Jouy, F., Lohmann, N., Wandel, E., Ruiz-Gómez, G., Pisabarro, M.T., Beck-Sickinger, A.G., Schnabelrauch, M., Möller, S., Simon, J.C., **Kalkhof, S.**, **von Bergen, M.**, Franz, S. (2017): Sulfated hyaluronan attenuates inflammatory signaling pathways in macrophages involving induction of antioxidants
Proteomics **17** (10), art. 1700082

http://dx.doi.org/10.1002/pmic.201700082

Sulfated hyaluronan attenuates inflammatory signaling pathways in macrophages involving induction of antioxidants

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Received: 28/02/2017; Revised: 28/02/2017; Accepted: 20/03/2017

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/pmic.201700082.

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Abbreviations:

AP-1 (activated protein-1), **CD36/CD44** (cluster of differentiation 36/44), **CS** (Chondroitinsulfate), **ctr** (control), **GAG** (glycosaminoglycan), **GLRX3** (glutaredoxin-3), **H-HA** (high molecular weight hyaluronan), **HA** (hyaluronan), **IL1RN** (interleukin-1 receptor antagonist protein), **iMΦ** (inflammatory macrophages), **IPA** (Ingenuity Pathway Analysis), **IRF5** (interferon regulatory factor 5), **L-HA** (low molecular weight hyaluronan), **Lox1** (lectin-like OxLDL receptor), **LPS** (lipopolysaccharide), **NFκB** (nuclear factor ‘’kappa-light-chain-enhancer’’ of activated B-cells), **NMB** (glycoprotein non-metastatic gene B), **OxLDL** (oxidized low-density lipoprotein), **PMA** (Phorbol-12-myristate-13-acetate), **sL-HA** (sulfated low molecular weight hyaluronan), **SOD** (superoxide dismutase), **STAT-1** (signal transducer and activator of transcription 1), **TLR** (Toll-like receptor), **TMT** (tandem mass tag)

Keywords:

Anti-inflammation / Antioxidants / Macrophages / Glycosaminoglycans / Sulfated hyaluronan

Total number of words: 6501

Abstract

It is well recognized that high molecular weight hyaluronan (H-HA) exerts potent anti-inflammatory effects while its fragmentation into low molecular weight HA (L-HA) is discussed to promote inflammation. Chemical modification of HA with sulfate groups has been shown to foster its anti-inflammatory activity which seems to be maintained in sulfated low molecular weight HA derivatives (sL-HA). However, the molecular mechanisms by which sL-HA produces its anti-inflammatory activity are not understood. In this study, we used global quantitative proteomics combined with targeted analysis of key proteins to characterize the effect of sL-HA on fully differentiated human inflammatory macrophages (iMΦ). Culture of iMΦ with sL-HA did not affect cell viability but resulted in a reduced pro-inflammatory cytokine response of iMΦ after activation indicating a profound counter-regulation of their initial inflammatory phenotype. Rapid internalization of sL-HA involving CD44 and scavenger receptors was observed. Furthermore, an upregulation of the antioxidants SOD2 and SOD3 was found while no oxidative stress was induced. Consequently, activity of transcription factors for inflammatory gene expression was downregulated in iMΦ with sL-HA after activation whereas anti-inflammatory proteins were induced.

This study proves anti-inflammatory properties of sL-HA and provides information on its regulatory mode of action on iMΦ.

Significance of the study

Chemical modification of hyaluronan (HA) with sulfate groups generates polysulfated HA derivatives with anti-inflammatory activities. Typically, immuno-regulatory properties of HA depend on its molecular size with high molecular weight HA (H-HA) being anti-inflammatory while the supposed pro-inflammatory activity of low molecular weight HA (L-HA) is controversially discussed. However, the therapeutic use of H-HA in inflammatory conditions remains limited by its degradation to L-HA which is accompanied by the loss of its anti-inflammatory functions. In this respect, it is of significant interest to characterize the immuno-regulatory activity of sulfated HA derivatives after degradation to low molecular sizes (sL-HA).

In this study, we demonstrate anti-inflammatory effects of sL-HA on fully differentiated, inflammatory macrophages (iMΦ). Comparing the effects of sL-HA and L-HA of the same molecular size using global quantitative proteomics, targeted assays and docking calculations we gained insights into the regulatory impact of sL-HA on functions of resting and activated iMΦ. Thereby, we revealed the uptake of sL-HA, induction of antioxidants and regulation of transcriptional activators as mechanisms of the anti-inflammatory activity of sL-HA. Our data prove potent anti-inflammatory properties of sL-HA independent of its molecular weight which should be further assessed in *in vivo* models of inflammation.

