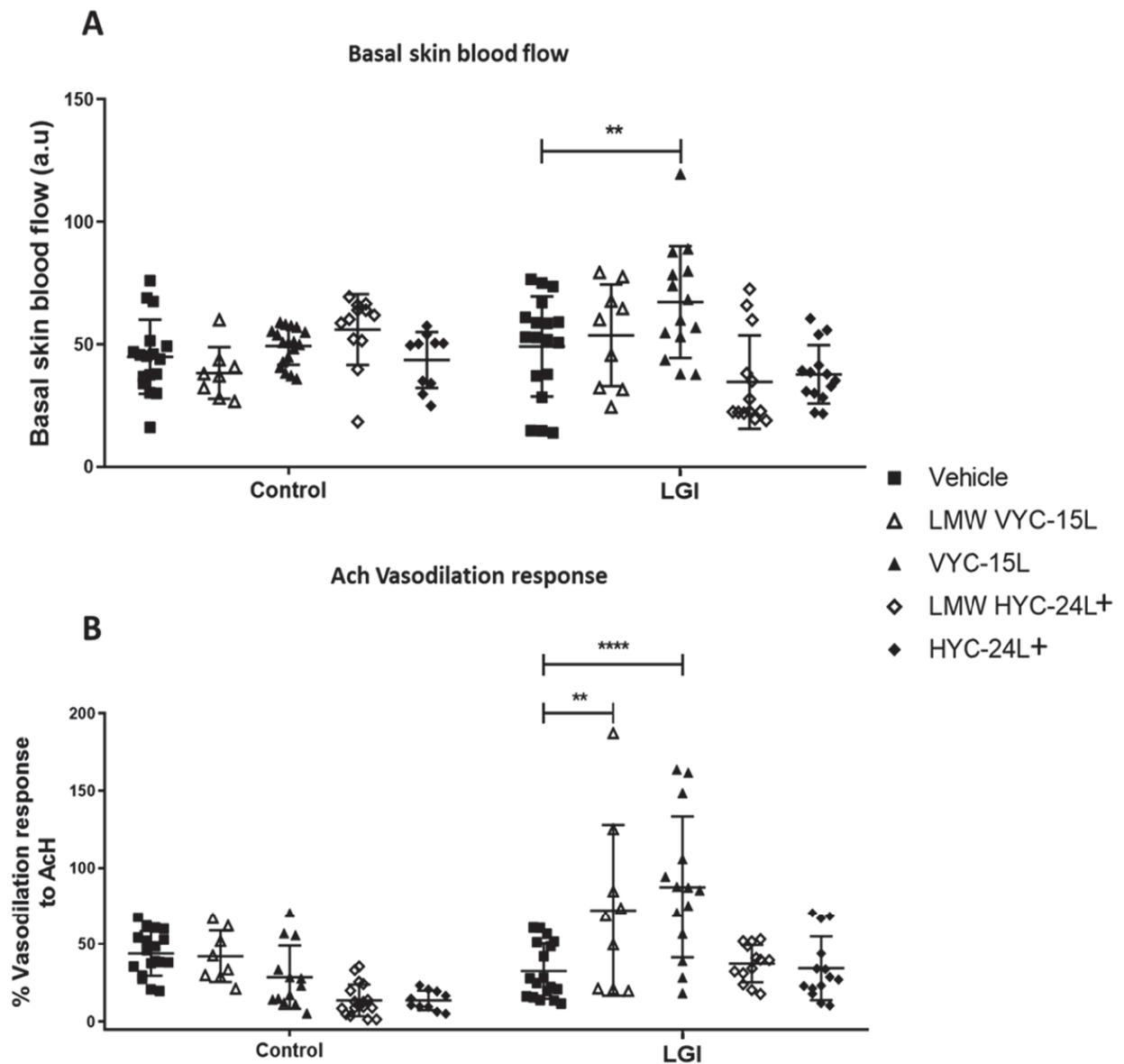


Figure 21: Vasodilation responses to VYC-15L and HYC-24L+ in a low-grade phenotype Basal skin blood flow (A) and ACh-mediated vasodilation (B) in control (PBS) and low grade inflammatory (LGI) mice in the presence of vehicle, LMW VYC-15L, VYC-15L, LMW HYC-24L+ and HYC-24L+. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs respective vehicle. Each point represents one measurement of iontophoresis. 5 mice were used for each condition.

**Basal skin blood flow (a.u): Mean ± SD**

Conditions	Control group (1)	LGI group (2)	P-value* <0.0,5 vs resp. vehicle
Vehicle control	45±13	49±11	
VYC-15L fragments	38±7	54±18	ns
VYC-15L	49±6	<b>67±18</b>	<b>&lt;0.048 (2)</b>
HYC-24L+ fragments	35±15	56±10	ns
HYC-24L+	38±9	44±10	ns
LPS	49±10	<b>114±39</b>	<b>&lt;0,0001 (2)</b>

*Table 5: Basal skin blood flow in control and LGI group*

**Vasodilation responses: Mean % ± SD**

Conditions	Control group (1)	LGI group (2)	P-value* <0.0,5 vs resp. vehicle
Vehicle control	43±12	32±15	
VYC-15L fragments	42±13	<b>72±40</b>	<b>&lt;0,0011 (2)</b>
VYC-15L	28±15	<b>87±33</b>	<b>&lt;0,0001 (2)</b>
HYC-24L+ fragments	13±7	37±9	ns
HYC-24L+	13±5	34±15	ns
LPS	<b>69±32</b>	8±6	<b>&lt;0,0261 (1)</b>

*Table 6: Vasodilation responses in control and LGI group*

#### 1.16.3.4 Vasodilation responses are not correlated with VCAM-1 and COX-2 expression

It appeared that VCAM-1 and COX-2 were not significantly overexpressed in the presence of VYC-15L and HYC-24L products compared to vehicle condition either in control or low-grade group after 24h stimulation (Figure 22A and B). In contrast, VCAM-1 was overexpressed in the presence of the positive LPS control in control group ( $13668 \pm 3400$  pg/mL) and LGI group ( $17380 \pm 3600$  pg/mL), compared to vehicle condition (less than 9000 pg/mL) (Figure 22).

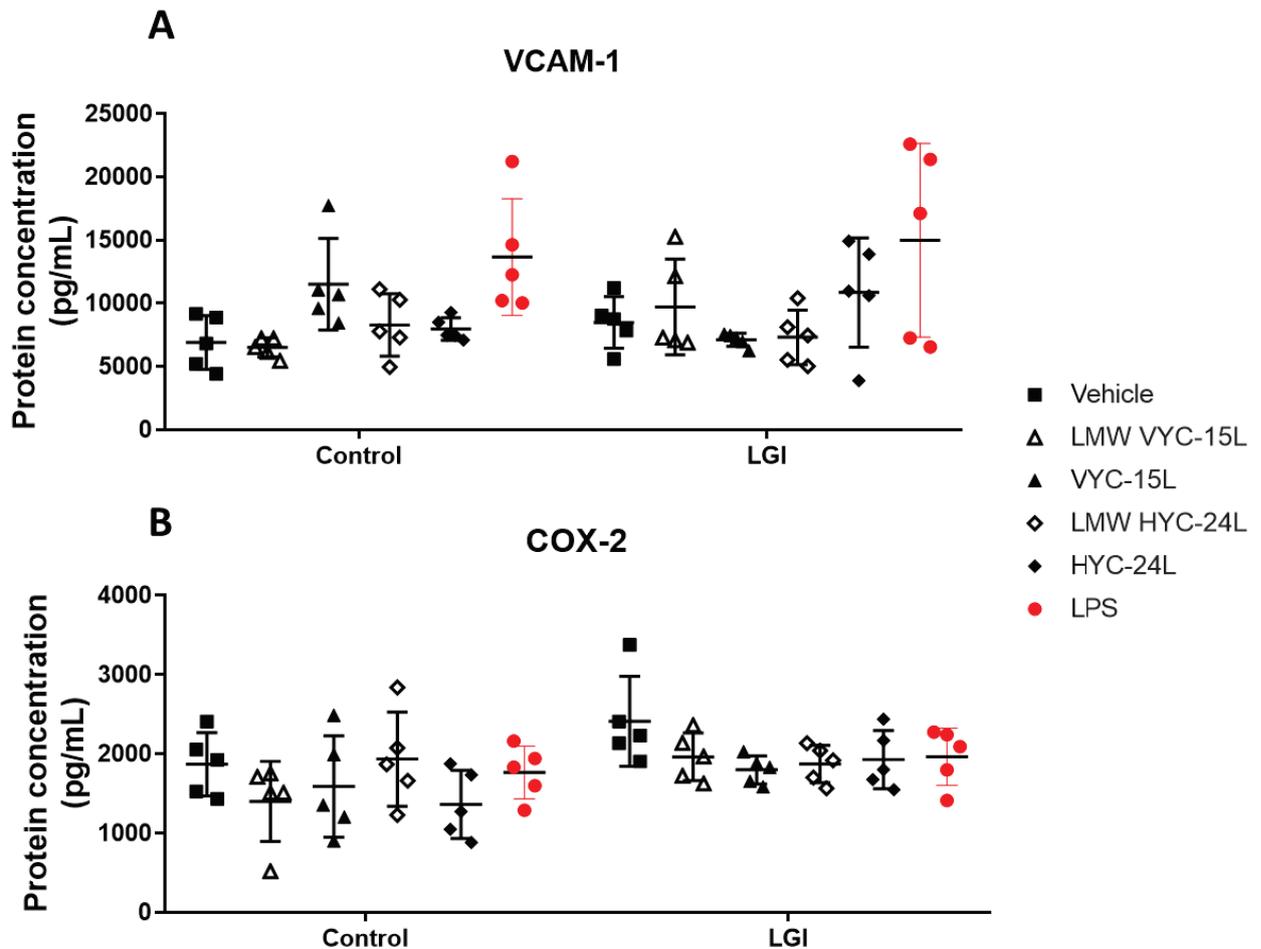


Figure 22: Vascular markers quantification

Vascular markers quantification. VCAM-1 and COX-2 were quantified after 24h of stimulation with vehicle control, LMW VYC-15L, VYC-15L, LMW HYC-24L+, HYC-24L+ and LPS as positive control by ELISA assay. Each condition has been compared to control condition (vehicle) in each group (Control or LGI group).

# Discussion

Hyaluronic acid fillers are designed to lift the tissue and add volume to improve the aesthetic appearance of wrinkles or loss of volume to counteract effect of aging. Nevertheless, some rare late adverse effect, as described in “Introduction”, lead to inflammatory and non-inflammatory nodule and granuloma development few month or year after the first injection (192). Link to the low percentage of late complication developed, we supposed, that degradation of crosslinked HA in time, inducing different range sizes of HA fragments, could be involved in these late complications. Furthermore, it has been reported that delayed onset nodules are more common in immune-reactive patients and particularly those with active autoimmune diseases (173). Main auto-immune disease such as rheumatoid arthritis or lupus have in common a low-grade inflammatory microenvironment (194). The possible inflammatory role of low molecular weight of HA and HA non degraded in a low-grade inflammatory environment, has never been explored.

In this context, the objective of this thesis was to evaluate the inflammatory and vascular responses of crosslinked hyaluronic acid and its degradation products in an inflammatory environment. For this purpose, we used two different crosslinked HA, from VYCROSS® technology and UltraPlus XS (HYC-24L+) from HYLACROSS® technology. In order to study the potential different behavior of dermal skin cells in high or low-grade inflammatory environment in presence of these crosslinked HA and their low molecular weight degradation product. Vascular aspect was assess using low grade inflammatory mice model.

## 1) Microenvironnement and cells interactions

Understanding the effect of hyaluronic acid and its degradation product, implies taking into account the microenvironment. Microenvironment, is complex environment involving all cell type in the skin. It can be modulated by phenotypes of cells, by intern and extern stimuli inducing inflammatory environment. In case of homeostasis dysregulation, altered microenvironment may become persistent leading to chronic inflammation as observed in aging (198). Immune cells have long been considered to be the major players in inflammatory deregulation. Nevertheless, it is now well known that other cell type such as fibroblasts and endothelial cell play a major role in immune responses. Dermal fibroblasts are the key cells to synthetize the ECM composed by many components including collagen and ELN fibers. But they are also known to stimulate vascular inflammation through cytokines and chemokines production (199), leading to endothelial activation and leukocytes attraction (187, 200, 201). This can amplify the pro-inflammatory effect on the microvascular system. Microvascular endothelial cells are the major components of dermal blood vessels and are clearly involved in skin inflammatory process. Endothelial cells participate to synthesize and secrete chemokine and cytokine in response due to different factor (202). IL-1 $\beta$  and TNF- $\alpha$  are specific inflammatory mediators, activating endothelium and permitting vessels hyperpermeability allowing leucocyte infiltration. And these vascular modulations are described in skin diseases linked to a chronic inflammation (66, 203). Thus, inflammation is a common condition that affects the vasculature, but is also a skin feature that occurs in long-term pathologies, as well as in short-term conditions at high or low-grade level (66). Inflammatory disorders can induce on endothelial cells, secretion of chemokine and cytokine leading to immune cells recruitment (186). This inside-out inflammatory response has been supplemented with an outside-in signaling involving activated pro-inflammatory fibroblasts interacting with immune cells to direct endothelial activation (187). In this context, fibroblasts play a critical role in modulating immune cells such as leukocytes and could be responsible for the establishment of chronic inflammation (200). We showed that activated endothelial cell, without any immune cell interaction, could impact dermal cell behaviors such as dermal fibroblast and the ECM formation. HDMEC secreted pro-inflammatory cytokines leading to a loss of vascular integrity which is involved in in the development of skin or autoimmune diseases, inducing inflammatory processes as chronic inflammatory conditions like psoriasis or lupus (204, 205).

We showed that the secretory microenvironment related to endothelial inflammation could trigger a perpetual fibroblast inflammatory phenotype that could be responsible for an exacerbation in ECM degradation with an increase in MMP1 and unstable collagen 1 network. In contact with endothelial inflammatory secretion, and no immune cells, it appears that fibroblasts switch from normal to pro-inflammatory phenotype and are less involved in ECM synthesis.

Vascular inflammation has long been considered an inside-out response, starting with endothelial activation and extravasation of leukocytes, and eventually leading to an inflammatory response that spreads outwardly inducing a change in the tissue microenvironment. While another signaling pathway has emerged: the microenvironment can modulate vascular inflammatory responses by way of outside-in signaling, whereby activated, proinflammatory fibroblasts and leukocytes may altered the endothelium (201, 206).

## 2) Low molecular weight of HA

Hyaluronic acid has different biological role, in homeostasis but also in pathologies as described in introduction (93). During healing process, cancer or aging, HA is degraded using hyaluronidase mainly, inducing HA fragments. Among these fragments, it has been established, that size matters in biological responses. Indeed, high molecular weight of HA fragments (>500kDa) are described to be anti-inflammatory, while oligomers (<10 kDa) are commonly described as pro-inflammatory and implicated in healing process (88). HA fragments are considered as low molecular weight when they weigh less than 500 kDa to 10 kDa.

In the present work, we have produced different range sizes of HA fragments coming from two different crosslinked HA. LMW of HA were obtained from 200kDa to 30 kDa depending on the time degradation and of the filler degraded. Indeed, we showed that VYC-15L induced smallest fragments than HYC-24L+ after long time degradation and correlated to mass molar distribution with two different profiles for each crosslinked HA. These results could be explained by the initial concentration of HA per filler and by their different crosslinking degree. Indeed, it has been shown that VYC-15L was composed by 15mg/mL of HA while HYC-24L+ was composed by 24mg/mL of HA (166). And that HYC-24L+ was more resistant to compression and then cohesive than VYC-15L. HYC-24L+ have also better capacities to take up water than VYC-15L (166).

These differences in composition induce, certainly, a resistance to degradation of HYC-24L+ using hyaluronidase PH-20 (HYLENEX) comparatively to VYC-15L. Furthermore, the difference in crosslinking degree, induce a different network of polymer, increasing elasticity and viscosity, giving chemical and physical barrier to enzyme. The enzyme then degrades more slowly than cross-linked HA, which has a higher degree of cross-linking (76). Nevertheless, after short time, medium time, or long-time degradation, we have obtained 3 range sizes of fragments with an average of molecular weight lower than 500kDa. Confirming that we have produced range sizes of low molecular weight of HA fragments. According to physiological degradation, we have then obtained a large spectrum of sizes of LMW of HA in order to evaluate their potential effect of dermal skin cells and in vivo model.

Another important aspect was the endotoxin contamination. We have shown that our samples were endotoxin free, with less than 0.03EU/mL inside samples degraded. In fact, it

has been reviewed that studies describing low molecular weight of HA fragments, as pro-inflammatory, used different sources of HA or enzyme contaminated by endotoxin leading to inflammation (103, 207). Caution is needed to ensure that the effects are not due to picogram levels of endotoxin contamination, from either the HA source or from the hyaluronidase used to generate the fragments (101). Nomenclature and methods to degrade HA must be considered, and endotoxin quantification must be plan. Nevertheless, choice of type hyaluronidase and type of degradation must be considered to be in the most physiological condition. PH-20 is a testicular enzyme encoded by the sperm adhesion molecule 1 (SPAM1), located on the human sperm surface and inner acrosomal surface. HYLENEX enzyme is produced by genetically engineered Chinese Hamster Ovary (CHO) cells containing a DNA plasmid encoding for a soluble fragment of human hyaluronidase (PH-20). But, it has been found that a similar hyaluronidase to PH-20 was expressed by chondrocytes, synoviocytes and dermal fibroblasts (208). To be in better skin physiological condition, it will be interesting to use different type of hyaluronidase, such as HYAL-1, HYAL-2 which mainly use in skin HA metabolism. Nevertheless, in the body, HA degradation is predominantly mediated by these hyaluronidases but, it has been reported that all human hyaluronidase have similar catalytic mechanisms (209).