1 Introduction

Glycosaminoglycans (GAGs) have been recognized as important regulators of biological processes controlling functions of a variety of cells including immune cells [1]. In particular, the anti-inflammatory effect of GAGs suggests their potential as an immunomodulating agent

to limit uncontrolled inflammatory processes [2]. In this respect, GAGs like Chondroitinsulfate (CS), heparin and hyaluronan (HA) have been shown to directly downregulate inflammatory cytokine production by immune cells and to reduce inflammation *in vivo* [3-6]. The regulatory activity of GAGs on cells is controlled by their structural conformation which is determined by their sulfation pattern and/or molecular weight [1, 2, 7]. For example, the immunomodulating impact of the sulfated GAG heparin appears to be predominantly influenced by its sulfation pattern. While both unfractionated and low molecular weight heparin have been shown to reduce inflammatory cytokine release in monocytes, differently sulfated heparin-like derivatives revealed abolished anti-inflammatory effects if selected sulfate groups were lacking [8, 9]. Since HA is the only naturally occurring non-sulfated GAG, its immuno-modulating action is dependent on its molecular weight [5]. It is well known that high molecular weight HA (H-HA) has potent anti-inflammatory and immunosuppressive functions promoting homeostasis in physiologic conditions [10, 11]. Due to its beneficial function, H-HA is applied as anti-inflammatory agent in the treatment of osteoarthritis [12]. In contrast, low molecular weight HA (L-HA) is discussed to induce pro-inflammatory responses. Several studies demonstrate profound pro-inflammatory effects of purified L-HA ranging from HA oligomers (2-18 mers) [10, 11] to HA fragments (5 to 500 kDa) [13] while other studies report of no pro-inflammatory activities of L-HA [14]. However, despite this controversy on the effect of purified L-HA, there is convincing evidence of L-HA accumulating in strong inflammatory environments due to de novo biosynthesis of L-HA and degradation of H-HA and that this shift from H-HA to L-HA favors progression of inflammation [13, 15]. Thus, the therapeutic success of anti-inflammatory H-HA applied in severe inflammatory conditions is limited by its degradation to L-HA discussed to be pro-inflammatory or at least to possess no anti-inflammatory activity. To circumvent this unwanted side effect, HA could be modified by the introduction of sulfate groups as sulfation seems to support the anti-inflammatory activity of GAGs and to prevail over any size effects. Indeed, there are first reports of sulfated HA or

sulfated semi-synthetic HA derivatives exerting anti-inflammatory effects and reducing inflammation *in vivo* [16, 17]. We have recently shown that sulfated L-HA (sL-HA) incorporated in an artificial extracellular matrix modulates the differentiation of blood monocytes towards a regulatory macrophage phenotype [18]. However, the molecular mechanisms by which sL-HA produces its anti-inflammatory activity remain unexplored and are therefore addressed in this study.

Using a global proteomics approach with tandem mass tag (TMT) labeling, the impact of purified sL-HA on fully differentiated human inflammatory macrophages (iMΦ) was elucidated by relative quantification of regulated proteins and characterization of involved signaling pathways. Additionally, targeted analysis of key proteins and cytokines of interest was performed as well as docking calculations with sL-HA.

2 Materials and Methods

2.1 Glycosaminoglycans

Native H-HA, from *Streptococcus* (Aqua Biochem Dessau, Germany) was degraded and sulfated to L-HA and sL-HA, respectively, as described [19]. All HA derivatives were tested endotoxin-free. Characteristics and structure of the different HA derivatives used are provided in **Table 1** and **Figure S9**. Further details are outlined in the **supporting information**. The HA derivatives were resolved in medium before being added into the macrophages culture.

2.2 Monocyte isolation, differentiation to inflammatory macrophages (iMΦ) and glycosaminoglycan (GAG) incubation

Human peripheral blood was taken from healthy volunteers after approval of the local ethics committee in compliance with the Declaration of Helsinki (# ethic vote: 064-2009). $CD14^+$ mononuclear cells were isolated using $CD14^+$ Cell Isolation Kit (Miltenyi, Germany) and differentiated for six days towards iMΦ using 50 U/ml GM-CSF (Leukine, USA) as previously described [18]. Mature iMΦ were treated for a further six days with a solution of HA derivatives (**Table 1**) which was directly added into the culture well, hence diluting the initial concentration of GM-CSF. Control iMΦ were sham-treated with a GAG-free medium for the same duration. After this timeline, iMΦ were either directly analyzed or stimulated with 100 ng/ml lipopolysaccharide (LPS) (Sigma-Aldrich, Germany) to trigger their inflammatory activation before analyses. Proteins were isolated for proteomic analysis or Western Blot, cell culture supernatant was harvested for ELISA, RNA was isolated from the cells for gene expression analysis and cell viability assays were performed. Western Blot, ELISA, gene expression analysis and cell viability assays were performed according to standard procedures. For details see **supporting information**.

2.3 Global quantitative proteomics study

Proteins were isolated from macrophages with a urea-based lysis buffer for 10 min at 4 °C, then 15 µg of protein per condition was reduced, alkylated, purified by acetone precipitation and digested by trypsin. The resulting peptides from the various experimental conditions were differently chemically labeled using the Tandem Mass Tag (TMT, Thermo Scientific, USA) approach before being combined.

Peptide samples were then analyzed on a nano-UHPLC system coupled to an Orbitrap Fusion mass spectrometer (**Fig. S1**). After trapping, TMT-labeled peptides were separated on a RP column using a 260 min non-linear gradient. Survey scans were recorded within the Orbitrap Fusion using top speed (5 sec) in data dependent mode. For MS2, scans were selected with a quadrupole mass filter width of 0.5 amu and fragmented with a normalized collision energy set to 35 % using high-energy collision.

MS raw data were processed using the Proteome Discoverer v.1.4.0.288 (Thermo Scientific, USA) with the algorithms SEQUEST and MS Amanda for peptide identification. Spectra were matched to the UniprotKB/Swissprot human protein database (06 Nov 2014). The peptide FDR was kept to < 1 % (using Percolator). High confidence unique and razor peptides were selected. Only proteins with quantitative data for at least three out of four biological replicates were kept. The $\log_2$ [ratio(HA vs. no GAG)] was calculated and normalized for each condition. For details see **supporting information**.

2.4 Pathway analysis

The proteomic data was further analyzed by IPA (Ingenuity Pathway Analysis, Ingenuity® Systems, www.ingenuity.com). Only proteins with a p-value < 0.1 and pathways related to human monocytes-derived macrophages and T lymphocytes were considered for IPA analysis.

2.5 Measurement of intracellular oxidative stress

Macrophages were treated with GAGs or PMA (100 nM) for the indicated time points or remained untreated before the addition of 7.5 µM of CellROX® Deep Red Reagent (Thermo Scientific, USA). After 30 min incubation cells were washed with PBS, detached using trypsin/EDTA and red fluorescence as mean for intracellular ROS induction immediately analyzed by flow cytometry.

2.6 Immunofluorescence microscopy

Macrophages were seeded in chambered coverslips (µ-slide, ibidi, Germany) and incubated with anti-Lox1 (BioLegend, UK) or anti-CD36 (BioLegend, UK) antibodies at 37 °C for 30 min. Cells were washed with PBS before simultaneous addition of the appropriate Alexa-488 secondary antibody (Thermo Scientific, USA) and fluorescent ATTO565-labeled sL-HA (Innovent e.V., Germany) for further 30 min at 37 °C. Co-localization of the different targets was visualized using a Keyence BZ-9000 microscope (Biorevo) or a confocal microscope (Axio Observer Z1, Zeiss, Germany) equipped with fluorescence and image capturing system. For details see **supporting information**.

2.7 Surface marker expression and blocking of CD44

Surface expression of different markers and uptake of ATTO565-labeled sL-HA in the presence and absence of anti-CD44-blocking antibody (eBioscience, Germany) were analyzed by flow cytometry. For details see **supporting information**.

2.8 Molecular modeling and docking

Hexameric (dp6) sulfated hyaluronan HA462' and HA463' were modeled using AMBER 14 [20] (the numbers indicate the sulfate group position within each disaccharide unit present in the polymeric sL-HA experimentally used). Their interaction with the protein targets CD44, Lox1, and CD36 (previously energy minimized) was studied by molecular docking with Autodock 3 [21]. For details see **supporting information**.

2.9 Statistics

All experiments were performed with at least three different donors. Data are presented as mean ± SD. Statistical analysis was performed by a two tailed t-test with unequal variance between two groups or by one-way ANOVA with multiple comparisons versus indicated control using Tukey's post-test. All p-values below 0.05 were considered to be statistically significant.

3 Results and discussion

3.1 Sulfated L-HA reduces pro-inflammatory cytokine release of inflammatory macrophages

The anti-inflammatory activity of sL-HA was assessed using resting inflammatory macrophages (iMΦ). Blood monocytes were differentiated with GM-CSF for six days into macrophages presenting a pro-inflammatory activation profile in response to an activating stimulus, like. LPS (**Fig. S2**), similar to macrophages found at inflammation sites [22, 23]. These resting iMΦ were cultured for another six days with sL-HA, L-HA (of the same