In addition, as previously mentioned, late complications may appear a few months to a year after the injection. And we cannot confirm that the products of degradation are not key player in adverse effects. Indeed, even if we produced range size of fragments, we never tried to stimulate dermal skin cells with a unique size of fragments purified. It would therefore be interesting to increase time of degradation, to assess if smaller fragments than 30kDa for VYC-15L and 80kDa for HYC-24L+ could induce inflammatory responses and ideally identify and extract size of fragments which could lead to inflammatory reaction.

### **3) Inflammation module cell inflammatory responses to crosslinked HA and their degradation products**

An important outcome of studies on molecular mechanisms that underlie inflammation was that an inflammatory response is not a specialized function of cells of hematopoietic origin (macrophages, neutrophils, lymphocytes etc.), but rather a fundamental attribute of all cellular types. Fibroblasts play a critical role in modulating immune cells and could be responsible for the establishment of chronic inflammation (200, 210). Furthermore, among skin immunity, endothelial cell plays a major role in homeostasis and pathogenesis, modulating leucocytes recruitment (41, 211). It appeared then that skin immunity and HA interaction is a complex model, involving a certain number of cells and mechanisms. In this way, we assessed crosslinked HA degraded and non-degraded on dermal fibroblasts, HDMEC, macrophages M1, mature DC and in an integrative model.

### **4) Crosslinked HA and immune cells**

In this work, exogenous LMW HA fragments obtained from crosslinked HA, and non-degraded HA exhibited different pattern of cytokines release depending on the cell type that was exposed to a previous inflammatory microenvironment. Macrophages and DC as an important antigen-presenting cell play a crucial role in inflammatory response and modulate the pathogenesis of diseases (212, 213). The macrophages M1 can secrete several proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-8 (CXCL8) and IL-6 in inflammatory pathogenesis (214). DC function as immune sentinels and instigators of T cell responses, secreting chemokine such as CCL5 and CXCL8/IL8 (213, 215).

In 2D model of M1, we showed, that both non-degraded HA induced an increased secretion of IL-1 $\beta$  and TNF- $\alpha$  at 24h. Nevertheless, this increase in inflammatory markers in the presence of the two non-degraded HA were not persistent in time, with no differences observed at 48h and 72h. These results are in accordance with Baeva et al. that reported no inflammation due to HMW HA added to M1 that were previously stimulated with LPS and IFN $\gamma$  at 72h (100). Another study, showed that murine macrophages under the inflammatory states, and in presence of HMW-HA suppressed nitric oxide production (191). Furthermore, the levels of cytokines induced by HA non degraded in M1 and at 24h were much lower than those observed after LPS stimulation, as positive control (Supplementary Data).

LMW HA fragments from the two HA did not increase cytokine level on M1 and mDC, except for IL-8 on mDC which was overexpressed at 24h in presence of the three range sizes of LMW-VYC15L. Nevertheless, these differences, as for HA non degraded, were not persistent in time with no differences at 72h. However, similar to the non-degraded HA results the pro-inflammatory effect did not last in time revealing possible feed-back mechanisms that allow cells to adapt from initial inflammatory response (216). It has been described by Avenoso et al. that miRNA, was involved in the inflammatory modulation in presence of HA oligosaccharides. miRNAs, are small noncoding RNA molecules (approximately 22 nucleotides in length) which regulate gene expression binding the 3'-UTRs of their target mRNAs, degrading mRNAs for cleavage or repressing their translation. In this study, they showed that mir146a reduced the inflammatory cascade by modulating TLR4 and NF-kB activation in presence of 6-mer oligosaccharides (216). Nevertheless, no studies have been done on implication of miRNA modulation in presence of LMW-HA and HA non degraded. In this way, it will be interesting to go further in their possible implication, to understand why inflammatory responses observed at 24h were not persistent in time. Another aspect to study, is the plasticity of macrophages to modulate their phenotypes and activation according to the inflammatory context and in presence of HA (217). Indeed, it has been shown that murine macrophages in presence of HMW-HA promoted an alternatively activated-like state with gene up regulation such as *arginase1*, IL-10 and *CCL2* while LMW-HA induced pro-inflammatory activation (137). Macrophages and dendritic cells can then modulate their response in presence of HA inducing different step of polarization. It has been also observed that SOD3 (Extracellular Superoxide Dismutase) plays a role in inhibiting skin inflammatory response induced by HA fragments with no implication of TLR4. SOD3 inhibited also DC maturation (218). Nevertheless, in this study, no information was given on the source and method of degradation of the fragments.

In this work, we supposed that the non-persistent differences between condition could be link to feed-back mechanisms that allow cells to adapt by anti-inflammatory cytokine secretion. IL-4 and IL-13, the two main cytokines involved in the switch M1 to M2, were undetectable on M1 and mDC supernatant, suggesting that these non-persistent inflammatory state with LMW HA and HA non degraded were not correlated to anti-inflammatory process.

Furthermore, direct LPS stimulation on M1 and mDC induced a strong and sustained inflammation up to 72h compared to the mild and transient response obtained with non-degraded and degraded HA products. LPS is one of the most potent biological response modifiers known. Indeed, picomolar concentrations are sufficient to stimulate cells of the immune/ inflammatory/ vascular systems (219, 220) compared to micromolar concentration of HA (100). LPS is involved in the development of chronic inflammation through massive increase in pro-inflammatory cytokines that promote the secretion of other inflammatory mediators and recruiting macrophages to the site of inflammation which contribute to the disease worsening (221). In addition, LPS has a higher affinity for the TLR4 receptor than HA (222).

A recent study highlighted that glycosylation, a reversible posttranslational modification, may regulates the activity of intracellular proteins on murine macrophages and human monocyte-derived macrophage via  $\beta$ -N-acetylglucosamine (one of the degradation products during HA degradation). Indeed, they showed that LPS treatment induces a marked increase in protein O-GlcNAcylation and this glycosylation may reduce inflammation (223).

We showed that M1 and mDC down expressed RANTES (CCL5) in presence of free-endotoxin HA contrary to literature (224, 225). According to Dong et al. that persistent inflammatory responses observed with LMW-HA was link to endotoxin contamination. Furthermore, no differences in CD44 protein expression was observed in presence of each condition on M1 and mDC while HA binding in response to HA is generally accompanied by an increase in CD44 expression and typically takes 2–3 days on immune cells (99). Suggesting that another receptor or mechanisms, implicating co-interaction with TLR4 for example, could explain these different inflammatory responses in presence of HA as illustrated below (Figure 23).

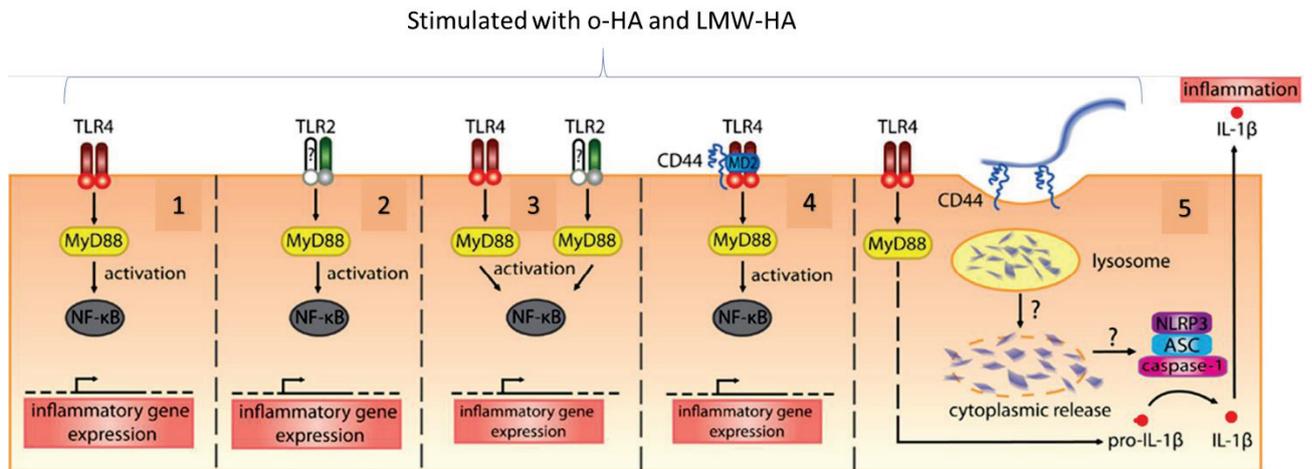


Figure 23: Receptor pathway implicated in proinflammatory signals by HA fragments (Adapted from (99))

Indeed, it has been reviewed that different receptors were implicated in mediating the proinflammatory signals induced by HA fragments from o-HA to LMW-HA. HA fragments lower than 500 kDa have been described to stimulate proinflammatory cytokine production via TLR4 alone (1), TLR2 alone (2), both TLR2 and TLR4 (3), a complex of TLR4 involving CD44 (4), or via TLR4 and CD44-mediated uptake that leads to inflammasome activation such as NLRP3 (129, 158, 188, 226, 227). Nevertheless, main publication describing these pathways used human umbilical cord and it has been reviewed that this type of HA sources was contaminated by nucleic acid endotoxin (103, 228). Indeed, they showed that HA from umbilical cord was endotoxin contaminated even after polymyxin B treatment. Furthermore, they showed that inflammatory responses observed was independent of CD44 using murine macrophages CD44<sup>-/-</sup> and IL-1 $\beta$  was not overexpressed in presence of HA (103), questioning a certain number of studies on HA and its fragments.

### 5) HA and dermal skin cells

Dermal fibroblasts are the main cell type involved in skin integrity through ECM synthesis. However, fibroblasts can also dysregulate ECM in the presence pro-inflammatory microenvironment (195) and are considered as sentinel cell in immune reaction (39, 42, 43). Microvascular endothelial cells as major components of dermal blood vessels participate to synthesize and secrete chemokine and cytokine and are implicated in recruiting immune cells in response to dysfunctions due to different factors (e.g. UV, heat, pathological and physiological processes) (202, 229). The crosstalk between endothelial cells and fibroblasts has been studied through fibroblasts influencing endothelial cells, since fibroblasts provide

ECM support as well as the production of several molecules like VEGF for angiogenesis (230-233). In addition, (230) it has been suggested that endothelial cells might also affect fibroblasts since cytokines, chemokines and other molecules present in the microenvironment could affect skin integrity altering ECM components (234). In skin aging, it has been shown that IL-8 and IL-6 are clearly linked to MMPs modulation and COL1A1 gene and protein expression (235-237). We showed that the low-grade inflammatory microenvironment produced by activated endothelial cells induced a strong up-regulation of *MMP1* in dermal fibroblasts suggesting collagen I degradation associated to a reduction in collagen I synthesis due to *COL1A1* gene downregulation.

In normal condition, we showed that dermal fibroblasts with LMW VYC-15L were able to upregulate collagen 1 and elastin gene expression showing that HA could promote fibroblast main function such as ECM synthesis. However, this LMW VYC-15L beneficial effect was lost in inflammatory fibroblasts (Appendices 6). Fibroblasts stimulation occurs by direct binding of filler to cellular receptors and addition of exogenous HA has been reported to trigger TGF- $\beta$  signaling and collagen production (238, 239). Collagen production following filler injection is also observed by enhancing structural support within dermal ECM through mechanical properties (240). While, endothelial cells in presence of LMW-HA has been reported to stimulate vascular proliferation, migration, and tubule formation *in vitro*, secreting cytokines, MMPs (93) and involved in EC barrier disruption (241) and HMW-HA as antiangiogenic, inhibiting proliferation and motility (242).

Most knowledge about the different effect of HA on fibroblasts and endothelial cells are based on endogenous HA and mainly in injuries context. Indeed, it has been reviewed that HA has both a pro- and anti-inflammatory effect *in vivo*, and these contradictory functions depend upon sizes of HA and which receptors is engage (93). Indeed, it is commonly known that o-HA and native HA can interact with CD44 and RHAMM in fibroblasts and endothelial cells.

Principal function of HA with fibroblast described are in proliferation, migration, inflammation, cancer, and wound healing. It has been shown that oligosaccharides of HA (o-HA) accelerated wound closure using CD44 and RHAMM receptor interaction and LMW-HA increased fibroblast proliferation in wound repair process (243, 244). While native HA induced a decrease in proliferation (245). And interestingly, in chronic dermal fibroblast, the LMW of HA fragment at 70kDa reduced fibroblast proliferation (245). Another study, have shown that

crosslinked HA could induce fibroblast proliferation activity, depending on the bioavailability of certain binding domains of the HA (246). As said previously, endothelial cells behaviors is modulated with HA (247). High molecular weight of HA is described as antiangiogenic while o-HA is a strong stimulator to endothelial cell proliferation using RHAMM receptor on EC membrane, but not CD44 (248).

In contrast, in the present work, we showed that high or low inflammatory preconditioned dermal fibroblasts and HDMEC did not induce strong pro-inflammatory cytokines when degraded and non-degraded HA were added, comparatively to a LPS stimulation at 1 $\mu$ g/mL (Appendices 3 and 4). Indeed, LPS stimulation induced higher amount of IL-6, IL-8 and IL-1 $\beta$  in these models. And specific inflammatory mediators are described as endothelium activators like IL-1 $\beta$  or TNF- $\alpha$ , permitting vessels hyper-permeability allowing leucocyte infiltration, while IL-6 and IL-8 are described as involved in ECM modulation. Vascular modulations are involved in skin diseases linked to a chronic inflammation (66, 203, 249, 250). Nevertheless, we showed that low level of inflammation at less than 10 IU/mL of cytokine can induce important vascular inflammation leading to ECM alteration.

Contrary to literature we did not observed important anti-inflammatory effect in presence of crosslinked HA non-degraded. Interestingly, we showed that HA degraded and non-degraded induce low differences in cytokines release depending on the degree of inflammation and the cell type. Indeed, we showed that IL-6 was overexpressed in presence of LMW VYC-15L on HDMEC in high-grade inflammatory state while a low-grade inflammatory state induced down expression of this cytokine.

These observations, could be explain by a different clustering of HA receptor leading to different biological effect. Indeed, it has been described that the clustering of CD44 at the cell membrane can be disrupted when HA is degraded under inflammatory conditions leading to fragments. CD44 signaling pathway is then altered by declustering, and this modification increased its availability to interact with other receptor proteins such as RHAMM, TLR4, or TLR2 (32). Nevertheless, no studies have identified if different degree of inflammation impact HA receptor clustering.

These in vitro experiments confirm that HA and their biological effects are modulated depending on the microenvironment (93). We confirmed that LMW-HA did not induced

strong inflammation on dermal skin and immune cells in endotoxin-free condition, as recently described and comparatively to an acute LPS stimulation (103). We demonstrated that depending on the microenvironment and cell types, degraded and non-degraded HA can induce opposite action to those reviewed in the literature in normal conditions (94). An unidentified mechanism seems to modulate the cytokine secretion in presence of crosslinked HA and their degradation products in 2D models from healthy donor, it will be therefore interesting to evaluate the inflammatory responses using primary cells from sick patients and more precisely with low-grade inflammatory pathologies. In order to evaluate if the low inflammatory modulation with crosslinked HA are correlated to altered phenotypes or if an altered phenotype induce an exacerbate inflammation in presence of HA degraded. Indeed, an increases in HA are associated with chronic disease processes with unremitting inflammation such as diabetes (251), rheumatoid arthritis (252), asthma (253) or lupus (254) have dysregulation of HA metabolism such as overexpression of HAS-2 or in activity of hyaluronidase leading to inflammatory development.

To conclude this part, we showed that using inflammatory in vitro model, dermal skin cells and immune cells may slightly respond to HA and their degradation product depending on the degree of inflammation, on the type of cells and on the type of crosslinked HA. According to recent study, we confirmed that LMW-HA did not induce exacerbate inflammation and we did not identify the pathway involved in inflammatory modulation in the different model. These inflammatory modulations have not been explained but different regulation mechanisms have been proposed. Previously, we have shown that low-grade inflammation induces a high inflammatory release of HDMEC which can alter dermal fibroblasts and the synthesis of extracellular matrix components. In this way, homeostasis and diseases involved different type of cells and mechanisms leading to regulation or dysregulation.

## 6) Crosslinked HA in a mouse low-grade inflammatory model

However, *in vitro* models present limitations and cannot reflect complexity of the skin whose homeostasis relies on a finely tuned equilibrium of well-regulated interactions between the different layers of the skin with their cellular and subcellular structures (255). To better characterize the cellular response observed after crosslinked HA injection in humans (93, 171), we also used an *in vivo* murine model. Super low dose endotoxin in the blood circulation is one of the emerging risk factors that can cause chronic low-grade inflammation in humans and animals (256, 257). In the present study, a low-grade inflammation phenotype was induced by repeated intraperitoneal injections of a super low dose of LPS for 6 weeks in healthy mice, based on previous studies (176, 177). Furthermore, no signs of suffering or pain were observed during the entire time of experience, according to NC3R guidelines. This chronic LPS treatment increased pro-inflammatory cytokines (IL-6 and CCL2) in the plasma, associated with a moderate skin inflammation revealed by a trend to increase inflammatory markers (TNF- $\alpha$ , IL-1 $\beta$  and COX-2) within the skin, validating our mouse model of low-grade inflammation. Indeed, it has been described that a prolonged low dose of LPS stimulation could induce alteration either on cells or *in vivo* (176, 258). It's also described that obese-diabetic mice, presenting a low-grade inflammation, had an increase of COX-2 level on skin biopsy correlated to systemic inflammation-associated (259), suggesting that vascular behavior is altered. In addition, low-grade inflammation is characterized by persistent micro-inflammation, which is difficult to detect, not symptomatic and could lead to the development of pathology in time (260, 261). Nevertheless, it's unclear if low-grade inflammation is cause of chronic diseases or chronic disease induce this low-grade inflammation. This *in vivo* mouse model of low-grade inflammation was then used to study the effects induced by intradermal injections of crosslinked HA and its degradation products and evaluate differences between the two crosslinking technologies on skin integrity and microvascular reactivity.