molecular weight and serving as size control) and with H-HA known to attenuate iMΦ responses [24] (thus serving as an anti-inflammatory positive control). Culture with the GAGs alone had neither an effect on the expression of macrophage activation markers, with results equivalent to untreated iMΦ (**Fig. S2A**), nor induced the release of cytokines (**Fig. S2B**), thus demonstrating no activation of resting iMΦ by either sL-HA, L-HA or H-HA. However, when iMΦ were stimulated with LPS (LPS stimulation for 5 h (TNFα) and 24 h (others)) in the presence of the GAGs a significantly reduced release of the pro-inflammatory cytokines TNFα, IL-12(p40), IL-6 and MCP-1 was detected for iMΦ cultured with sL-HA compared to LPS-stimulated control iMΦ (**Fig. 1**). The observed anti-inflammatory activity of sL-HA is in line with previous studies showing reduced inflammatory cytokine production by mesenchymal stromal cells and monocytes after treatment with sL-HA [17, 18]. A similar decrease in the cytokine release of TNFα and MCP-1 was found for H-HA, confirming data from other studies [24], whereas L-HA showed no significant effect on iMΦ activation (**Fig. 1**). Although several reports describe the inflammatory activation of macrophages and dendritic cells by L-HA [13, 24, 25] we found no pro-inflammatory activity of L-HA paralleling recent findings of others [14, 26]. It has been suggested that the source of HA and endotoxin levels might explain the divergent data on the pro-inflammatory effects of purified HA fragments [14]. Our study using bacterial-derived endotoxin-free HA derivatives may support this notion. We acknowledge that further studies are required to clarify this controversy on L-HA activity especially since degradation of H-HA and accumulation of L-HA following injury or insult *in vivo* has been proven to impact progression of inflammation [13, 15]. However, this is beyond the scope of this study which addresses the molecular basis responsible for the anti-inflammatory activity of sulfated L-HA.

3.2 Sulfated L-HA induces a strong global effect on protein regulation in inflammatory macrophages

To reveal the molecular mechanisms by which sL-HA counter-regulates iMΦ functions, a global quantitative proteomics study was performed as per workflow (**Fig. S1**), in which effects on resting iMΦ (no LPS stimulation) and on short-term (30 min LPS) and long-term (4h LPS) activated iMΦ were assessed. Firstly, we confirmed the pro-inflammatory properties of LPS on macrophages alone (with no GAG). We demonstrated in the absence of GAG an increase in pro-inflammatory cytokines (TNFα, IL-12, IL-6, MCP-1) in macrophages activated with LPS over resting macrophages (untreated) (**Fig. S2B**) and similarly, a pathway analysis of the regulated proteins predicted strong activation of the pro-inflammatory RhoA signaling pathway [27] in activated versus resting macrophages (**Fig. S3**).

Interestingly, a clear difference in the number of significantly regulated proteins in resting and activated iMΦ was observed between sL-HA and its size control L-HA (**Fig. 2A**). While a very low number of significantly regulated proteins (< 5) was detected when cells were cultured with L-HA, incubation with sL-HA resulted in about 60 proteins being significantly regulated. These findings indicate a strong global effect of HA sulfation on iMΦ activity, which was further substantiated in the pathway analysis.

An overrepresentation analysis of the regulated proteins revealed five main pathways with a p-value < 0.01 and an absolute z-score > 2 (**Fig. 2B**). In resting and activated iMΦ the 'EIF2 signaling pathway' was strongly inhibited when cultured with sL-HA but only slightly reduced with L-HA. Pathway related to oxidative stress like 'NRF2-mediated oxidative stress response' was found activated with sL-HA after 4 h LPS, while no information were obtained for L-HA. Pathways linked to inflammatory response including 'fMLP signaling' and 'Acute phase response signaling' were also more activated in sL-HA than in L-HA conditions. No

constant differences between L-HA and sL-HA cultured iMΦ were found in regulated pathway related to cell movement and maintenance ('Regulation of Actin based motility by Rho').

3.3 Sulfated L-HA is internalized by inflammatory macrophages involving CD44 and scavenger receptors

Binding to CD44 has been implicated in the anti-inflammatory activity of H-HA [28]. In-depth analysis of regulated proteins revealed a clear upregulation of CD44 for all sL-HA conditions (**supporting data1**) which was confirmed by flow cytometry analysis of CD44 expression in resting iMΦ (**Fig. 3A**). Therefore, the possible interaction of sL-HA with CD44 was addressed. Using a blind docking approach, a binding region for sL-HA was predicted, which overlaps with residues distributed along the HA binding domain of CD44 [29] (**Fig. 3B, Fig. S4A**). Thus, a functional blocking CD44 antibody would compete for the same recognition site precluding interaction of sL-HA with iMΦ as demonstrated in **Fig. 3C**. In line with the reported uptake of HA after CD44 binding [28] rapid internalization and intracellular accumulation of sL-HA in iMΦ was detected which started with vesicular clustering of sL-HA on the iMΦ surface (**Fig. S5**). However, CD44 blocking did not completely prevent sL-HA interaction with iMΦ (**Fig. 3C**) suggesting the involvement of other receptors in the uptake of sL-HA. Scavenger receptors are widely expressed by macrophages and have been reported to mediate the uptake of polyanionic macromolecules [30], which prompted us to investigate their role in sL-HA internalization. Monitoring sL-HA uptake by fluorescence microscopy, the scavenger receptors lectin-like OxLDL receptor 1 (Lox1) and CD36 were found to co-localize with sL-HA clusters at the cell surface (**Fig. 3D**) suggesting the implication of both receptors in the sL-HA uptake by iMΦ. This was further supported by blind docking studies. Our results suggest that there is, from the two sites involved in OxLDL recognition [31], a sL-HA

sulfation pattern dependent preference for the Lox1 recognition site with residues in the basic spin (R208, R229, R231 and R248) over a second region near the C-terminal (K266 and K267) (**Fig. 3E, Fig. S4C**). For CD36, most of the binding poses obtained for sL-HA did not overlap with the CD36 functional domain (residues 155-183) [32] (**Fig. 3F, Fig. S4B**). Therefore, sL-HA would be able to interact with iMΦ in the presence of functional blocking antibodies against Lox1 and CD36, as they recognize different receptor regions. Interestingly, the recognition of OxLDL by scavenger receptors like Lox1 activates an oxidative stress response by increasing ROS production, NADPH oxidase expression and superoxide anion ($O_2^{\bullet-}$) formation [33, 34]. Thus, binding of sL-HA to the OxLDL recognition site of Lox1 may induce the activation of such oxidative-acting molecules in iMΦ which will be addressed in the following section.

3.4 Sulfated L-HA induces proteins involved in regulating oxidative stress responses in inflammatory macrophages

The induction of an oxidative stress response is also supported by the global proteomics study through the regulated pathway: 'NRF2-mediated oxidative stress response'. An oxidative stress response involves the formation of oxidants and subsequent upregulation of antioxidants counter-regulating the oxidants' activity [35]. Our proteomics analysis revealed the selective upregulation of both oxidative-acting proteins and anti-oxidative-acting proteins in iMΦ treated with sL-HA in contrast to iMΦ treated with L-HA (**Fig. 4A**).

The multicomponent enzyme complex NADPH oxidase, responsible for the production of $O_2^{\bullet-}$, is composed of two transmembrane and four cytosolic proteins including p47phox and p67phox [36]. These two proteins were clearly upregulated in all sL-HA conditions (except for p67phox at 4 h) (**Fig. 4A**). The intracellular increase of $O_2^{\bullet-}$ production is counter-

regulated by superoxide dismutases (SOD) converting it into less harmful H_2O_2 [37]. Accordingly, a strong upregulation of mitochondrial SOD2 was found in all sL-HA conditions (**Fig. 4A**), while cytosolic SOD1 was not significantly regulated (**supporting data1**). As SODs are key regulators of cellular oxidative stress, their regulation was confirmed exemplarily. Significant induction of SOD2 and SOD3 (**Fig. 4B/C, Fig. S6**) but not of SOD1 (not shown) was detected in resting iMΦ after one and six days culture with sL-HA compared to iMΦ treated with no GAG and L-HA which maintained when sL-HA-treated iMΦ were activated with LPS (**Fig. S6, S7**). SOD3 requires copper for its catalytic activity, which is made available by enzymes like antioxidant-1 (ATOX1) [38]. In the proteomics analysis, ATOX1 was upregulated in all sL-HA conditions (**Fig. 4A**). Together with the observed increase of SOD3 expression, this indicates a functional activation of SOD3 in iMΦ by sL-HA. Glutaredoxin-3 (GLRX3) is another antioxidant enzyme that was slightly upregulated in sL-HA treated iMΦ along with the glutamate-cysteine ligase catalytic subunit (GCLC) (**Fig. 4A**). GCLC is part of the glutamate-cysteine ligase (GCL), an enzyme required for *de novo* synthesis of glutathione (GSH) [39]. Glutaredoxins, along with GSH, maintain redox homeostasis in cells and protect against oxidative stress-induced cell death [40]. Finally, the antioxidant stress sensitive cytokine osteopontin was found significantly upregulated in resting iMΦ with sL-HA (**Fig. 4A**). This protein has been described to counter-regulate inducible nitric oxide synthase (iNOS) in macrophages [41]. Altogether, our data strongly indicate the induction of an oxidative stress response in iMΦ by sL-HA which indicates an upstream formation of reactive nitrogen species (RNS) and ROS [35]. Therefore, we monitored the expression of the NO producing enzyme iNOS [42] and the production of intracellular ROS in resting iMΦ during the time course of culture with sL-HA and observed no induction of iNOS (data not shown) and no increased ROS levels in iMΦ as short-term (1 h, 6 h, 24 h) and long-term (6 days) response to sL-HA treatment (**Fig. 4D**). This is surprising as the upregulation of antioxidants like SODs or GSH are typically triggered by the

generation of RNS/ROS [43]. In turn, RNS/ROS are immediately neutralized by the fast activity of antioxidants, a crucial defense mechanism of cells to prevent RNS/ROS-mediated damage which – if the antioxidant effect is not efficient – can lead to cell death [35]. In this respect, we observed equal viability between iMΦ cultured with sL-HA, L-HA or without GAG (**Fig. 4E**). Thus, the lack of RNS/ROS detection despite the induction of antioxidants in iMΦ after sL-HA treatment may result from their counter-regulation by an effective antioxidant response. On the other hand, antioxidants might also be upregulated in the absence of RNS/ROS formation by an unknown mechanism to be elucidated in continuing studies. Of note, heparin and CS were also described to induce SOD3 expression in dermal fibroblasts but whether this involved the formation of RNS/ROS was not investigated [44].