Histological analysis revealed intact skin tissue architecture around the injection site in all mice that received vehicle, even in the presence of low-grade inflammation, revealing the non-traumatic nature of the intradermal injection in healthy adult mice. In addition, IL-6 protein concentration in the skin was not increased 24h after intradermal injection of vehicle in healthy and inflammatory microenvironment, excluding confounding effects from native HA degraded by a chronic low-grade inflammation. Nevertheless, it has been shown, that low

dose of LPS can induce higher production of reactive oxygen species (ROS) (262), and these products are involved in HA degradation (83). We considered that after 24h of intradermal injection, native and crosslinked HA cannot be totally degraded at this kinetics in addition to the non-differences observed in ECM structure between control and low-grade inflammatory group.

Regarding the injection of exogenous HA and its degradation products, we showed that an inflammatory infiltration into the skin was more pronounced with VYC-15L compared to HYC-24L+, particularly in mice with a low-grade inflammatory phenotype. Likewise, non-degraded VYC-15L was the only product to increase the IL-6 concentration in the skin in healthy conditions (more than 1.6 respect to corresponding vehicle, without reaching significance) and in the presence of low-grade inflammation (more than 2.3-fold than vehicle), confirming the results highlighted by histological analyses. Indeed, it has been shown that IL-6 was essential in recruitment of leucocyte, regulating their infiltration, angiogenesis, and collagen accumulation (263). Nevertheless, skin labeling of CD31 and Collagen IV did not show differences in vessels quantity between VYC-15L and HYC-24L+ after 24h intradermal injection (Data not shown), suggesting that after 24h, IL-6 produced by VYC-15L, does not induce higher angiogenesis process or that 24h time stimulation was not enough to observed vessels formation. Nevertheless, IL-1 $\beta$  levels were not different between each condition. Even if, we observed that low-grade inflammatory group present a certain heterogeneity in inflammatory responses respect to control condition. It has been reported that patient with low-grade inflammation respond differently to the same treatment suggesting a possible individual alteration in response (264). Moreover, working on vivo models, many parameters have to be taken into account that can lead to heterogeneous responses, such as the stress of the mice, the environment, population in the cages or the individual factors of each mouse even if these mice come from the same lineage.

We suggest that this cutaneous inflammation induced by VYC-15L injection was more due to mechanical effects due to filler injection and/or tissue integration rather than its biological effects, since LMW degradation products did not increase IL-6 protein concentration. In contrast, HYC-24L+ did not modulate IL-6 concentration in the skin independently of inflammatory microenvironment. These different observations between VYC-15L and HYC-24L+ can be explained by their different compositions, structure, or rheological properties

known to influence filler performance (166). Indeed, it has been described that rheological properties, degree of cross-linking and concentration of HA inside the gel may have different biological and physical effects, but mainly on degradation process (265, 266) as explained above.

In contrast to our results, native HA, crosslinked HA and HMW degraded products of HA are commonly described as anti-inflammatory and in inflammatory model of interstitial cystitis, these HA decreased level of IL-6, IL-8 and permeability (267), and thus used as treatment in osteoarthritis (268) to reduce inflammation. While LMW-HA, commonly described as pro-inflammatory can also be used as anti-inflammatory, modifying their chemical structure (269). Furthermore, it has been shown that depending on the type of chemical modification, LMW-HA can be highly inflammatory or anti-inflammatory (269). This highlight different effects of HA and/or its degradation products according to the chemical modifications and properties, to the tissue (skin vs cartilage) and the inflammatory phenotype of the microenvironment, as observed on in vitro study.

### **7) Crosslinked HA modulate vascular function in low-grade inflammatory group**

About vascular function, we showed that VYC-15L was the only product capable of modulating this function of the skin in the presence of a low-grade inflammation. Indeed, basal vascular tone of the skin was not changed by the injection of HA and their degradation products in all mice, except following the VYC-15L injection in low-grade inflammatory mice. In addition, we showed that endothelium-dependent vasodilation in response to acetylcholine (ACh) were increased by VYC-15L and its degradation product compared to vehicle in low-grade inflammatory mice, associated with an enhanced intragroup heterogeneity. This enhanced ACh-mediated vasodilation illustrates an abnormal endothelial function, which was not observed with HYC-24L+. We showed that higher vasodilation responses were not correlated to higher number of vessels in these conditions by histological observation (Data not shown). Nevertheless, it has been shown that crosslinked HA can induce angiogenesis (240). After 1, 2, 4 and 12 weeks post intradermal injection on aged individual, no inflammation by histological analysis was observed (240) but an increase number of blood vessels in the mid to deep dermis in filler-injected skin, using endothelial cell marker CD31 quantification (240) have been reported. While in this work we did not see differences in vessels density after 24h injection. It will be therefore interesting, to evaluate in a low-grade phenotype if an increase number of vessels can be observed in presence of VYC-15L in a long time post intradermal injection.

ACh is the most widely used drug to assess the endothelial L-arginine/nitric oxide (NO) pathway. In normal condition, it has been shown that vasodilation in response to ACh administration predominantly involves the production of NO (178, 270). Inhibition of endothelial NOS abolishes ACh-induced vasodilatation whereas specific and non-specific inhibitions of COX-1 and COX-2 have no effect on this vasodilatation (271). While in obese-diabetic mice associated with low-grade inflammation ACh-stimulated vasodilation could rely on COX-2 pathway, as previously reported (259). We showed a slight COX-2 overexpression in low-grade inflammatory mice respect to control that could contributes to this inflammation-linked adaptation in dermal microvascular reactivity. However, how VYC-15L and its degradation products could interact with COX-2 or the related vasodilators PGI<sub>2</sub> and PGE<sub>2</sub> remains unknown in the skin, although a link between the hyaluronan-mediated inflammation and arachidonic acid metabolism via the COX pathway was identified for monocytes and

macrophages (272). Moreover, interaction of HA with arachidonic metabolism and COX-2 was recently identified in other cell types like astrocytes involved in neuroinflammation (104).

The VCAM-1 protein mediates the adhesion of immune cells to vascular endothelium and is involved in endothelial cells permeability. VCAM-1 is also used as marker with ICAM-1 to evaluate endothelial cells activation (273). Furthermore, it has been shown that an upregulation of VCAM-1 may contribute to impaired endothelium-dependent relaxation (274) and it can be activated by pro-inflammatory cytokines and also by ROS or toll-like receptor agonists. VCAM-1 expression may be modulated by HA. Indeed, it has been shown that LMW-HA at 40 kDa and o-HA at 6.9-kDa induced overexpression of VCAM-1 while native HA induced less VCAM-1 expression on synovial cells from patient with rheumatoid arthritis (275). In this work, similarly to COX-2, VCAM-1 was not overexpressed in presence of HA degraded and non-degraded even if a heterogeneity intra-group have been observed either on control and low-grade inflammatory group. Therefore, besides endothelial cells, we cannot rule out the possible involvement of other cells such as macrophages in the enhanced response to Ach in inflammatory context. Among vasodilation pathway, other markers have to be evaluated to determine what mechanisms is involved. Indeed, vasodilation can be induced by different pathway. Endothelial cells in presence of ACh induce eNOS upregulation and its activity is also regulated at the transcriptional level by VEGF (Vascular Endothelial Growth Factor), insulin and bFGF (basic Fibroblast Growth Factor) leading to NO production (276). And in this work we did not quantified VEGF and NO production in presence of HA products.

To conclude, we showed that depending on the microenvironment, HA crosslinked and more precisely VYC-15L induced higher ACh-mediated vasodilation respect to control group with no direct implication of COX-2 and VCAM-1, suggesting that another pathway is involved. Furthermore, vasodilation response is correlated to an increase of IL-6 in these conditions suggesting that low level inflammation seems to be involved in vasodilation response. Low-grade inflammatory phenotypes induced a sensitivity to ACh in presence of VYC-15L while HYC-24L+ did not, suggesting that vascular modulation could be link to mechanical and rheological properties of the filler.

## Conclusion & Perspectives

For almost 20 years, hyaluronic acid has been the main filler used in corrective aesthetics to counteract the effect of time on the skin. It is described as non-immunogenic and biocompatible. However, as with any medical device, certain complications may appear to be mainly related to a technical problem. Nevertheless, some rare late adverse effect has been reported inducing delayed complication such as late-onset inflammatory nodules reactions.

In this preliminary project in collaboration with AbbVie (Legacy Allergan), we hypothesized that the complication could be related to a predisposition of patients to have a low-grade inflammatory environment and that degradation products of crosslinked HA could be the key of complication in this microenvironment. We evaluated two different crosslinked HA using different crosslinked technology VYCROSS® and HYLACROSS®. We highlighted that, range sizes of LMW-HA did not induce directly exacerbate inflammation on dermal skin cells and immune cells under different degree of inflammation as described recently. Furthermore, inflammatory modulation observed on these models were not persistent and were specific to HA degraded but also HA non-degraded. We have shown that a highly inflammatory microenvironment induced reverse responses in a low-grade inflammatory environment. Suggesting that depending on the cellular inflammatory phenotype, degraded and non-degraded HA induced different mechanisms. Nevertheless, apart from immune cells, we used primary dermal fibroblasts and HDMEC coming from healthy donors. In this way, it will be interesting to used primary cells, such as PBMC, fibroblasts and EC coming from patient having a low-grade inflammatory phenotype to evaluate in these condition HA metabolism and pathway which could react differently in presence of HA degraded and non-degraded. We can underline that range size of fragments could be lower than an average at 30kDa for VYC-15L and at 80 kDa for HYC-24L+. It will be interesting to increase time of degradation to produce o-HA which are clearly involved and described as pro-inflammatory. We decided to used range size of fragments to be the more in physiological condition but it will be interesting to purify and concentrate LMW-HA of the same size and treat cells to assess if LMW-HA effect is dependent of the concentration.

The development of an integrative model using the LPS mouse model induced by a super-low dose of LPS, has shown that LMW-HA according to in vitro results did not induce any inflammatory or vascular reaction while VYC-15L and in a low-grade inflammatory phenotype induced higher ACh-mediated vasodilation correlated with an increase of IL-6. Highlighting,

that VYC-15L non-degraded induced a sensitivity to ACh follow by a basal skin blood flow increased while the other crosslinked HA did not modulate vasodilation responses, illustrating an abnormal endothelial function depending on the type of crosslinked HA. To go further in the comprehension of this differences, it will be necessary to do the link between IL-6 increase in skin biopsy, the physico-chemical properties and ACh-mediated vasodilation. To establish if the vascular response and inflammatory response observed in low-grade inflammatory phenotype, is correlated to the difference in crosslinked technologies, it will be interesting to evaluate other VYCROSS® products and compare it to VYC-15L. Furthermore, we evaluated vascular and inflammatory responses, with no increase in vessels density. In this sense, evaluating the effect of cross-linked HA in a low-grade inflammatory model on angiogenesis and inflammation over a long period of time could answer a certain number of points.

furthermore, it would be useful to evaluate the degradation of HA in an in vivo inflammatory model by injecting hyaluronidase leading to the production of different fragments directly into the tissue and thus assess during degradation whether the degraded products may lead to an alteration of vascular functions correlated with exacerbated inflammation. Furthermore, LPS mice model is an experimental model and according to our hypothesis, suggesting that complication could be related to pathologies undiagnosed such as auto-immune diseases. It will be therefore necessary to use chronic inflammatory model or auto-immune models such as diabetic mice (NOD), Lupus mice model (NZM2410) or intestinal hyperpermeability mice model leading to systemic endotoxemia in order to evaluate if HA mechanism altered induce inflammatory response to crosslinked HA.

# Bibliography

1. Swann G. The skin is the body's largest organ. *J Vis Commun Med*. 2010;33(4):148-9.
2. Chuong CM, Nickoloff BJ, Elias PM, Goldsmith LA, Macher E, Maderson PA, et al. What is the 'true' function of skin? *Experimental dermatology*. 2002;11(2):159-87.
3. Patton KT, Thibodeau GA. *Anatomy & Physiology 8th*: Elsevier; 15th March 2012. 352 p.
4. Wilkinson. *Skin*: Cambridge University Press; 2009.
5. Nicol NH. Anatomy and physiology of the skin. *Dermatol Nurs*. 2005;17(1):62.
6. Lawton S. Anatomy and function of the skin. Part 2--The epidermis. *Nurs Times*. 2006;102(32):28-9.
7. Warog. Particules Janus pour des applications industrielles : stabilités et incorporation de principes actifs 2017.
8. Lawton S. Anatomy and function of the skin. Part 3--dermis and adjacent structures. *Nurs Times*. 2006;102(33):26-7.
9. Droual R. [Modesto Junior College]. Available from: <http://droualb.faculty.mjc.edu/Lecture%20Notes/Unit%201/Integumentary%20with%20figures.htm>.
10. Dr. R. K. Freinkel DTW. *The Biology of the Skin*: The Parthenon Publishing group; 2001.
11. Kanitakis J. Merkel cell carcinoma. *Cancer Treat Res*. 2009;146:329-41.
12. Zimmerman A, Bai L, Ginty DD. The gentle touch receptors of mammalian skin. (1095-9203 (Electronic)).
13. Ye H, De S. Thermal injury of skin and subcutaneous tissues: A review of experimental approaches and numerical models. *Burns : journal of the International Society for Burn Injuries*. 2017;43(5):909-32.
14. Boink MA, van den Broek LJ, Roffel S, Nazmi K, Bolscher JG, Gefen A, et al. Different wound healing properties of dermis, adipose, and gingiva mesenchymal stromal cells. *Wound Repair Regen*. 2016;24(1):100-9.
15. Park K. Role of micronutrients in skin health and function. *Biomol Ther (Seoul)*. 2015;23(3):207-17.
16. Bailey AJ. Molecular mechanisms of ageing in connective tissues. *Mech Ageing Dev*. 2001;122(7):735-55.
17. Marcos-Garcés V, Molina Aguilar P, Bea Serrano C, García Bustos V, Benavent Seguí J, Ferrández Izquierdo A, et al. Age-related dermal collagen changes during development, maturation and ageing - a morphometric and comparative study. *J Anat*. 2014;225(1):98-108.
18. Varani J, Dame MK, Rittie L, Fligel SE, Kang S, Fisher GJ, et al. Decreased collagen production in chronologically aged skin: roles of age-dependent alteration in fibroblast function and defective mechanical stimulation. *Am J Pathol*. 2006;168(6):1861-8.
19. Bennett MF, Robinson MK, Baron ED, Cooper KD. Skin immune systems and inflammation: protector of the skin or promoter of aging? *J Investig Dermatol Symp Proc*. 2008;13(1):15-9.
20. Shin J-W, Kwon S-H, Choi J-Y, Na J-I, Huh C-H, Choi H-R, et al. Molecular Mechanisms of Dermal Aging and Antiaging Approaches. *International journal of molecular sciences*. 2019;20(9):2126.
21. Zhao X, Kwan JYY, Yip K, Liu PP, Liu FF. Targeting metabolic dysregulation for fibrosis therapy. *Nat Rev Drug Discov*. 2020;19(1):57-75.
22. Oliver N, Sternlicht M, Gerritsen K, Goldschmeding R. Could aging human skin use a connective tissue growth factor boost to increase collagen content? *J Invest Dermatol*. 2010;130(2):338-41.
23. Uitto J, Li Q, Urban Z. The complexity of elastic fibre biogenesis in the skin--a perspective to the clinical heterogeneity of cutis laxa. *Exp Dermatol*. 2013;22(2):88-92.
24. Tamburro AM, Bochicchio B, Pepe A. The dissection of human tropoelastin: from the molecular structure to the self-assembly to the elasticity mechanism. *Pathol Biol (Paris)*. 2005;53(7):383-9.

25. Sproul EP, Argraves WS. A cytokine axis regulates elastin formation and degradation. *Matrix Biol.* 2013;32(2):86-94.
26. Kuang PP, Zhang XH, Rich CB, Foster JA, Subramanian M, Goldstein RH. Activation of elastin transcription by transforming growth factor-beta in human lung fibroblasts. *Am J Physiol Lung Cell Mol Physiol.* 2007;292(4):L944-52.
27. Pittayapruek P, Meehansan J, Prapapan O, Komine M, Ohtsuki M. Role of Matrix Metalloproteinases in Photoaging and Photocarcinogenesis. *Int J Mol Sci.* 2016;17(6).
28. Tsukahara K, Takema Y, Moriwaki S, Tsuji N, Suzuki Y, Fujimura T, et al. Selective inhibition of skin fibroblast elastase elicits a concentration-dependent prevention of ultraviolet B-induced wrinkle formation. *J Invest Dermatol.* 2001;117(3):671-7.
29. Fallacara A, Baldini E, Manfredini S, Vertuani S. Hyaluronic Acid in the Third Millennium. *Polymers (Basel).* 2018;10(7).
30. Papakonstantinou E, Roth M, Karakiulakis G. Hyaluronic acid: A key molecule in skin aging. *Dermatoendocrinol.* 2012;4(3):253-8.
31. Cowman MK, Lee HG, Schwertfeger KL, McCarthy JB, Turley EA. The Content and Size of Hyaluronan in Biological Fluids and Tissues. *Front Immunol.* 2015;6:261.
32. Cowman MK. Hyaluronan and Hyaluronan Fragments. *Adv Carbohydr Chem Biochem.* 2017;74:1-59.
33. Tammi R, Agren UM, Tuhkanen AL, Tammi M. Hyaluronan metabolism in skin. *Prog Histochem Cytochem.* 1994;29(2):1-81.
34. Holmes MW, Bayliss MT, Muir H. Hyaluronic acid in human articular cartilage. Age-related changes in content and size. *Biochem J.* 1988;250(2):435-41.
35. Ghatak S, Maytin EV, Mack JA, Hascall VC, Atanelishvili I, Moreno Rodriguez R, et al. Roles of Proteoglycans and Glycosaminoglycans in Wound Healing and Fibrosis. *Int J Cell Biol.* 2015;2015:834893.
36. Carrino DA, Calabro A, Darr AB, Dours-Zimmermann MT, Sandy JD, Zimmermann DR, et al. Age-related differences in human skin proteoglycans. *Glycobiology.* 2011;21(2):257-68.
37. Driskell RR, Watt FM. Understanding fibroblast heterogeneity in the skin. *Trends Cell Biol.* 2015;25(2):92-9.
38. Meigel WN, Gay S, Weber L. Dermal architecture and collagen type distribution. *Arch Dermatol Res.* 1977;259(1):1-10.
39. Silzle T, Randolph GJ, Kreutz M, Kunz-Schughart LA. The fibroblast: sentinel cell and local immune modulator in tumor tissue. *Int J Cancer.* 2004;108(2):173-80.
40. Yaar M, Gilchrist BA. Photoaging: mechanism, prevention and therapy. *Br J Dermatol.* 2007;157(5):874-87.
41. Nguyen AV, Soulika AM. The Dynamics of the Skin's Immune System. *Int J Mol Sci.* 2019;20(8).
42. Smith RS, Smith T, Blieden T, Phipps RP. Fibroblasts as Sentinel Cells: Synthesis of Chemokines and Regulation of Inflammation. *American Journal of Pathology*,; 1997.
43. Bautista-Hernández LA, Gómez-Olivares JL, Buentello-Volante B, Bautista-de Lucio VM. Fibroblasts: The Unknown Sentinels Eliciting Immune Responses Against Microorganisms. *Eur J Microbiol Immunol (Bp).* 2017;7(3):151-7.
44. Dongari-Bagtzoglou A, Wen K, Lamster IB. *Candida albicans* triggers interleukin-6 and interleukin-8 responses by oral fibroblasts in vitro. *Oral Microbiol Immunol.* 1999;14(6):364-70.
45. Méliopoulos A, Levacher C. La peau : Structure et physiologie 2012.
46. van den Oord JJ, de Wolf-Peeters C. Epithelium-lining macrophages in psoriasis. *Br J Dermatol.* 1994;130(5):589-94.
47. van den Oord JJ, De Ley M. Distribution of metallothionein in normal and pathological human skin. *Arch Dermatol Res.* 1994;286(1):62-8.
48. Krzyszczyk P, Schloss R, Palmer A, Berthiaume F. The Role of Macrophages in Acute and Chronic Wound Healing and Interventions to Promote Pro-wound Healing Phenotypes. *Front Physiol.* 2018;9:419.

49. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol.* 2000;164(12):6166-73.
50. Gleissner CA. Macrophage Phenotype Modulation by CXCL4 in Atherosclerosis. *Front Physiol.* 2012;3:1.
51. Kadl A, Meher AK, Sharma PR, Lee MY, Doran AC, Johnstone SR, et al. Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. *Circ Res.* 2010;107(6):737-46.
52. Finn AV, Nakano M, Polavarapu R, Karmali V, Saeed O, Zhao X, et al. Hemoglobin directs macrophage differentiation and prevents foam cell formation in human atherosclerotic plaques. *J Am Coll Cardiol.* 2012;59(2):166-77.
53. Mills CD. Anatomy of a discovery: m1 and m2 macrophages. *Front Immunol.* 2015;6:212.
54. Gros E, Novak N. Cutaneous dendritic cells in allergic inflammation. *Clin Exp Allergy.* 2012;42(8):1161-75.
55. Valladeau J, Saeland S. Cutaneous dendritic cells. *Semin Immunol.* 2005;17(4):273-83.
56. Nestle FO, Zheng XG, Thompson CB, Turka LA, Nickoloff BJ. Characterization of dermal dendritic cells obtained from normal human skin reveals phenotypic and functionally distinctive subsets. *J Immunol.* 1993;151(11):6535-45.
57. Clausen BE, Stoitzner P. Functional Specialization of Skin Dendritic Cell Subsets in Regulating T Cell Responses. *Front Immunol.* 2015;6:534.
58. Subramanian N, Bray MA. Interleukin 1 releases histamine from human basophils and mast cells in vitro. *The Journal of Immunology.* 1987;138(1):271.
59. Driskell RR, Jahoda CA, Chuong CM, Watt FM, Horsley V. Defining dermal adipose tissue. *Exp Dermatol.* 2014;23(9):629-31.
60. Dréno B. [Anatomy and physiology of skin and cutaneous annexes]. *Ann Dermatol Venereol.* 2009;136 Suppl 6:S247-51.
61. Deegan AJ, Wang RK. Microvascular imaging of the skin. *Phys Med Biol.* 2019;64(7):07TR1.
62. Pober JS, Sessa WC. Inflammation and the blood microvascular system. *Cold Spring Harb Perspect Biol.* 2014;7(1):a016345.
63. Tansey EA, Johnson CD. Recent advances in thermoregulation. *Adv Physiol Educ.* 2015;39(3):139-48.
64. Mai J, Virtue A, Shen J, Wang H, Yang X-F. An evolving new paradigm: endothelial cells--conditional innate immune cells. *Journal of hematology & oncology.* 2013;6:61-.
65. Michiels C. Endothelial cell functions. *J Cell Physiol.* 2003;196(3):430-43.
66. Huggenberger R, Detmar M. The cutaneous vascular system in chronic skin inflammation. *J Invest Dermatol Symp Proc.* 2011;15(1):24-32.
67. Butler LM, Rainger GE, Nash GB. A role for the endothelial glycosaminoglycan hyaluronan in neutrophil recruitment by endothelial cells cultured for prolonged periods. *Exp Cell Res.* 2009;315(19):3433-41.
68. Genasetti A, Vigetti D, Viola M, Karousou E, Moretto P, Rizzi M, et al. Hyaluronan and human endothelial cell behavior. *Connect Tissue Res.* 2008;49(3):120-3.
69. Taylor KR, Gallo RL. Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. *Faseb j.* 2006;20(1):9-22.
70. Sandoo A, van Zanten JJCSV, Metsios GS, Carroll D, Kitis GD. The endothelium and its role in regulating vascular tone. *The open cardiovascular medicine journal.* 2010;4:302-12.
71. Olchanheski LR, Sordi R, Oliveira JG, Alves GF, Mendes RT, Santos FA, et al. The role of potassium channels in the endothelial dysfunction induced by periodontitis. *J Appl Oral Sci.* 2018;26:e20180048.
72. Davidge ST. Prostaglandin H synthase and vascular function. *Circ Res.* 2001;89(8):650-60.
73. Meyer K. PJ. The polysaccharide of the vitreous of humour. *Journal of Biological Chemistry;* 1934.
74. DORFMAN A. The kinetics of the enzymatic hydrolysis of hyaluronic acid. *J Biol Chem.* 1948;172(2):377-87.

75. OGDON AG, STANIER JE. The dimensions of the particle of hyaluronic acid complex in synovial fluid. *Biochem J.* 1951;49(5):585-90.
76. LAURENT TC, GERGELY J. Light scattering studies on hyaluronic acid. *J Biol Chem.* 1955;212(1):325-33.
77. BALAZS EA, HOGBERG B, LAURENT TC. The biological activity of hyaluron sulfuric acid. *Acta Physiol Scand.* 1951;23(2-3):168-78.
78. Caputo A. Depolymerization of Hyaluronic Acid by X-rays. *Nature.* 1957;179(4570):1133-4.
79. Tezel A, Fredrickson GH. The science of hyaluronic acid dermal fillers. *J Cosmet Laser Ther.* 2008;10(1):35-42.
80. Kuehl C, Zhang T, Kaminskas LM, Porter CJH, Davies NM, Forrest L, et al. Hyaluronic Acid Molecular Weight Determines Lung Clearance and Biodistribution after Instillation. *Molecular pharmaceutics.* 2016;13(6):1904-14.
81. Lagendijk AK, Szabó A, Merks RM, Bakkers J. Hyaluronan: a critical regulator of endothelial-to-mesenchymal transition during cardiac valve formation. *Trends Cardiovasc Med.* 2013;23(5):135-42.
82. Slevin M, Krupinski J, Gaffney J, Matou S, West D, Delisser H, et al. Hyaluronan-mediated angiogenesis in vascular disease: uncovering RHAMM and CD44 receptor signaling pathways. *Matrix Biol.* 2007;26(1):58-68.
83. Soltés L, Mendichi R, Kogan G, Schiller J, Stankovska M, Arnhold J. Degradative action of reactive oxygen species on hyaluronan. *Biomacromolecules.* 2006;7(3):659-68.
84. Chanmee T, Ontong P, Kimata K, Itano N. Key Roles of Hyaluronan and Its CD44 Receptor in the Stemness and Survival of Cancer Stem Cells. *Front Oncol.* 2015;5:180.
85. Toole BP, Zoltan-Jones A, Misra S, Ghatak S. Hyaluronan: a critical component of epithelial-mesenchymal and epithelial-carcinoma transitions. *Cells Tissues Organs.* 2005;179(1-2):66-72.
86. Knudson CB, Knudson W. Hyaluronan and CD44: modulators of chondrocyte metabolism. *Clin Orthop Relat Res.* 2004(427 Suppl):S152-62.
87. Noble PW. Hyaluronan and its catabolic products in tissue injury and repair. *Matrix Biol.* 2002;21(1):25-9.
88. Petrey AC, de la Motte CA. Hyaluronan, a crucial regulator of inflammation. *Frontiers in immunology.* 2014;5:101-.
89. Termeer C, Sleeman JP, Simon JC. Hyaluronan--magic glue for the regulation of the immune response? *Trends Immunol.* 2003;24(3):112-4.
90. Garantziotis S, Savani RC. Hyaluronan biology: A complex balancing act of structure, function, location and context. *Matrix Biol.* 2019;78-79:1-10.
91. Walker K, Basehore B, Goyal A, Bansal P, Zito P. Hyaluronic acid: StatPearls; 2020 [Available from: <https://www.ncbi.nlm.nih.gov/books/NBK482440/>].
92. De Bouille K, Glogau R Fau - Kono T, Kono T Fau - Nathan M, Nathan M Fau - Tezel A, Tezel A Fau - Roca-Martinez J-X, Roca-Martinez Jx Fau - Paliwal S, et al. A review of the metabolism of 1,4-butanediol diglycidyl ether-crosslinked hyaluronic acid dermal fillers. (1524-4725 (Electronic)).
93. Monslow J, Govindaraju P, Puré E. Hyaluronan - a functional and structural sweet spot in the tissue microenvironment. *Front Immunol.* 2015;6:231.
94. Cyphert JM, Trempus CS, Garantziotis S. Size Matters: Molecular Weight Specificity of Hyaluronan Effects in Cell Biology. *Int J Cell Biol.* 2015;2015:563818.
95. Tavianatou AG, Caon I, Franchi M, Piperigkou Z, Galesso D, Karamanos NK. Hyaluronan: molecular size-dependent signaling and biological functions in inflammation and cancer. *FEBS J.* 2019;286(15):2883-908.
96. Litwiniuk M, Krejner A, Speyrer MS, Gauto AR, Grzela T. Hyaluronic Acid in Inflammation and Tissue Regeneration. *Wounds.* 2016;28(3):78-88.
97. Mummert ME. Immunologic roles of hyaluronan. (0257-277X (Print)).
98. Jiang D, Liang J, Noble PW. Hyaluronan as an immune regulator in human diseases. *Physiol Rev.* 2011;91(1):221-64.
99. Lee-Sayer SS, Dong Y, Arif AA, Olsson M, Brown KL, Johnson P. The where, when, how, and why of hyaluronan binding by immune cells. *Front Immunol.* 2015;6:150.

100. Baeva LF, Lyle Db Fau - Rios M, Rios M Fau - Langone JJ, Langone Jj Fau - Lightfoote MM, Lightfoote MM. Different molecular weight hyaluronic acid effects on human macrophage interleukin 1 $\beta$  production. (1552-4965 (Electronic)).
101. Dong Y, Arif A, Olsson M, Cali V, Hardman B, Dosanjh M, et al. Endotoxin free hyaluronan and hyaluronan fragments do not stimulate TNF- $\alpha$ , interleukin-12 or upregulate co-stimulatory molecules in dendritic cells or macrophages. *Scientific Reports*. 2016;6(1):36928.
102. Huang Z. The Activity of Hyaluronan and Hyaluronidase PH20 in Inflammation-A Role by Reagent Contaminants? *Journal of Clinical and Cellular Immunology*. 2015;06.
103. Dong Y, Arif A, Olsson M, Cali V, Hardman B, Dosanjh M, et al. Endotoxin free hyaluronan and hyaluronan fragments do not stimulate TNF- $\alpha$ , interleukin-12 or upregulate co-stimulatory molecules in dendritic cells or macrophages. *Sci Rep*. 2016;6:36928.
104. Chistyakov DV, Astakhova AA, Azbukina NV, Goriainov SV, Chistyakov VV, Sergeeva MG. High and Low Molecular Weight Hyaluronic Acid Differentially Influences Oxylipins Synthesis in Course of Neuroinflammation. *Int J Mol Sci*. 2019;20(16).
105. Itano N, Kimata K. Mammalian hyaluronan synthases. *IUBMB Life*. 2002;54(4):195-9.
106. Weigel PH, Hascall VC, Tammi M. Hyaluronan synthases. *J Biol Chem*. 1997;272(22):13997-4000.
107. Nicola V, Juergen S, Robert Stern and Ladislav S. Role, Metabolism, Chemical Modifications and Applications of Hyaluronan. *Current Medicinal Chemistry*. 2009;16(14):1718-45.
108. Itano N, Sawai T, Yoshida M, Lenas P, Yamada Y, Imagawa M, et al. Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. *J Biol Chem*. 1999;274(35):25085-92.
109. Spicer AP, McDonald JA. Characterization and molecular evolution of a vertebrate hyaluronan synthase gene family. *J Biol Chem*. 1998;273(4):1923-32.
110. Stuhlmeier KM, Pollaschek C. Differential effect of transforming growth factor beta (TGF-beta) on the genes encoding hyaluronan synthases and utilization of the p38 MAPK pathway in TGF-beta-induced hyaluronan synthase 1 activation. *J Biol Chem*. 2004;279(10):8753-60.
111. Wilkinson TS, Potter-Perigo S, Tsoi C, Altman LC, Wight TN. Pro- and anti-inflammatory factors cooperate to control hyaluronan synthesis in lung fibroblasts. *Am J Respir Cell Mol Biol*. 2004;31(1):92-9.
112. Chien L-J, Lee C-K. Hyaluronic acid production by recombinant *Lactococcus lactis*. *Applied Microbiology and Biotechnology*. 2007;77(2):339-46.
113. Yu H, Stephanopoulos G. Metabolic engineering of *Escherichia coli* for biosynthesis of hyaluronic acid. *Metabolic Engineering*. 2008;10(1):24-32.
114. Widner B, Behr R, Von Dollen S, Tang M, Heu T, Sloma A, et al. Hyaluronic Acid Production in *Bacillus subtilis*. *Applied and Environmental Microbiology*. 2005;71(7):3747.
115. DeLorenzi C, Weinberg M, Solish N, Swift A. The long-term efficacy and safety of a subcutaneously injected large-particle stabilized hyaluronic acid-based gel of nonanimal origin in esthetic facial contouring. *Dermatol Surg*. 2009;35 Suppl 1:313-21.
116. Liu L, Liu Y, Li J, Du G, Chen J. Microbial production of hyaluronic acid: current state, challenges, and perspectives. *Microb Cell Fact*. 2011;10:99.
117. Sze JH, Brownlie JC, Love CA. Biotechnological production of hyaluronic acid: a mini review. *3 Biotech*. 2016;6(1):67.
118. Khunmanee S, Jeong Y, Park H. Crosslinking method of hyaluronic-based hydrogel for biomedical applications. *J Tissue Eng*. 2017;8:2041731417726464.
119. Fagien S, Bertucci V, von Grote E, Mashburn JH. Rheologic and Physicochemical Properties Used to Differentiate Injectable Hyaluronic Acid Filler Products. *Plast Reconstr Surg*. 2019;143(4):707e-20e.
120. Stern R. Hyaluronan catabolism: a new metabolic pathway. *Eur J Cell Biol*. 2004;83(7):317-25.
121. Schiller J, Fuchs B, Arnhold J, Arnold K. Contribution of reactive oxygen species to cartilage degradation in rheumatic diseases: molecular pathways, diagnosis and potential therapeutic strategies. *Curr Med Chem*. 2003;10(20):2123-45.