3.5 Anti-inflammatory effect of sulfated L-HA is mediated by counter-regulation of transcriptional factors and activation of negative regulators of inflammatory gene transcription

Antioxidants have been shown to impact inflammatory functions of cells through the regulation of transcription factor activities [45]. Thus, the anti-inflammatory activity of SOD3 was linked to the inhibition of NFκB activation [46]. Reduced activation of NFκB and of activated protein-1 (AP-1) was also found in cells after overexpression of SOD2 and GLRX3 [47, 48], while the induction of osteopontin supported the degradation of STAT-1/pSTAT-1 [41]. Since these transcription factors regulate the expression of inflammatory genes [49, 50] and since we observed the upregulation of SOD2, SOD3, GLRX3 and osteopontin in iMΦ in response to sL-HA (**Fig. 4A-C**), transcription factor activation in the LPS-stimulated iMΦ was analyzed. Reduced levels of IRF5, phosphorylated NFκB and phosphorylated STAT-1 found in the sL-HA-treated activated iMΦ (**Fig. 4F, Fig. S8**) clearly demonstrate the decreased pro-inflammatory activation of the iMΦ which is also reflected in the reduced production of

inflammatory cytokines as observed in **Fig. 1**. The mechanism by which antioxidants impact transcription factor activities is not completely understood. It has been shown that sequence-specific DNA binding and transactivation activities of transcription factors are differentially regulated in reducing or oxidizing environments [45]. It is suggested that such redox regulation processes occur in cells in localized parts where particular oxidants or antioxidants interact with and modulate the activity of transcription factors such as NFκB [45]. Whether impaired transcription factor activity in the sL-HA-treated iMΦ arises from the direct interaction with antioxidants remains to be explored. An interesting notion in this respect is that similar to our sL-HA, both heparin and CS also block the activation of transcription factors (e.g. NFκB) reducing the expression of inflammatory cytokines [51, 52] and induce SOD3 [44]. This might hint at a common molecular mechanism by which sulfated GAGs mediate their anti-inflammatory activity but further studies are required to substantiate this hypothesis.

Furthermore, the global proteomics study revealed a strong upregulation of the anti-inflammatory proteins interleukin-1 receptor antagonist protein (IL1RN) and transmembrane glycoprotein NMB in activated iMΦ treated with sL-HA compared to L-HA (**Fig. 4A**). IL1RN is known to inhibit IL-1β activity, one of the leading pro-inflammatory cytokines in inflammatory processes [53]. NMB is described to negatively regulate inflammatory responses by downregulating pro-inflammatory cytokines including TNFα, IL-1β, IL-6 and IL-12(p40) [54]. Upregulation of these anti-inflammatory proteins therefore suggests an additional autocrine/paracrine counter-regulation of the inflammatory activity of the sL-HA treated iMΦ.

4 Concluding remarks

Using a quantitative global proteomics approach, we were able to demonstrate and characterize the capacity of sL-HA to influence inflammatory signaling in fully differentiated iMΦ and to diminish their pro-inflammatory activation profile. Events that may contribute to the anti-inflammatory activity of sL-HA on iMΦ are illustrated in **Fig. 5**. They involve the recognition of sL-HA by CD44 and by the scavenger receptors CD36 and Lox1, probably mediating the uptake of sL-HA into the macrophages. Antioxidants like SOD2 and SOD3 are up-regulated in the presence of sL-HA by an unresolved mechanism. These antioxidants could be linked to the reduced activation of the transcription factors NFκB, STAT1 and IRF5 responsible for inflammatory gene transcription in activated iMΦ. Consequently, the production of pro-inflammatory cytokines like TNFα, IL-12(p40), MCP-1 and IL-6 decreased in favor of an upregulation of anti-inflammatory proteins (IL1RN, NMB). Altogether, our data provide a basis for further investigations aiming at deciphering the molecular mechanisms involved in the anti-inflammatory mode of action of sL-HA. Although our results point toward a direct effect of sL-HA on iMΦ, an indirect modulatory impact of sL-HA via interactions with regulating mediators of iMΦ cannot be ruled out. For example, the sL-HA binding of GM-CSF and the subsequent modification of its bioactivity, as it has been shown for heparin sulfate proteoglycans, might also be a mechanism by which sL-HA exerts its anti-inflammatory activity on iMΦ and should be addressed in further studies.