122. Lapčák L, Schurz J. Photochemical degradation of hyaluronic acid by singlet oxygen. *Colloid and Polymer Science*. 1991;269(6):633-5.
123. Tokita Y, Okamoto A. Degradation of hyaluronic acid—Kinetic study and thermodynamics. *European Polymer Journal*. 1996;32(8):1011-4.
124. Chen H, Qin J, Hu Y. Efficient Degradation of High-Molecular-Weight Hyaluronic Acid by a Combination of Ultrasound, Hydrogen Peroxide, and Copper Ion. *Molecules*. 2019;24(3).
125. Simulescu V, Kalina M, Mondek J, Pekař M. Long-term degradation study of hyaluronic acid in aqueous solutions without protection against microorganisms. *Carbohydrate Polymers*. 2016;137:664-8.
126. Girish KS, Kemparaju K. The magic glue hyaluronan and its eraser hyaluronidase: a biological overview. *Life Sci*. 2007;80(21):1921-43.
127. Csoka AB, Frost GI, Stern R. The six hyaluronidase-like genes in the human and mouse genomes. *Matrix Biol*. 2001;20(8):499-508.
128. Fieber C, Baumann P, Vallon R, Termeer C, Simon JC, Hofmann M, et al. Hyaluronan-oligosaccharide-induced transcription of metalloproteases. *J Cell Sci*. 2004;117(Pt 2):359-67.
129. Taylor KR, Trowbridge JM, Rudisill JA, Termeer CC, Simon JC, Gallo RL. Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. *J Biol Chem*. 2004;279(17):17079-84.
130. Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, et al. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med*. 2002;195(1):99-111.
131. Hemming R, Martin DC, Slominski E, Nagy JJ, Halayko AJ, Pind S, et al. Mouse Hyal3 encodes a 45- to 56-kDa glycoprotein whose overexpression increases hyaluronidase 1 activity in cultured cells. *Glycobiology*. 2008;18(4):280-9.
132. Stern R, Asari AA, Sugahara KN. Hyaluronan fragments: an information-rich system. *Eur J Cell Biol*. 2006;85(8):699-715.
133. Liu L, Du G, Chen J, Zhu Y, Wang M, Sun J. Microbial production of low molecular weight hyaluronic acid by adding hydrogen peroxide and ascorbate in batch culture of *Streptococcus zooepidemicus*. *Bioresour Technol*. 2009;100(1):362-7.
134. Bothner H, Waaler T, Wik O. Limiting viscosity number and weight average molecular weight of hyaluronate samples produced by heat degradation. *International Journal of Biological Macromolecules*. 1988;10(5):287-91.
135. Sall I, Féraud G. Comparison of the sensitivity of 11 crosslinked hyaluronic acid gels to bovine testis hyaluronidase. *Polymer Degradation and Stability*. 2007;92(5):915-9.
136. Itano N. Simple primary structure, complex turnover regulation and multiple roles of hyaluronan. *J Biochem*. 2008;144(2):131-7.
137. Rayahin JE, Buhrman JS, Zhang Y, Koh TJ, Gemeinhart RA. High and low molecular weight hyaluronic acid differentially influence macrophage activation. *ACS Biomater Sci Eng*. 2015;1(7):481-93.
138. Jokela T, Oikari S, Takabe P, Rilla K, Kärnä R, Tammi M, et al. Interleukin-1 $\beta$ -induced Reduction of CD44 Ser-325 Phosphorylation in Human Epidermal Keratinocytes Promotes CD44 Homomeric Complexes, Binding to Ezrin, and Extended, Monocyte-adhesive Hyaluronan Coats. *The Journal of biological chemistry*. 2015;290(19):12379-93.
139. Misra S, Heldin P, Hascall VC, Karamanos NK, Skandalis SS, Markwald RR, et al. Hyaluronan-CD44 interactions as potential targets for cancer therapy. *FEBS J*. 2011;278(9):1429-43.
140. Khan AI, Kerfoot SM, Heit B, Liu L, Andonegui G, Ruffell B, et al. Role of CD44 and hyaluronan in neutrophil recruitment. *J Immunol*. 2004;173(12):7594-601.
141. Tamer TM. Hyaluronan and synovial joint: function, distribution and healing. *Interdiscip Toxicol*. 2013;6(3):111-25.
142. Sherman L, Sleeman J, Herrlich P, Ponta H. Hyaluronate receptors: key players in growth, differentiation, migration and tumor progression. *Curr Opin Cell Biol*. 1994;6(5):726-33.
143. Bajorath J. Molecular organization, structural features, and ligand binding characteristics of CD44, a highly variable cell surface glycoprotein with multiple functions. *Proteins*. 2000;39(2):103-11.

144. Müller S, Sindikubwabo F, Cañeque T, Lafon A, Versini A, Lombard B, et al. CD44 regulates epigenetic plasticity by mediating iron endocytosis. *Nature Chemistry*. 2020.
145. Al-Rekabi Z, Fura AM, Juhlin I, Yassin A, Popowics TE, Sniadecki NJ. Hyaluronan-CD44 interactions mediate contractility and migration in periodontal ligament cells. *Cell adhesion & migration*. 2019;13(1):138-50.
146. Kobayashi K, Hernandez LD, Galán JE, Janeway CA, Medzhitov R, Flavell RA. IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell*. 2002;110(2):191-202.
147. Liang J, Jiang D, Griffith J, Yu S, Fan J, Zhao X, et al. CD44 is a negative regulator of acute pulmonary inflammation and lipopolysaccharide-TLR signaling in mouse macrophages. *J Immunol*. 2007;178(4):2469-75.
148. Muto J, Yamasaki K, Taylor KR, Gallo RL. Engagement of CD44 by hyaluronan suppresses TLR4 signaling and the septic response to LPS. *Mol Immunol*. 2009;47(2-3):449-56.
149. Wang Q, Teder P, Judd NP, Noble PW, Doerschuk CM. CD44 deficiency leads to enhanced neutrophil migration and lung injury in *Escherichia coli* pneumonia in mice. *Am J Pathol*. 2002;161(6):2219-28.
150. Suwannakul N, Ma N, Thanan R, Pinlaor S, Ungarreevittaya P, Midorikawa K, et al. Overexpression of CD44 Variant 9: A Novel Cancer Stem Cell Marker in Human Cholangiocarcinoma in Relation to Inflammation. *Mediators Inflamm*. 2018;2018:4867234.
151. Kim Y, Lee Y-S, Hahn J-H, Choe J, Kwon HJ, Ro JY, et al. Hyaluronic acid targets CD44 and inhibits FcεpsilonRI signaling involving PKCdelta, Rac1, ROS, and MAPK to exert anti-allergic effect. *Molecular immunology*. 2008;45(9):2537-47.
152. Naor D, Nedvetzki S. CD44 in rheumatoid arthritis. *Arthritis Res Ther*. 2003;5(3):105-15.
153. Zaman A, Cui Z, Foley JP, Zhao H, Grimm PC, Delisser HM, et al. Expression and role of the hyaluronan receptor RHAMM in inflammation after bleomycin injury. *Am J Respir Cell Mol Biol*. 2005;33(5):447-54.
154. Tolg C, Poon R, Fodde R, Turley EA, Alman BA. Genetic deletion of receptor for hyaluronan-mediated motility (Rhamm) attenuates the formation of aggressive fibromatosis (desmoid tumor). *Oncogene*. 2003;22(44):6873-82.
155. Salomão R, Martins PS, Brunialti MK, Fernandes MaL, Martos LS, Mendes ME, et al. TLR signaling pathway in patients with sepsis. *Shock*. 2008;30 Suppl 1:73-7.
156. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol*. 2003;21:335-76.
157. McInturff JE, Modlin RL, Kim J. The role of toll-like receptors in the pathogenesis and treatment of dermatological disease. *J Invest Dermatol*. 2005;125(1):1-8.
158. Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, et al. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nature Medicine*. 2005;11(11):1173-9.
159. del Fresno C, Otero K, Gómez-García L, González-León MC, Soler-Ranger L, Fuentes-Prior P, et al. Tumor cells deactivate human monocytes by up-regulating IL-1 receptor associated kinase-M expression via CD44 and TLR4. *J Immunol*. 2005;174(5):3032-40.
160. Voelcker V, Gebhardt C, Averbek M, Saalbach A, Wolf V, Weih F, et al. Hyaluronan fragments induce cytokine and metalloprotease upregulation in human melanoma cells in part by signalling via TLR4. *Exp Dermatol*. 2008;17(2):100-7.
161. Lucey P, Goldberg DJ. Complications of collagen fillers. *Facial Plast Surg*. 2014;30(6):615-22.
162. Baumann LS, Shamban AT, Lupo MP, Monheit GD, Thomas JA, Murphy DK, et al. Comparison of smooth-gel hyaluronic acid dermal fillers with cross-linked bovine collagen: a multicenter, double-masked, randomized, within-subject study. *Dermatol Surg*. 2007;33 Suppl 2:S128-35.
163. Necas J, Bartosikova L, Brauner P, Kolar J. Hyaluronic acid (hyaluronan): a review. *Veterinarni Medicina*; 2008.
164. Dechert TA, Ducale AE, Ward SI, Yager DR. Hyaluronan in human acute and chronic dermal wounds. *Wound Repair Regen*. 2006;14(3):252-8.
165. Shumate GT, Chopra R, Jones D, Messina DJ, Hee CK. In Vivo Degradation of Crosslinked Hyaluronic Acid Fillers by Exogenous Hyaluronidases. *Dermatol Surg*. 2018;44(8):1075-83.

166. Hee CK, Shumate GT, Narurkar V, Bernardin A, Messina DJ. Rheological Properties and In Vivo Performance Characteristics of Soft Tissue Fillers. *Dermatol Surg.* 2015;41 Suppl 1:S373-81.
167. Funt D, Pavicic T. Dermal fillers in aesthetics: an overview of adverse events and treatment approaches. *Clin Cosmet Investig Dermatol.* 2013;6:295-316.
168. Fallacara A, Manfredini S, Durini E, Vertuani S. Hyaluronic Acid Fillers in Soft Tissue Regeneration. *Facial Plast Surg.* 2017;33(1):87-96.
169. De Boule K, Heydenrych I. Patient factors influencing dermal filler complications: prevention, assessment, and treatment. *Clin Cosmet Investig Dermatol.* 2015;8:205-14.
170. Heydenrych I, Kapoor KM, De Boule K, Goodman G, Swift A, Kumar N, et al. A 10-point plan for avoiding hyaluronic acid dermal filler-related complications during facial aesthetic procedures and algorithms for management. *Clin Cosmet Investig Dermatol.* 2018;11:603-11.
171. Bhojani-Lynch T. Late-Onset Inflammatory Response to Hyaluronic Acid Dermal Fillers. *Plast Reconstr Surg Glob Open.* 2017;5(12):e1532.
172. Lemperle G, Gauthier-Hazan N, Wolters M, Eisemann-Klein M, Zimmermann U, Duffy DM. Foreign body granulomas after all injectable dermal fillers: part 1. Possible causes. *Plast Reconstr Surg.* 2009;123(6):1842-63.
173. King M, Bassett S, Davies E, King S. Management of Delayed Onset Nodules. *J Clin Aesthet Dermatol.* 2016;9(11):E1-E5.
174. Li C, Levin M, Kaplan DL. Bioelectric modulation of macrophage polarization. *Sci Rep.* 2016;6:21044.
175. Berges C, Naujokat C, Tinapp S, Wiczorek H, Höh A, Sadeghi M, et al. A cell line model for the differentiation of human dendritic cells. *Biochem Biophys Res Commun.* 2005;333(3):896-907.
176. Baker B, Geng S, Chen K, Diao N, Yuan R, Xu X, et al. Alteration of lysosome fusion and low-grade inflammation mediated by super-low-dose endotoxin. *J Biol Chem.* 2015;290(10):6670-8.
177. Guo H, Diao N, Yuan R, Chen K, Geng S, Li M, et al. Subclinical-Dose Endotoxin Sustains Low-Grade Inflammation and Exacerbates Steatohepatitis in High-Fat Diet-Fed Mice. *J Immunol.* 2016;196(5):2300-8.
178. Gohin S, Sigauco-Roussel D, Conjard-Duplany A, Dubourg L, Saumet JL, Fromy B. What can current stimulation tell us about the vascular function of endogenous prostacyclin in healthy rat skin in vivo? *J Invest Dermatol.* 2011;131(1):237-44.
179. Medow MS, Glover JL, Stewart JM. Nitric oxide and prostaglandin inhibition during acetylcholine-mediated cutaneous vasodilation in humans. *Microcirculation.* 2008;15(6):569-79.
180. Eljarrat-Binstock E, Domb AJ. Iontophoresis: a non-invasive ocular drug delivery. *J Control Release.* 2006;110(3):479-89.
181. Kalia YN, Naik A, Garrison J, Guy RH. Iontophoretic drug delivery. *Adv Drug Deliv Rev.* 2004;56(5):619-58.
182. Kvernmo HD, Stefanovska A, Kirkeboen KA, Kvernebo K. Oscillations in the human cutaneous blood perfusion signal modified by endothelium-dependent and endothelium-independent vasodilators. *Microvasc Res.* 1999;57(3):298-309.
183. Caballero AE, Arora S, Saouaf R, Lim SC, Smakowski P, Park JY, et al. Microvascular and macrovascular reactivity is reduced in subjects at risk for type 2 diabetes. *Diabetes.* 1999;48(9):1856-62.
184. Roustit M, Blaise S, Cracowski JL. Trials and tribulations of skin iontophoresis in therapeutics. *Br J Clin Pharmacol.* 2014;77(1):63-71.
185. Slany A, Bileck A, Kreutz D, Mayer RL, Muqaku B, Gerner C. Contribution of Human Fibroblasts and Endothelial Cells to the Hallmarks of Inflammation as Determined by Proteome Profiling. *Mol Cell Proteomics.* 2016;15(6):1982-97.
186. Qazi BS, Tang K, Qazi A. Recent advances in underlying pathologies provide insight into interleukin-8 expression-mediated inflammation and angiogenesis. *Int J Inflamm.* 2011;2011:908468.
187. Enzerink A, Vaheri A. Fibroblast activation in vascular inflammation. *J Thromb Haemost.* 2011;9(4):619-26.

188. Scheibner KA, Lutz MA, Boodoo S, Fenton MJ, Powell JD, Horton MR. Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J Immunol*. 2006;177(2):1272-81.
189. Kavasi RM, Berdiaki A, Spyridaki I, Corsini E, Tsatsakis A, Tzanakakis G, et al. HA metabolism in skin homeostasis and inflammatory disease. *Food Chem Toxicol*. 2017;101:128-38.
190. Baeva LF, Lyle DB, Fau - Rios M, Rios M, Fau - Langone JJ, Langone JJ, Fau - Lightfoote MM, Lightfoote MM. Different molecular weight hyaluronic acid effects on human macrophage interleukin 1 $\beta$  production. 2014(1552-4965 (Electronic)).
191. Lyle DB, Breger JC, Baeva LF, Shallcross JC, Durfor CN, Wang NS, et al. Low molecular weight hyaluronic acid effects on murine macrophage nitric oxide production. *J Biomed Mater Res A*. 2010;94(3):893-904.
192. Belezney K, Carruthers JD, Carruthers A, Mummert ME, Humphrey S. Delayed-onset nodules secondary to a smooth cohesive 20 mg/mL hyaluronic acid filler: cause and management. *Dermatol Surg*. 2015;41(8):929-39.
193. Philipp-Dormston WG, Goodman GJ, De Boulle K, Swift A, Delorenzi C, Jones D, et al. Global Approaches to the Prevention and Management of Delayed-onset Adverse Reactions with Hyaluronic Acid-based Fillers. *Plast Reconstr Surg Glob Open*. 2020;8(4):e2730.
194. Duan L, Rao X, Sigdel KR. Regulation of Inflammation in Autoimmune Disease. *J Immunol Res*. 2019;2019:7403796.
195. Sanchez B, Li L, Dulong J, Amond G, Lamartine J, Liu G, et al. Impact of Human Dermal Microvascular Endothelial Cells on Primary Dermal Fibroblasts in Response to Inflammatory Stress. *Front Cell Dev Biol*. 2019;7:44.
196. Sattar N, McCarey DW, Capell H, McInnes IB. Explaining how "high-grade" systemic inflammation accelerates vascular risk in rheumatoid arthritis. *Circulation*. 2003;108(24):2957-63.
197. Jeppesen J. Low-grade Chronic Inflammation and Vascular Damage in Patients with Rheumatoid Arthritis: Don't Forget "Metabolic Inflammation". *The Journal of Rheumatology*. 2011;38(4):595.
198. Ferrucci L, Fabbri E. Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty. *Nat Rev Cardiol*. 2018;15(9):505-22.
199. Singer AJ, Clark RA. Cutaneous wound healing. *N Engl J Med*. 1999;341(10):738-46.
200. Buckley CD, Pilling D, Lord JM, Akbar AN, Scheel-Toellner D, Salmon M. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol*. 2001;22(4):199-204.
201. McGettrick HM, Smith E, Filer A, Kissane S, Salmon M, Buckley CD, et al. Fibroblasts from different sites may promote or inhibit recruitment of flowing lymphocytes by endothelial cells. *European journal of immunology*. 2009;39(1):113-25.
202. Swerlick RA, Lawley TJ. Role of microvascular endothelial cells in inflammation. *J Invest Dermatol*. 1993;100(1):111S-5S.
203. McGee DW, Bamberg T, Vitkus SJ, McGhee JR. A synergistic relationship between TNF-alpha, IL-1 beta, and TGF-beta 1 on IL-6 secretion by the IEC-6 intestinal epithelial cell line. *Immunology*. 1995;86(1):6-11.
204. Heidenreich R, Röcken M, Ghoreschi K. Angiogenesis drives psoriasis pathogenesis. *Int J Exp Pathol*. 2009;90(3):232-48.
205. Achtman JC, Werth VP. Pathophysiology of cutaneous lupus erythematosus. *Arthritis Res Ther*. 2015;17:182.
206. Maiellaro K, Taylor WR. The role of the adventitia in vascular inflammation. *Cardiovasc Res*. 2007;75(4):640-8.
207. Huang Z, Zhao C, Chen Y, Cowell JA, Wei G, Kultti A, et al. Recombinant human hyaluronidase PH20 does not stimulate an acute inflammatory response and inhibits lipopolysaccharide-induced neutrophil recruitment in the air pouch model of inflammation. (1550-6606 (Electronic)).
208. El Hajjaji H, Cole AA, Manicourt D-H. Chondrocytes, synoviocytes and dermal fibroblasts all express PH-20, a hyaluronidase active at neutral pH. *Arthritis research & therapy*. 2005;7(4):R756-R68.
209. Stern R, Jedrzejewski MJ. Hyaluronidases: their genomics, structures, and mechanisms of action. *Chemical reviews*. 2006;106(3):818-39.