The application of H-HA as an anti-inflammatory agent is limited as it is prone to be degraded into pro-inflammatory or non-effective L-HA in inflammatory environments. Our results prove a profound anti-inflammatory activity of L-HA after modification with sulfate groups, suggesting that sulfation of HA may overcome the adverse effect of HA degradation.

Acknowledgements

We acknowledge funding through Helmholtz Interdisciplinary Graduate School for Environmental Research (HIGRADE) and the German Research Council (DFG SFB-TR67, projects A4, A7, B3, Z4 and DFG FR2671/4-1). We also acknowledge the ProVis platform. We thank Jörg Flemmig for the vital discussions regarding the oxidative stress topic as well as Stephanie Möller and Matthias Schnabelrauch from Innovent e.V. Jena for providing the GAGs. We also thank Benjamin Scheer and Inka Forstreuter for their excellent technical support and Kristin Löbner and Karin Mörl for their help with the microscopy analyses.

Conflict of interest

The authors have declared no conflict of interest.

5. References

[1] Gandhi, N. S., Mancera, R. L., The structure of glycosaminoglycans and their interactions with proteins. *Chem Biol Drug Des* 2008, *72*, 455-482.

[2] Taylor, K. R., Gallo, R. L., Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. *FASEB J* 2006, *20*, 9-22.

[3] Masuko, K., Murata, M., Yudoh, K., Kato, T., Nakamura, H., Anti-inflammatory effects of hyaluronan in arthritis therapy: Not just for viscosity. *Int J Gen Med* 2009, *2*, 77-81.

[4] Nelson, R. M., Cecconi, O., Roberts, W. G., Aruffo, A.*, et al.*, Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. *Blood* 1993, *82*, 3253-3258.

[5] Petrey, A. C., de la Motte, C. A., Hyaluronan, a crucial regulator of inflammation. *Front Immunol* 2014, *5*, 101.

[6] du Souich, P., Garcia, A. G., Verges, J., Montell, E., Immunomodulatory and anti-inflammatory effects of chondroitin sulphate. *J Cell Mol Med* 2009, *13*, 1451-1463.

[7] Gama, C. I., Tully, S. E., Sotogaku, N., Clark, P. M.*, et al.*, Sulfation patterns of glycosaminoglycans encode molecular recognition and activity. *Nat Chem Biol* 2006, *2*, 467-473.

[8] Hochart, H., Jenkins, P. V., Smith, O. P., White, B., Low-molecular weight and unfractionated heparins induce a downregulation of inflammation: decreased levels of proinflammatory cytokines and nuclear factor-kappaB in LPS-stimulated human monocytes. *Br J Haematol* 2006, *133*, 62-67.

[9] Gori, A. M., Attanasio, M., Gazzini, A., Rossi, L.*, et al.*, Cytokine gene expression and production by human LPS-stimulated mononuclear cells are inhibited by sulfated heparin-like semi-synthetic derivatives. *J Thromb Haemost* 2004, *2*, 1657-1662.

[10] Litwiniuk, M., Krejner, A., Speyrer, M. S., Gauto, A. R., Grzela, T., Hyaluronic Acid in Inflammation and Tissue Regeneration. *Wounds* 2016, *28*, 78-88.

[11] Termeer, C., Benedix, F., Sleeman, J., Fieber, C.*, et al.*, Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med* 2002, *195*, 99-111.

[12] Ishijima, M., Nakamura, T., Shimizu, K., Hayashi, K.*, et al.*, Intra-articular hyaluronic acid injection versus oral non-steroidal anti-inflammatory drug for the treatment of knee

osteoarthritis: a multi-center, randomized, open-label, non-inferiority trial. *Arthritis Res Ther* 2014, *16*, R18.

[13] McKee, C. M., Penno, M. B., Cowman, M., Burdick, M. D.*, et al.*, Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. *J Clin Invest* 1996, *98*, 2403-2413.

[14] Dong, Y., Arif, A., Olsson, M., Cali, V.*, 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.

[15] Teder, P., Vandivier, R. W., Jiang, D., Liang, J.*, et al.*, Resolution of lung inflammation by CD44. *Science* 2002, *296*, 155-158.

[16] Zhang, J., Xu, X., Rao, N. V., Argyle, B.*, et al.*, Novel sulfated polysaccharides disrupt cathelicidins, inhibit RAGE and reduce cutaneous inflammation in a mouse model of rosacea. *PLoS One* 2011, *6*, e16658.

[17] Hempel, U., Matthaus, C., Preissler, C., Moller, S.*, et al.*, Artificial matrices with high-sulfated glycosaminoglycans and collagen are anti-inflammatory and pro-osteogenic for human mesenchymal stromal cells. *J Cell Biochem* 2014, *115*, 1561-1571.

[18] Franz, S., Allenstein, F., Kajahn, J., Forstreuter, I.*, et al.*, Artificial extracellular matrices composed of collagen I and high-sulfated hyaluronan promote phenotypic and functional modulation of human pro-inflammatory M1 macrophages. *Acta Biomater* 2013, *9*, 5621-5629.