210. Buckley CD. Why does chronic inflammation persist: An unexpected role for fibroblasts. *Immunol Lett.* 2011;138(1):12-4.
211. Yang X, Chang Y, Wei W. Endothelial Dysfunction and Inflammation: Immunity in Rheumatoid Arthritis. *Mediators Inflamm.* 2016;2016:6813016.
212. Schultze JL, Freeman T, Hume DA, Latz E. A transcriptional perspective on human macrophage biology. *Semin Immunol.* 2015;27(1):44-50.
213. Haniffa M, Gunawan M, Jardine L. Human skin dendritic cells in health and disease. *J Dermatol Sci.* 2015;77(2):85-92.
214. Atri C, Guerfali FZ, Laouini D. Role of Human Macrophage Polarization in Inflammation during Infectious Diseases. *Int J Mol Sci.* 2018;19(6).
215. Sumpter TL, Balmert SC, Kaplan DH. Cutaneous immune responses mediated by dendritic cells and mast cells. *JCI Insight.* 2019;4(1).
216. Avenoso A, D'Ascola A, Scuruchi M, Mandraffino G, Campo S, Campo GM. miR146a up-regulation is involved in small HA oligosaccharides-induced pro-inflammatory response in human chondrocytes. *Biochim Biophys Acta Gen Subj.* 2020:129731.
217. Das A, Sinha M, Datta S, Abas M, Chaffee S, Sen CK, et al. Monocyte and macrophage plasticity in tissue repair and regeneration. *Am J Pathol.* 2015;185(10):2596-606.
218. Kwon MJ, Han J Fau - Kim BH, Kim Bh Fau - Lee YS, Lee Ys Fau - Kim T-Y, Kim TY. Superoxide dismutase 3 suppresses hyaluronic acid fragments mediated skin inflammation by inhibition of toll-like receptor 4 signaling pathway: superoxide dismutase 3 inhibits reactive oxygen species-induced trafficking of toll-like receptor 4 to lipid rafts. (1557-7716 (Electronic)).
219. Tynan GA, McNaughton A, Jarnicki A, Tsuji T, Lavelle EC. Polymyxin B inadequately quenches the effects of contaminating lipopolysaccharide on murine dendritic cells. *PLoS One.* 2012;7(5):e37261.
220. Rice JB, Stoll LL, Li WG, Denning GM, Weydert J, Charipar E, et al. Low-level endotoxin induces potent inflammatory activation of human blood vessels: inhibition by statins. *Arterioscler Thromb Vasc Biol.* 2003;23(9):1576-82.
221. Kany S, Vollrath JT, Relja B. Cytokines in Inflammatory Disease. *International journal of molecular sciences.* 2019;20(23):6008.
222. Akashi S, Saitoh S Fau - Wakabayashi Y, Wakabayashi Y Fau - Kikuchi T, Kikuchi T Fau - Takamura N, Takamura N Fau - Nagai Y, Nagai Y Fau - Kusumoto Y, et al. Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. (0022-1007 (Print)).
223. Al-Mukh H, Baudoin L, Bouaboud A, Sanchez-Salgado J-L, Maraqa N, Khair M, et al. Lipopolysaccharide Induces GFAT2 Expression to Promote  $\alpha$ -Linked  $\beta$ -N-Acetylglucosaminylation and Attenuate Inflammation in Macrophages. *The Journal of Immunology.* 2020:ji2000345.
224. Hodge-Dufour J, Noble Pw Fau - Horton MR, Horton Mr Fau - Bao C, Bao C Fau - Wysoka M, Wysoka M Fau - Burdick MD, Burdick Md Fau - Strieter RM, et al. Induction of IL-12 and chemokines by hyaluronan requires adhesion-dependent priming of resident but not elicited macrophages. (0022-1767 (Print)).
225. Horton MR, McKee CM, Bao C, Liao F, Farber JM, Hodge-DuFour J, et al. Hyaluronan fragments synergize with interferon-gamma to induce the C-X-C chemokines mig and interferon-inducible protein-10 in mouse macrophages. *J Biol Chem.* 1998;273(52):35088-94.
226. Taylor KR, Yamasaki K, Radek KA, Di Nardo A, Goodarzi H, Golenbock D, et al. Recognition of hyaluronan released in sterile injury involves a unique receptor complex dependent on Toll-like receptor 4, CD44, and MD-2. *J Biol Chem.* 2007;282(25):18265-75.
227. Yamasaki K, Muto J, Taylor KR, Cogen AL, Audish D, Bertin J, et al. NLRP3/cryopyrin is necessary for interleukin-1beta (IL-1beta) release in response to hyaluronan, an endogenous trigger of inflammation in response to injury. *The Journal of biological chemistry.* 2009;284(19):12762-71.
228. Shiedlin A, Bigelow R Fau - Christopher W, Christopher W Fau - Arbabi S, Arbabi S Fau - Yang L, Yang L Fau - Maier RV, Maier Rv Fau - Wainwright N, et al. Evaluation of hyaluronan from different sources: *Streptococcus zooepidemicus*, rooster comb, bovine vitreous, and human umbilical cord. (1525-7797 (Print)).

229. Swerlick RA, Lee KH, Li LJ, Sepp NT, Caughman SW, Lawley TJ. Regulation of vascular cell adhesion molecule 1 on human dermal microvascular endothelial cells. *J Immunol.* 1992;149(2):698-705.
230. Villaschi S, Nicosia RF. Paracrine interactions between fibroblasts and endothelial cells in a serum-free coculture model. Modulation of angiogenesis and collagen gel contraction. *Lab Invest.* 1994;71(2):291-9.
231. Martin TA, Harding K, Jiang WG. Matrix-bound fibroblasts regulate angiogenesis by modulation of VE-cadherin. *Eur J Clin Invest.* 2001;31(11):931-8.
232. Berthod F, Germain L, Tremblay N, Auger FA. Extracellular matrix deposition by fibroblasts is necessary to promote capillary-like tube formation in vitro. *J Cell Physiol.* 2006;207(2):491-8.
233. Stunova A, Vistejnova L. Dermal fibroblasts-A heterogeneous population with regulatory function in wound healing. *Cytokine Growth Factor Rev.* 2018;39:137-50.
234. Van Linthout S, Miteva K, Tschöpe C. Crosstalk between fibroblasts and inflammatory cells. *Cardiovasc Res.* 2014;102(2):258-69.
235. Dasu MR, Barrow RE, Spies M, Herndon DN. Matrix metalloproteinase expression in cytokine stimulated human dermal fibroblasts. *Burns.* 2003;29(6):527-31.
236. Kolář M, Szabo P, Dvořánková B, Lacina L, Gabius HJ, Strnad H, et al. Upregulation of IL-6, IL-8 and CXCL-1 production in dermal fibroblasts by normal/malignant epithelial cells in vitro: Immunohistochemical and transcriptomic analyses. *Biol Cell.* 2012;104(12):738-51.
237. Quan T, Fisher GJ. Role of Age-Associated Alterations of the Dermal Extracellular Matrix Microenvironment in Human Skin Aging: A Mini-Review. *Gerontology.* 2015;61(5):427-34.
238. David-Raoudi M, Tranchepain F, Deschrevel B, Vincent J-C, Bogdanowicz P, Boumediene K, et al. Differential effects of hyaluronan and its fragments on fibroblasts: Relation to wound healing. *Wound Repair and Regeneration.* 2008;16(2):274-87.
239. Mast BA, Diegelmann Rf Fau - Krummel TM, Krummel Tm Fau - Cohen IK, Cohen IK. Hyaluronic acid modulates proliferation, collagen and protein synthesis of cultured fetal fibroblasts. (0934-8832 (Print)).
240. Quan T, Wang F, Shao Y, Rittié L, Xia W, Orringer JS, et al. Enhancing structural support of the dermal microenvironment activates fibroblasts, endothelial cells, and keratinocytes in aged human skin in vivo. *J Invest Dermatol.* 2013;133(3):658-67.
241. Singleton PA. Hyaluronan regulation of endothelial barrier function in cancer. *Adv Cancer Res.* 2014;123:191-209.
242. Toole BP. Hyaluronan: from extracellular glue to pericellular cue. *Nat Rev Cancer.* 2004;4(7):528-39.
243. Tolg C, Telmer P, Turley E. Specific sizes of hyaluronan oligosaccharides stimulate fibroblast migration and excisional wound repair. *PLoS One.* 2014;9(2):e88479.
244. Ghazi K, Deng-Pichon U, Warnet J-M, Rat P. Hyaluronan Fragments Improve Wound Healing on In Vitro Cutaneous Model through P2X7 Purinoreceptor Basal Activation: Role of Molecular Weight. *PLOS ONE.* 2012;7(11):e48351.
245. Ferguson EL, Roberts JL, Moseley R, Griffiths PC, Thomas DW. Evaluation of the physical and biological properties of hyaluronan and hyaluronan fragments. *Int J Pharm.* 2011;420(1):84-92.
246. Wohlrab J, Wohlrab D, Neubert RH. Comparison of noncross-linked and cross-linked hyaluronic acid with regard to efficacy of the proliferative activity of cutaneous fibroblasts and keratinocytes in vitro. *J Cosmet Dermatol.* 2013;12(1):36-40.
247. Slevin M, West D, Kumar P, Rooney P, Kumar S. Hyaluronan, angiogenesis and malignant disease. *Int J Cancer.* 2004;109(5):793-4; author reply 5-6.
248. Gao F, Yang CX, Mo W, Liu YW, He YQ. Hyaluronan oligosaccharides are potential stimulators to angiogenesis via RHAMM mediated signal pathway in wound healing. *Clin Invest Med.* 2008;31(3):E106-16.
249. Fahey E, Doyle SL. IL-1 Family Cytokine Regulation of Vascular Permeability and Angiogenesis. *Front Immunol.* 2019;10:1426.

250. Jiang WG, Sanders AJ, Ruge F, Harding KG. Influence of interleukin-8 (IL-8) and IL-8 receptors on the migration of human keratinocytes, the role of PLC- $\gamma$  and potential clinical implications. *Experimental and therapeutic medicine*. 2012;3(2):231-6.
251. Mine S, Okada Y, Kawahara C, Tabata T, Tanaka Y. Serum hyaluronan concentration as a marker of angiopathy in patients with diabetes mellitus. *Endocr J*. 2006;53(6):761-6.
252. Yoshioka Y, Kozawa E, Urakawa H, Arai E, Futamura N, Zhuo L, et al. Suppression of hyaluronan synthesis alleviates inflammatory responses in murine arthritis and in human rheumatoid synovial fibroblasts. *Arthritis Rheum*. 2013;65(5):1160-70.
253. Cheng G, Swaidani S, Sharma M, Lauer ME, Hascall VC, Aronica MA. Hyaluronan deposition and correlation with inflammation in a murine ovalbumin model of asthma. *Matrix Biol*. 2011;30(2):126-34.
254. Suarez-Fueyo A, Tsokos MG, Kwok SK, Maeda K, Katsuyama E, Lapchak PH, et al. Hyaluronic Acid Synthesis Contributes to Tissue Damage in Systemic Lupus Erythematosus. *Front Immunol*. 2019;10:2172.
255. Ghallab A. In vitro test systems and their limitations. *EXCLI journal*. 2013;12:1024-6.
256. Lassenius MI, Pietiläinen KH, Kaartinen K, Pussinen PJ, Syrjänen J, Forsblom C, et al. Bacterial endotoxin activity in human serum is associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation. *Diabetes Care*. 2011;34(8):1809-15.
257. Wiesner P, Choi Sh Fau - Almazan F, Almazan F Fau - Benner C, Benner C Fau - Huang W, Huang W Fau - Diehl CJ, Diehl Cj Fau - Gonen A, et al. Low doses of lipopolysaccharide and minimally oxidized low-density lipoprotein cooperatively activate macrophages via nuclear factor kappa B and activator protein-1: possible mechanism for acceleration of atherosclerosis by subclinical endotoxemia. (1524-4571 (Electronic)).
258. Geng S, Chen K, Yuan R, Peng L, Maitra U, Diao N, et al. The persistence of low-grade inflammatory monocytes contributes to aggravated atherosclerosis. *Nat Commun*. 2016;7:13436.
259. Nguyen-Tu MS, Nivoit P, Oréa V, Lemoine S, Acquaviva C, Pagnon-Minot A, et al. Inflammation-linked adaptations in dermal microvascular reactivity accompany the development of obesity and type 2 diabetes. *Int J Obes (Lond)*. 2019;43(3):556-66.
260. Minihane AM, Vinoy S, Russell WR, Baka A, Roche HM, Tuohy KM, et al. Low-grade inflammation, diet composition and health: current research evidence and its translation. *Br J Nutr*. 2015;114(7):999-1012.
261. Castro AM, Macedo-de la Concha LE, Pantoja-Meléndez CA. Low-grade inflammation and its relation to obesity and chronic degenerative diseases. *Revista Médica del Hospital General de México*. 2017;80(2):101-5.
262. Maitra U, Deng H, Glaros T, Baker B, Capelluto DG, Li Z, et al. Molecular mechanisms responsible for the selective and low-grade induction of proinflammatory mediators in murine macrophages by lipopolysaccharide. *J Immunol*. 2012;189(2):1014-23.
263. Lin ZQ, Kondo T, Ishida Y, Takayasu T, Mukaida N. Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. *J Leukoc Biol*. 2003;73(6):713-21.
264. Lasselín J, Kemani MK, Kanstrup M, Olsson GL, Axelsson J, Andreasson A, et al. Low-grade inflammation may moderate the effect of behavioral treatment for chronic pain in adults. *J Behav Med*. 2016;39(5):916-24.
265. La Gatta A, Salzillo R, Catalano C, D'Agostino A, Pirozzi AVA, De Rosa M, et al. Hyaluronan-based hydrogels as dermal fillers: The biophysical properties that translate into a "volumetric" effect. *PLoS One*. 2019;14(6):e0218287.
266. Molliard SG, Bétemps JB, Hadjab B, Topchian D, Micheels P, Salomon D. Key rheological properties of hyaluronic acid fillers: from tissue integration to product degradation. *Plastic and Aesthetic Research*. 2018;5:17.
267. Rooney P, Srivastava A, Watson L, Quinlan LR, Pandit A. Hyaluronic acid decreases IL-6 and IL-8 secretion and permeability in an inflammatory model of interstitial cystitis. *Acta Biomater*. 2015;19:66-75.

268. Zhang Z, Wei X, Gao J, Zhao Y, Guo L, Chen C, et al. Intra-Articular Injection of Cross-Linked Hyaluronic Acid-Dexamethasone Hydrogel Attenuates Osteoarthritis: An Experimental Study in a Rat Model of Osteoarthritis. *Int J Mol Sci.* 2016;17(4):411.
269. Babasola O, Rees-Milton KJ, Bebe S, Wang J, Anastassiades TP. Chemically modified N-acylated hyaluronan fragments modulate proinflammatory cytokine production by stimulated human macrophages. *The Journal of biological chemistry.* 2014;289(36):24779-91.
270. Warren JB. Nitric oxide and human skin blood flow responses to acetylcholine and ultraviolet light. *The FASEB Journal.* 1994;8(2):247-51.
271. Morris SJ, Shore AC. Skin blood flow responses to the iontophoresis of acetylcholine and sodium nitroprusside in man: possible mechanisms. *J Physiol.* 1996;496 ( Pt 2):531-42.
272. Sokolowska M, Chen L-Y, Eberlein M, Martinez-Anton A, Liu Y, Alsaaty S, et al. Low molecular weight hyaluronan activates cytosolic phospholipase A2 $\alpha$  and eicosanoid production in monocytes and macrophages. *The Journal of biological chemistry.* 2014;289(7):4470-88.
273. Kong DH, Kim YK, Kim MR, Jang JH, Lee S. Emerging Roles of Vascular Cell Adhesion Molecule-1 (VCAM-1) in Immunological Disorders and Cancer. *Int J Mol Sci.* 2018;19(4).
274. Zhang R, Jia G, Bao J, Zhang Y, Bai Y, Lin L, et al. Increased vascular cell adhesion molecule-1 was associated with impaired endothelium-dependent relaxation of cerebral and carotid arteries in simulated microgravity rats. *J Physiol Sci.* 2008;58(1):67-73.
275. Fujii K, Tanaka Y, Hubscher S, Saito K, Ota T, Eto S. Cross-linking of CD44 on rheumatoid synovial cells up-regulates VCAM-1. *J Immunol.* 1999;162(4):2391-8.
276. Sase K, Michel T. Expression and regulation of endothelial nitric oxide synthase. *Trends Cardiovasc Med.* 1997;7(1):28-37.

# Appendices

**Review: “Biological effect of hyaluronic acid products and wound healing”**

Benjamin Sanchez, Dominique Sigaud-Roussel and Bérengère Fromy; Submitted in International Journal of Cosmetic Science.



**Biological effect of hyaluronic acid products and wound healing**

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## Biological effect of hyaluronic acid products and wound healing

### Abstract

Hyaluronic acid is a molecule whose constant synthesis and degradation allow a multitude of biological processes. It is a finely regulated process to maintain tissue regulation and homeostasis. In the human body, different studies demonstrated that endogenous and exogenous HA were mainly degraded or altered inducing modulation of their physico-chemical properties and biological effects such as wound healing, and inflammation according to different mechanisms, described in this review.

### Keywords

Hyaluronic acid, metabolism, inflammation, wound healing

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## 1. Introduction

In 1934 Karl Meyer and John Palmer discovered hyaluronic acid, a combination of uronic acid and an aminoglycan, in the vitreous humour of eyes. It is by associating the terms uronic acid and the word hyaloid, from the greek "hyalos" meaning vitreous, that they proposed the term hyaluronic acid (1). It was only in the 1950s that HA structure and its properties were discovered (2-5). They have shown that HA was composed of a repeating disaccharide units and they highlighted the sensitivity of HA to UV or X-ray inducing its complete degradation (5, 6). Each disaccharide has a molecular weight around 400 Da but depending on the number of repeating units in a polymer it can reach to 20 MDa (7, 8). As mentioned above in the body, the proportion of HA and its size depends on the tissue and the type of synthesis.

More than 50% of the total HA content of the whole body is located in the skin. Endogenous HA has other important functions in the skin but also in other tissues playing a role in organogenesis (9), angiogenesis (10), reactive oxygen species (11), cancer (12, 13), chondrocytes (14), lung injury (15), inflammation (16) and immune regulation (17). It exists in many forms of HA: circulating, associated within tissue and inserted into the extracellular matrix. This hydrophilic molecule is used in the treatment of osteoarthritis, giving elasticity at the cartilage and increasing the viscosity of the synovial fluid using exogenous HA (18). Hyaluronic acid thus plays a valuable role in the joints as a lubricant and shock absorber. These various effects have been studied in recent years, highlighting the complexity of this regulation (19, 20) .

These commercial products use HA crosslinked with crosslinking agent to increase the longevity of HA and their rheological properties. The most common agent used and studied over 15 years in industry is the BDDE (21) according to its biodegradability and stability (21). Contrary to endogenous HA which has a half-life of 24 to 48 hours in the skin, exogenous crosslinked HA can have a clinical duration that can reach or exceed 1 year (21). The interest of these fillers are their properties to counteract the loss of moisture and elasticity in the skin and the development of wrinkles improving skin quality and appearances.

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## 2. HA metabolism

### 2.1 Intrinsic synthesis

Hyaluronic acid is a molecule whose constant synthesis and degradation allow a multitude of biological processes to be precisely modulated. It is a finely regulated process to maintain tissue regulation and homeostasis.

The mechanism of synthesis of hyaluronic acid is a fast and efficient process (19). Hyaluronic acid is produced mainly on the surface of the fibroblasts by extrusion into the extracellular matrix in association with numerous receptors such as CD44. Unlike other GAGs, hyaluronic acid is not synthesized in the Golgi apparatus but on the inner surface of the plasma membrane of the cell by transmembrane proteins: the HAS (Hyaluronan synthases). The intrinsic production of HA is provided by three mammalian hyaluronan synthases: HAS1, HAS2 and HAS3 (22, 23). The sequence homology between the three HA is very high (24) but these enzymes differ in their stability and in the length of the HA chain produced. Indeed, HAS1 and HAS2 produce a hyaluronic acid twice as long as HAS3. In this way, long linear chains of HA are formed on the cell surface and play an essential role in the cell microenvironment in keratinocytes, fibroblasts, endothelial cells and cells of the immune system (25).

It has been reported that HAS plays different roles in the development according to the different tissues or cells types, synthesizing different sizes for different functions (26, 27). The different activations of HAS have been reported *in vitro* to be modulated by cytokines, growth factor or chemokines in homeostasis and diseases like rheumatoid arthritis. For example, TGF- $\beta$  activates HAS1 while it suppresses HAS3 activities in synoviocytes (28); and IL-1 $\beta$  and TNF- $\alpha$  induces HAS2 mRNA on lung fibroblasts (29).

Intrinsic hyaluronic acid is finely regulated leading to different size and proportion of HA dependent of the tissue. Indeed, in healthy condition, HA in human synovial fluid has an average molecular weight around 6 to 7MDa, in normal blood serum is mainly relatively low from 100 to 300 kDa, while in human normal skin the average of molecular weight is around 4 to 6 MDa (30).

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## 2.2 Extrinsic synthesis

Hyaluronic acid can be obtained by tissue extraction or produced by bacterial fermentation. Tissue extraction can be carried out from tissues containing large amounts of HA such as the rooster crests. With tissue extraction from animals, 10 000–15 000 monomeric units per chain corresponding to an average of 4–6 MDa can be extracted (7). Nevertheless, these methods have drawbacks such as cost and difficulty in purification, which increases the risk of allergies and infections.

Recombinant HA production has emerged as an alternative using Gram-positive and Gram-negative bacteria as hosts, including *Bacillus* sp, *Lactococcus lactis* and *Escherichia coli* (31-33). Bacterial fermentation is the most common process to produce HA (34). HA is synthesized in the cell membranes and excreted into the media to facilitate manipulation and purification. With this technique, some risks remain with the existence of a possible contamination by endotoxins (35, 36). Bacterially produced HA can vary in molecular weight, producing low molecular weight of HA to very high molecular weight, depending on the monomeric units per chain (7).

Then, some chemical modifications are carried out in order to increase the half-life of HA in the body or modify its rheological properties according to the desired indications (Figure 1) (37, 38).

The main chemical modification is the use of crosslinking agents, inducing the formation of the gel producing a network of polymer, imposing a chemical and physical barrier to enzymatic and free radical breakdown. Indeed, enzymes and free radicals degraded then slowly crosslinked HA than native HA due to the large size of crosslinked HA and their difficulties to penetrate the gel (7).

## 3. Crosslinked HA and its properties

Crosslinked hyaluronic acid is a biocompatible, viscoelastic, and hygroscopic compound (39, 40). One of the main properties of HA is its capacity to surround itself with water molecules, being a highly hydrophilic polymer, preventing dehydration and maintains skin structure. These properties can be modulated by the length, the molecular weight and the different degree of polymerization of HA (7) and give different rheological properties, clinical indications and durations depending on the crosslinking (38, 41).

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4 Their characteristics can be modulated depending on the cross linker used, its degree of  
5 crosslinking, the gel hardness, its consistency and the total HA concentration, which is  
6 the amount of HA per milliliter of finished product. All these characteristic modulations  
7 change the lifetime of HA in the skin (7, 42).

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10 According to its capacity to have different rheological properties and actions, hyaluronic  
11 acid has been studied in many fields in order to understand its action on the organism,  
12 its metabolic role but also its interest in therapeutics and corrective aesthetics.

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15 Its properties give the possibility to develop a large variety of filler.

#### 16 17 18 19 20 **4. Degradation**

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22 In the human body, *in vivo* and *in vitro* experiments demonstrated that endogenous and  
23 exogenous HA were mainly degraded or altered inducing modulation of their physico-  
24 chemical properties and biological effects (5) according to different mechanisms: by  
25 enzymatic degradation by hyaluronidases and  $\beta$ -glucuronidases and  $\beta$ -  
26 acetylglucosaminidases (43), by action of hydroxyl radicals (44), UV (45), PH in solution  
27 (46), Heat (46, 47) or combination of sonication, ultrasound, H<sub>2</sub>O<sub>2</sub>, and copper Ion (48).

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30 Hyaluronidases cleave the  $\beta$ 1-4 bond Hyaluronic acid and generate hyaluronic acid  
31 fragments on variable sizes. There are six genes in the human genome coding for  
32 hyaluronidases of varying tissue distribution (49). In humans, genes coding for  
33 hyaluronidases include HYAL-1, HYAL-2, HYAL-3, HYAL-4, HYALP1 and PH-20 and form  
34 two clusters on two different chromosomes (50). These enzymes are found in organs  
35 such as the spleen, skin, eyes, liver and in some body fluids.

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HYAL-1 and HYAL-2 are the predominant isoforms to cleave HA and have ubiquitous expression. HYAL-2 begins by cleaving high molecular weight HA into intermediate sized fragments (about 20 kDa) that is mostly bound to the CD44 receptor. Its expression is limited to lysosomes. HYAL-1 cleaves the biopolymer into monosaccharides follow by lysosomal enzymes ( $\beta$ -glucuronidases and  $\beta$ -acetylglucosaminidases) leading to the formation of tetrasaccharides (800 Da) and then ending by single sugars formation (Figure 2) (50).

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The fragments of HA during the enzymatic process have been reported to induce transcription of MMPs (51) and stimulated endothelial cell activation (52) while oligomers (6 to 20 kDa) have been reported to be proangiogenic, pro-inflammatory and immune-stimulatory (53).

The role of HYAL3 in the degradation is not completely elucidated, but it seems to contribute to HA metabolisms by increasing the HYAL1 activity (54). PH-20 is a testicular enzyme encoded by the sperm adhesion molecule 1 (SPAM1), located on the human sperm surface and inner acrosomal surface. These enzymes are not totally specific to hyaluronic acid, HYAL4 can cleave chondroitin sulfate (50) for instance.

The progressive degradation of large polymers of hyaluronic acid by enzymatic reactions generates polymers of variable sizes. It has been reported that these different sizes of HA exert extremely diverse and often opposing biological functions. The mechanisms underlying this opposite signaling are still poorly defined but could involve competition for membrane receptors and induce inflammatory, immuno-stimulatory and angiogenic responses (55).

Nevertheless, hyaluronic acid can be degraded by other factors than enzymes like UV rays,  $\gamma$ , a combination of ultrasounds, ultrasound,  $H_2O_2$ , and copper Ion, by oxidation-reduction depolymerization (by hydrogen peroxide and or ascorbate) or by heat and/or under pressure (48, 56, 57).

The use of HA crosslinked as filler is also subject to be degraded. Crosslinking agents used to increase the HA stability in the skin have been shown not to interfere with the natural enzymatic process described above (58). In fact, it has been reviewed by De Boule et al. 2013 that crosslinked HA have the same enzymatic, hydrolytic and oxidative degradation process as native HA in the body (21) .

### 5. HA Receptors

Secreted into the extracellular matrix, HA molecules and fragments are incorporated into the extracellular matrix and are capable of interacting with receptors in order to transmit different cellular signals and thereby influence the behavior of the cells, which could in turn modify their differentiation, migration and phenotypes (59, 60).

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4 HA acts through its binding to various cellular receptors of the transmembrane type.  
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6 CD44 is the main receptor, the most studied today. Its state of glycosylation is one of  
7  
8 the elements conditioning its ability to bind with HA. The other receptors are: RHAMM,  
9  
10 TLR (Toll-Like-Receptors) 4, HARE and LYVE-1.  
11

### 12 **5.1 CD44**

13  
14 CD44 is the major hyaluronic acid binding protein and its function has been extensively  
15  
16 studied. CD44 is widely expressed in almost all types of cells in humans and mice. Within  
17  
18 this family, there are many post-translational modifications that can modulate the  
19  
20 affinity of CD44 to HA (61). The smallest isoform, without variant, is called "standard"  
21  
22 CD44s and is expressed in most tissues. Its main role is that of receptor for hyaluronic  
23  
24 acid. This molecule is involved in cell/cell and cell/matrix extracellular adhesion (62). It  
25  
26 is involved in many biological processes, such as lymphocyte recruitment,  
27  
28 hematopoiesis, embryogenesis and wound healing (63-65). CD44-HA binding is  
29  
30 important not only for intercellular interactions, but also for interactions between the  
31  
32 cells and the ECM and affects numerous functions, including inflammation, cancer and  
33  
34 metastasis (66, 67) as well as cell migration (68). This receptor is essential for the  
35  
36 inflammatory process in correlation with TLR4 in presence of lipopolysaccharides (LPS)  
37  
38 (69-72). Furthermore, it has been shown that CD44 and its isoforms are overexpressed  
39  
40 or involved in some inflammatory pathologies, such as cancer (73), asthma (74) or  
41  
42 rheumatoid arthritis (75).  
43

### 44 **5.2 RHAMM**

45  
46 The second major HA receptor is the Receptor for Hyaluronan Mediated Motility  
47  
48 (RHAMM). RHAMM can be located on the cell surface, the cytoplasm, the nucleus, or  
49  
50 the ECM. RHAMM is a functional receptor in many cell types including endothelial cells.  
51  
52 In interaction with HA, RHAMM plays an important role in tissue injury and repair (76).  
53  
54 Furthermore, it has been shown that RHAMM was not an absolute requirement for  
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56 normal development (77) and RHAMM is not directly involved in the inflammatory  
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58 process in presence of HA and HA fragments unlike CD44.  
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### 60 **5.3 Toll-Like Receptor 2 and 4**

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The TLRs are involved in the different inflammatory process. There are about 10 different TLRs. Among them, TLR2 and TLR4 have been identified as initiators of immune responses in presence of lipopolysaccharides, mycobacteria and gram-positive organisms (69, 78, 79).

Many types of cells have Toll-like receptors (TLRs) on their surfaces such as macrophages, dermal fibroblasts and endothelial cells (80). The activation of TLRs by binding to their natural ligand leads to signaling cascade resulting in the nuclear translocation of the NF- $\kappa$ B transcription factor, which modulates the transcription of different target genes. Thus, genes coding for pro-inflammatory chemokines and cytokines will be activated such as TNF- $\alpha$  and IL-6, resulting in the local recruitment of various cells such as polynuclear cells and macrophages. The activation of TLRs also leads to the release of certain natural antimicrobial molecules and to an increased phagocytosis process of the cells.

Among all its interactions, TLR2 and TLR4 seem to be receptors of HA. Some studies show that in the absence of TLRs, and in the presence of HA fragments, there is no induction of cytokines and chemokines (81) and that HA oligomers induce dendritic cells maturation via TLR4 (53).

Del Fresno et al. showed that hyaluronic acid upregulates IL-1R-associated Kinase-M, a negative regulator of TLR signaling inhibiting IRAK4/1 complex, to deactivate human monocytes via CD44 and TLR4 (82). In a melanoma, small HA fragments stimulate expression of MMP2 and IL-8, leading to NF- $\kappa$ B activation, enhanced the motility of cells, in part in a TLR4-dependent manner (83).

Nevertheless, this part is controversial. In fact, there is no direct evidence that LMW of HA binds to TLR2 or TLR4, and some reports have suggested that the inflammatory response in the presence of fragments of HA was linked to samples contamination by endotoxin during the degradation process done *in vitro* using hyaluronidase (84).

#### 5.4 Other receptors

Other receptors are known to interact with hyaluronic acid such as: HARE and LYVE1 regulating lymphatic endothelial cells functions, Brevican and neurocan modulating

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4 neuronal development and brain tumor invasiveness and SHAP-1 and TSG-6 involved in  
5 osteoblastic differentiation, in fertility, in complement activation and injury and fibrosis.  
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#### 10 11 **6. Biological effect of HA and its degradation products in wound healing**

12 Physical properties and size distribution induce different signaling pathways leading to  
13 different biological effects. According to its high molecular weight, hyaluronic acid  
14 possesses structural effect allowing its association with the fibers of the extracellular  
15 matrix in the tissues. It contributes to the hydration of the extracellular matrix and  
16 tissues through its hydrophilic and viscoelastic properties due to its helical structure and  
17 negative charges. But during the different process of ECM remodeling, pathologies,  
18 aging or healing, hyaluronic acid can be degraded using specific enzymes named  
19 hyaluronidase and some lysosomal enzymes. These enzymes can produce different  
20 range sizes of molecular weight of hyaluronic acid fragments, including oligomers of HA  
21 (o-HA) from 10 Da to 10kDa, low molecular weight of HA (LMW-HA) from 10kDa to  
22 250kDa, medium molecular weight of fragments (85) from 250kDa to 1000kDa and high  
23 molecular weight of HA (HMW-HA) from 1000 kDa to ~6000 kDa (20, 86, 87) (Table 1).  
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36 Endogenous hyaluronic acid and its degradation products have different biological  
37 effects. The two main effects are its role in wound healing process and the second and  
38 most controversial is its role in regulating the immune response.  
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42 Hyaluronic acid plays a role in the 3 phases of wound healing. During the first phase,  
43 specific hyaluronidases and lysosomal enzymes degrade hyaluronic acid into oligomers  
44 of variable sizes. The high concentration of oligomers, which interact with CD44, causes  
45 diapedesis of leukocytes and macrophages and stimulates the production of pro-  
46 inflammatory cytokines (88). During the proliferative phase, the increase in the  
47 concentration of oligomers allows the organization and stabilization of the extracellular  
48 matrix that facilitates the migration of fibroblasts, leading to the formation of capillaries  
49 and a granuloma in the wound. During the remodeling phase, the cells produce native  
50 hyaluronic acid and it is this that binds to CD44 stopping the signaling pathway leading  
51 to stop the proliferation of endothelial cells.  
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Concerning high molecular weight of HA, it is considered as anti-inflammatory. By inhibiting the activation of the nuclear factor NF-kappa B in wound healing process, Baeva et al. observed that monocytes and macrophages under different inflammatory stimuli and in presence of high molecular weight of HA suppressed IL-1 $\beta$  production (89). Indeed, it has been discovered that HA macromolecules mask the membrane receptors of the cells involved in the inflammatory reaction, thus preventing their activation and contributing to the anti-inflammatory action (20, 90).

Numerous studies have evaluated HA in inflammation link to their degradation products but the recent controversy suggest that inflammatory responses are correlated to o-HA size dependent and not link to low molecular weight of HA.

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## References

1. Meyer K. PJ. The polysaccharide of the vitreous of humour. *Journal of Biological Chemistry*; 1934.
2. DORFMAN A. The kinetics of the enzymatic hydrolysis of hyaluronic acid. *J Biol Chem*. 1948;172(2):377-87.
3. OGSTON AG, STANIER JE. The dimensions of the particle of hyaluronic acid complex in synovial fluid. *Biochem J*. 1951;49(5):585-90.
4. LAURENT TC, GERGELY J. Light scattering studies on hyaluronic acid. *J Biol Chem*. 1955;212(1):325-33.
5. BALAZS EA, HOGBERG B, LAURENT TC. The biological activity of hyaluron sulfuric acid. *Acta Physiol Scand*. 1951;23(2-3):168-78.
6. Caputo A. Depolymerization of Hyaluronic Acid by X-rays. *Nature*. 1957;179(4570):1133-4.
7. Tezel A, Fredrickson GH. The science of hyaluronic acid dermal fillers. *J Cosmet Laser Ther*. 2008;10(1):35-42.
8. Kuehl C, Zhang T, Kaminskas LM, Porter CJH, Davies NM, Forrest L, et al. Hyaluronic Acid Molecular Weight Determines Lung Clearance and Biodistribution after Instillation. *Molecular pharmaceutics*. 2016;13(6):1904-14.
9. Lagendijk AK, Szabó A, Merks RM, Bakkers J. Hyaluronan: a critical regulator of endothelial-to-mesenchymal transition during cardiac valve formation. *Trends Cardiovasc Med*. 2013;23(5):135-42.
10. Slevin M, Krupinski J, Gaffney J, Matou S, West D, Delisser H, et al. Hyaluronan-mediated angiogenesis in vascular disease: uncovering RHAMM and CD44 receptor signaling pathways. *Matrix Biol*. 2007;26(1):58-68.
11. Soltés L, Mendichi R, Kogan G, Schiller J, Stankovska M, Arnhold J. Degradative action of reactive oxygen species on hyaluronan. *Biomacromolecules*. 2006;7(3):659-68.
12. Chanmee T, Ontong P, Kimata K, Itano N. Key Roles of Hyaluronan and Its CD44 Receptor in the Stemness and Survival of Cancer Stem Cells. *Front Oncol*. 2015;5:180.
13. Toole BP, Zoltan-Jones A, Misra S, Ghatak S. Hyaluronan: a critical component of epithelial-mesenchymal and epithelial-carcinoma transitions. *Cells Tissues Organs*. 2005;179(1-2):66-72.
14. Knudson CB, Knudson W. Hyaluronan and CD44: modulators of chondrocyte metabolism. *Clin Orthop Relat Res*. 2004(427 Suppl):S152-62.
15. Noble PW. Hyaluronan and its catabolic products in tissue injury and repair. *Matrix Biol*. 2002;21(1):25-9.
16. Petrey AC, de la Motte CA. Hyaluronan, a crucial regulator of inflammation. *Frontiers in immunology*. 2014;5:101-.
17. Termeer C, Sleeman JP, Simon JC. Hyaluronan--magic glue for the regulation of the immune response? *Trends Immunol*. 2003;24(3):112-4.
18. Stern R, Jedrzejewski MJ. Hyaluronidases: their genomics, structures, and mechanisms of action. *Chemical reviews*. 2006;106(3):818-39.

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19. Garantziotis S, Savani RC. Hyaluronan biology: A complex balancing act of structure, function, location and context. *Matrix Biol.* 2019;78-79:1-10.
20. Cyphert JM, Trempus CS, Garantziotis S. Size Matters: Molecular Weight Specificity of Hyaluronan Effects in Cell Biology. *Int J Cell Biol.* 2015;2015:563818.
21. De Boulle K, Glogau R Fau - Kono T, Kono T Fau - Nathan M, Nathan M Fau - Tezel A, Tezel A Fau - Roca-Martinez J-X, Roca-Martinez Jx Fau - Paliwal S, et al. A review of the metabolism of 1,4-butanediol diglycidyl ether-crosslinked hyaluronic acid dermal fillers. (1524-4725 (Electronic)).
22. Itano N, Kimata K. Mammalian hyaluronan synthases. *IUBMB Life.* 2002;54(4):195-9.
23. Weigel PH, Hascall VC, Tammi M. Hyaluronan synthases. *J Biol Chem.* 1997;272(22):13997-4000.
24. Walter J, Hutter B, Khare T, Paulsen M. Repetitive elements in imprinted genes. *Cytogenet Genome Res.* 2006;113(1-4):109-15.
25. Nicola V, Juergen S, Robert Stern and Ladislav S. Role, Metabolism, Chemical Modifications and Applications of Hyaluronan. *Current Medicinal Chemistry.* 2009;16(14):1718-45.
26. Itano N, Sawai T, Yoshida M, Lenas P, Yamada Y, Imagawa M, et al. Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. *J Biol Chem.* 1999;274(35):25085-92.
27. Spicer AP, McDonald JA. Characterization and molecular evolution of a vertebrate hyaluronan synthase gene family. *J Biol Chem.* 1998;273(4):1923-32.
28. Stuhlmeier KM, Pollaschek C. Differential effect of transforming growth factor beta (TGF-beta) on the genes encoding hyaluronan synthases and utilization of the p38 MAPK pathway in TGF-beta-induced hyaluronan synthase 1 activation. *J Biol Chem.* 2004;279(10):8753-60.
29. Wilkinson TS, Potter-Perigo S, Tsoi C, Altman LC, Wight TN. Pro- and anti-inflammatory factors cooperate to control hyaluronan synthesis in lung fibroblasts. *Am J Respir Cell Mol Biol.* 2004;31(1):92-9.
30. Cowman MK, Lee HG, Schwertfeger KL, McCarthy JB, Turley EA. The Content and Size of Hyaluronan in Biological Fluids and Tissues. *Front Immunol.* 2015;6:261.
31. Chien L-J, Lee C-K. Hyaluronic acid production by recombinant *Lactococcus lactis*. *Applied Microbiology and Biotechnology.* 2007;77(2):339-46.
32. Yu H, Stephanopoulos G. Metabolic engineering of *Escherichia coli* for biosynthesis of hyaluronic acid. *Metabolic Engineering.* 2008;10(1):24-32.
33. Widner B, Behr R, Von Dollen S, Tang M, Heu T, Sloma A, et al. Hyaluronic Acid Production in *Bacillus subtilis*. *Applied and Environmental Microbiology.* 2005;71(7):3747.
34. DeLorenzi C, Weinberg M, Solish N, Swift A. The long-term efficacy and safety of a subcutaneously injected large-particle stabilized hyaluronic acid-based gel of nonanimal origin in esthetic facial contouring. *Dermatol Surg.* 2009;35 Suppl 1:313-21.
35. Liu L, Liu Y, Li J, Du G, Chen J. Microbial production of hyaluronic acid: current state, challenges, and perspectives. *Microb Cell Fact.* 2011;10:99.
36. Sze JH, Brownlie JC, Love CA. Biotechnological production of hyaluronic acid: a mini review. *3 Biotech.* 2016;6(1):67.

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37. Khunmanee S, Jeong Y, Park H. Crosslinking method of hyaluronic-based hydrogel for biomedical applications. *J Tissue Eng.* 2017;8:2041731417726464.
38. Fagien S, Bertucci V, von Grote E, Mashburn JH. Rheologic and Physicochemical Properties Used to Differentiate Injectable Hyaluronic Acid Filler Products. *Plast Reconstr Surg.* 2019;143(4):707e-20e.
39. Necas J, Bartosikova L, Brauner P, Kolar J. Hyaluronic acid (hyaluronan): a review. *Veterinarni Medicina;* 2008.
40. Dechert TA, Ducale AE, Ward SI, Yager DR. Hyaluronan in human acute and chronic dermal wounds. *Wound Repair Regen.* 2006;14(3):252-8.
41. Shumate GT, Chopra R, Jones D, Messina DJ, Hee CK. In Vivo Degradation of Crosslinked Hyaluronic Acid Fillers by Exogenous Hyaluronidases. *Dermatol Surg.* 2018;44(8):1075-83.
42. Hee CK, Shumate GT, Narurkar V, Bernardin A, Messina DJ. Rheological Properties and In Vivo Performance Characteristics of Soft Tissue Fillers. *Dermatol Surg.* 2015;41 Suppl 1:S373-81.
43. Stern R. Hyaluronan catabolism: a new metabolic pathway. *Eur J Cell Biol.* 2004;83(7):317-25.
44. Schiller J, Fuchs B, Arnhold J, Arnold K. Contribution of reactive oxygen species to cartilage degradation in rheumatic diseases: molecular pathways, diagnosis and potential therapeutic strategies. *Curr Med Chem.* 2003;10(20):2123-45.
45. Lapčík L, Schurz J. Photochemical degradation of hyaluronic acid by singlet oxygen. *Colloid and Polymer Science.* 1991;269(6):633-5.
46. Tokita Y, Okamoto A. Degradation of hyaluronic acid—Kinetic study and thermodynamics. *European Polymer Journal.* 1996;32(8):1011-4.
47. Simulescu V, Kalina M, Mondek J, Pekař M. Long-term degradation study of hyaluronic acid in aqueous solutions without protection against microorganisms. *Carbohydrate Polymers.* 2016;137:664-8.
48. Chen H, Qin J, Hu Y. Efficient Degradation of High-Molecular-Weight Hyaluronic Acid by a Combination of Ultrasound, Hydrogen Peroxide, and Copper Ion. *Molecules.* 2019;24(3).
49. Girish KS, Kemparaju K. The magic glue hyaluronan and its eraser hyaluronidase: a biological overview. *Life Sci.* 2007;80(21):1921-43.
50. Csoka AB, Frost GI, Stern R. The six hyaluronidase-like genes in the human and mouse genomes. *Matrix Biol.* 2001;20(8):499-508.
51. Fieber C, Baumann P, Vallon R, Termeer C, Simon JC, Hofmann M, et al. Hyaluronan-oligosaccharide-induced transcription of metalloproteases. *J Cell Sci.* 2004;117(Pt 2):359-67.
52. Taylor KR, Trowbridge JM, Rudisill JA, Termeer CC, Simon JC, Gallo RL. Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. *J Biol Chem.* 2004;279(17):17079-84.
53. Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, et al. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med.* 2002;195(1):99-111.

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- 2
- 3
- 4
- 5 54. Hemming R, Martin DC, Slominski E, Nagy JI, Halayko AJ, Pind S, et al. Mouse
- 6 Hyal3 encodes a 45- to 56-kDa glycoprotein whose overexpression increases
- 7 hyaluronidase 1 activity in cultured cells. *Glycobiology*. 2008;18(4):280-9.
- 8 55. Stern R, Asari AA, Sugahara KN. Hyaluronan fragments: an information-rich
- 9 system. *Eur J Cell Biol*. 2006;85(8):699-715.
- 10 56. Liu L, Du G, Chen J, Zhu Y, Wang M, Sun J. Microbial production of low
- 11 molecular weight hyaluronic acid by adding hydrogen peroxide and ascorbate in batch
- 12 culture of *Streptococcus zooepidemicus*. *Bioresour Technol*. 2009;100(1):362-7.
- 13 57. Bothner H, Waaler T, Wik O. Limiting viscosity number and weight average
- 14 molecular weight of hyaluronate samples produced by heat degradation. *International*
- 15 *Journal of Biological Macromolecules*. 1988;10(5):287-91.
- 16 58. Sall I, Féraud G. Comparison of the sensitivity of 11 crosslinked hyaluronic acid
- 17 gels to bovine testis hyaluronidase. *Polymer Degradation and Stability*. 2007;92(5):915-
- 18 9.
- 19 59. Itano N. Simple primary structure, complex turnover regulation and multiple
- 20 roles of hyaluronan. *J Biochem*. 2008;144(2):131-7.
- 21 60. Rayahin JE, Buhrman JS, Zhang Y, Koh TJ, Gemeinhart RA. High and low
- 22 molecular weight hyaluronic acid differentially influence macrophage activation. *ACS*
- 23 *Biomater Sci Eng*. 2015;1(7):481-93.
- 24 61. Jokela T, Oikari S, Takabe P, Rilla K, Kärnä R, Tammi M, et al. Interleukin-1 $\beta$ -
- 25 induced Reduction of CD44 Ser-325 Phosphorylation in Human Epidermal
- 26 Keratinocytes Promotes CD44 Homomeric Complexes, Binding to Ezrin, and Extended,
- 27 Monocyte-adhesive Hyaluronan Coats. *The Journal of biological chemistry*.
- 28 2015;290(19):12379-93.
- 29 62. Misra S, Heldin P, Hascall VC, Karamanos NK, Skandalis SS, Markwald RR, et al.
- 30 Hyaluronan-CD44 interactions as potential targets for cancer therapy. *FEBS J*.
- 31 2011;278(9):1429-43.
- 32 63. Khan AI, Kerfoot SM, Heit B, Liu L, Andonegui G, Ruffell B, et al. Role of CD44
- 33 and hyaluronan in neutrophil recruitment. *J Immunol*. 2004;173(12):7594-601.
- 34 64. Tamer TM. Hyaluronan and synovial joint: function, distribution and healing.
- 35 *Interdiscip Toxicol*. 2013;6(3):111-25.
- 36 65. Sherman L, Sleeman J, Herrlich P, Ponta H. Hyaluronate receptors: key players
- 37 in growth, differentiation, migration and tumor progression. *Curr Opin Cell Biol*.
- 38 1994;6(5):726-33.
- 39 66. Bajorath J. Molecular organization, structural features, and ligand binding
- 40 characteristics of CD44, a highly variable cell surface glycoprotein with multiple
- 41 functions. *Proteins*. 2000;39(2):103-11.
- 42 67. Müller S, Sindikubwabo F, Cañeque T, Lafon A, Versini A, Lombard B, et al. CD44
- 43 regulates epigenetic plasticity by mediating iron endocytosis. *Nat Chem*. 2020.
- 44 68. Al-Rekabi Z, Fura AM, Juhlin I, Yassin A, Popowics TE, Sniadecki NJ. Hyaluronan-
- 45 CD44 interactions mediate contractility and migration in periodontal ligament cells.
- 46 *Cell adhesion & migration*. 2019;13(1):138-50.
- 47 69. Kobayashi K, Hernandez LD, Galán JE, Janeway CA, Medzhitov R, Flavell RA.
- 48 IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell*. 2002;110(2):191-
- 49 202.
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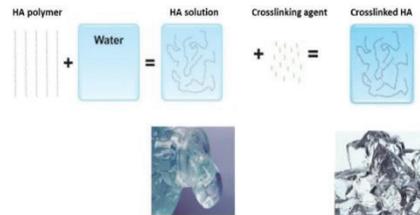
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70. Liang J, Jiang D, Griffith J, Yu S, Fan J, Zhao X, et al. CD44 is a negative regulator of acute pulmonary inflammation and lipopolysaccharide-TLR signaling in mouse macrophages. *J Immunol.* 2007;178(4):2469-75.
71. Muto J, Yamasaki K, Taylor KR, Gallo RL. Engagement of CD44 by hyaluronan suppresses TLR4 signaling and the septic response to LPS. *Mol Immunol.* 2009;47(2-3):449-56.
72. Wang Q, Teder P, Judd NP, Noble PW, Doerschuk CM. CD44 deficiency leads to enhanced neutrophil migration and lung injury in *Escherichia coli* pneumonia in mice. *Am J Pathol.* 2002;161(6):2219-28.
73. Suwannakul N, Ma N, Thanan R, Pinlaor S, Ungarreevittaya P, Midorikawa K, et al. Overexpression of CD44 Variant 9: A Novel Cancer Stem Cell Marker in Human Cholangiocarcinoma in Relation to Inflammation. *Mediators Inflamm.* 2018;2018:4867234.
74. Kim Y, Lee Y-S, Hahn J-H, Choe J, Kwon HJ, Ro JY, et al. Hyaluronic acid targets CD44 and inhibits FcεpsilonRI signaling involving PKCdelta, Rac1, ROS, and MAPK to exert anti-allergic effect. *Molecular immunology.* 2008;45(9):2537-47.
75. Naor D, Nedvetzki S. CD44 in rheumatoid arthritis. *Arthritis Res Ther.* 2003;5(3):105-15.
76. Zaman A, Cui Z, Foley JP, Zhao H, Grimm PC, Delisser HM, et al. Expression and role of the hyaluronan receptor RHAMM in inflammation after bleomycin injury. *Am J Respir Cell Mol Biol.* 2005;33(5):447-54.
77. Tolg C, Poon R, Fodde R, Turley EA, Alman BA. Genetic deletion of receptor for hyaluronan-mediated motility (Rhamm) attenuates the formation of aggressive fibromatosis (desmoid tumor). *Oncogene.* 2003;22(44):6873-82.
78. Salomão R, Martins PS, Brunialti MK, Fernandes MaL, Martos LS, Mendes ME, et al. TLR signaling pathway in patients with sepsis. *Shock.* 2008;30 Suppl 1:73-7.
79. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol.* 2003;21:335-76.
80. McInturff JE, Modlin RL, Kim J. The role of toll-like receptors in the pathogenesis and treatment of dermatological disease. *J Invest Dermatol.* 2005;125(1):1-8.
81. Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, et al. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nature Medicine.* 2005;11(11):1173-9.
82. del Fresno C, Otero K, Gómez-García L, González-León MC, Soler-Ranger L, Fuentes-Prior P, et al. Tumor cells deactivate human monocytes by up-regulating IL-1 receptor associated kinase-M expression via CD44 and TLR4. *J Immunol.* 2005;174(5):3032-40.
83. Voelcker V, Gebhardt C, Averbek M, Saalbach A, Wolf V, Weih F, et al. Hyaluronan fragments induce cytokine and metalloprotease upregulation in human melanoma cells in part by signalling via TLR4. *Exp Dermatol.* 2008;17(2):100-7.
84. Dong Y, Arif A, Olsson M, Cali V, Hardman B, Dosanjh M, et al. Endotoxin free hyaluronan and hyaluronan fragments do not stimulate TNF- $\alpha$ , interleukin-12 or upregulate co-stimulatory molecules in dendritic cells or macrophages. *Sci Rep.* 2016;6:36928.

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85. Ghazi K, Deng-Pichon U, Warnet J-M, Rat P. Hyaluronan Fragments Improve Wound Healing on In Vitro Cutaneous Model through P2X7 Purinoreceptor Basal Activation: Role of Molecular Weight. PLOS ONE. 2012;7(11):e48351.
86. Monslow J, Govindaraju P, Puré E. Hyaluronan - a functional and structural sweet spot in the tissue microenvironment. Front Immunol. 2015;6:231.
87. Tavianatou AG, Caon I, Franchi M, Piperigkou Z, Galesso D, Karamanos NK. Hyaluronan: molecular size-dependent signaling and biological functions in inflammation and cancer. FEBS J. 2019;286(15):2883-908.
88. Litwiniuk M, Krejner A, Speyrer MS, Gauto AR, Grzela T. Hyaluronic Acid in Inflammation and Tissue Regeneration. Wounds. 2016;28(3):78-88.
89. Baeva LF, Lyle Db Fau - Rios M, Rios M Fau - Langone JJ, Langone Jj Fau - Lightfoote MM, Lightfoote MM. Different molecular weight hyaluronic acid effects on human macrophage interleukin 1 $\beta$  production. (1552-4965 (Electronic)).
90. Chistyakov DV, Astakhova AA, Azbukina NV, Goriainov SV, Chistyakov VV, Sergeeva MG. High and Low Molecular Weight Hyaluronic Acid Differentially Influences Oxylipins Synthesis in Course of Neuroinflammation. Int J Mol Sci. 2019;20(16).

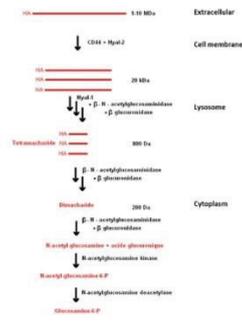
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HA solution to crosslinked HA (Adapted from Tezel (7))

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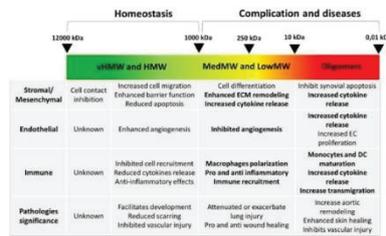
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HA enzymatic catabolism (Adapted from Stern et al. 2004 (43))

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: Biological and pathological significance of endogenous HA size (Adapted from Ghazi (85))

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