[19] Moller, S., Schmidtke, M., Weiss, D., Schiller, J.*, et al.*, Synthesis and antiherpetic activity of carboxymethylated and sulfated hyaluronan derivatives. *Carbohydr Polym* 2012, *90*, 608-615.

[20] Case, D. A., Berryman, J. T., Betz, R. M., Cerutti, D. S., *et al.*, AMBER 14. *University of California, San Francisco* 2014.

[21] Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., *et al.*, Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J Comput Chem* 1998, *19*, 1639-1662.

[22] Fleetwood, A. J., Dinh, H., Cook, A. D., Hertzog, P. J., Hamilton, J. A., GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling. *J Leukoc Biol* 2009, *86*, 411-421.

[23] Murray, P. J., Allen, J. E., Biswas, S. K., Fisher, E. A.*, et al.*, Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* 2014, *41*, 14-20.

[24] Rayahin, J. E., Buhrman, J. S., Zhang, Y., Koh, T. J., Gemeinhart, R. A., High and low molecular weight hyaluronic acid differentially influence macrophage activation. *ACS Biomater Sci Eng* 2015, *1*, 481-493.

[25] Termeer, C. C., Hennies, J., Voith, U., Ahrens, T.*, et al.*, Oligosaccharides of hyaluronan are potent activators of dendritic cells. *J Immunol* 2000, *165*, 1863-1870.

[26] Krejcova, D., Pekarova, M., Safrankova, B., Kubala, L., The effect of different molecular weight hyaluronan on macrophage physiology. *Neuro Endocrinol Lett* 2009, *30 Suppl 1*, 106-111.

[27] Wang, C., Song, S., Zhang, Y., Ge, Y.*, et al.*, Inhibition of the Rho/Rho kinase pathway prevents lipopolysaccharide-induced hyperalgesia and the release of TNF-alpha and IL-1beta in the mouse spinal cord. *Sci Rep* 2015, *5*, 14553.

[28] Misra, S., Hascall, V. C., Markwald, R. R., Ghatak, S., Interactions between Hyaluronan and Its Receptors (CD44, RHAMM) Regulate the Activities of Inflammation and Cancer. *Front Immunol* 2015, *6*, 201.

[29] Banerji, S., Wright, A. J., Noble, M., Mahoney, D. J.*, et al.*, Structures of the Cd44-hyaluronan complex provide insight into a fundamental carbohydrate-protein interaction. *Nat Struct Mol Biol* 2007, *14*, 234-239.

[30] Szabo, R., Peiser, L., Pluddemann, A., Bosze, S.*, et al.*, Uptake of branched polypeptides with poly[L-lys] backbone by bone-marrow culture-derived murine macrophages: the role of the class a scavenger receptor. *Bioconjug Chem* 2005, *16*, 1442-1450.

[31] Ohki, I., Ishigaki, T., Oyama, T., Matsunaga, S.*, et al.*, Crystal structure of human lectin-like, oxidized low-density lipoprotein receptor 1 ligand binding domain and its ligand recognition mode to OxLDL. *Structure* 2005, *13*, 905-917.

[32] Kar, N. S., Ashraf, M. Z., Valiyaveettil, M., Podrez, E. A., Mapping and characterization of the binding site for specific oxidized phospholipids and oxidized low density lipoprotein of scavenger receptor CD36. *J Biol Chem* 2008, *283*, 8765-8771.

[33] Chen, X. P., Xun, K. L., Wu, Q., Zhang, T. T.*, et al.*, Oxidized low density lipoprotein receptor-1 mediates oxidized low density lipoprotein-induced apoptosis in human umbilical vein endothelial cells: role of reactive oxygen species. *Vascul Pharmacol* 2007, *47*, 1-9.

[34] Rueckschloss, U., Galle, J., Holtz, J., Zerkowski, H. R., Morawietz, H., Induction of NAD(P)H oxidase by oxidized low-density lipoprotein in human endothelial cells: antioxidative potential of hydroxymethylglutaryl coenzyme A reductase inhibitor therapy. *Circulation* 2001, *104*, 1767-1772.

[35] Fulda, S., Gorman, A. M., Hori, O., Samali, A., Cellular stress responses: cell survival and cell death. *Int J Cell Biol* 2010, *2010*, 214074.

[36] El-Benna, J., Dang, P. M., Gougerot-Pocidalo, M. A., Elbim, C., Phagocyte NADPH oxidase: a multicomponent enzyme essential for host defenses. *Arch Immunol Ther Exp (Warsz)* 2005, *53*, 199-206.

[37] Fukai, T., Ushio-Fukai, M., Superoxide dismutases: role in redox signaling, vascular function, and diseases. *Antioxid Redox Signal* 2011, *15*, 1583-1606.

[38] Itoh, S., Ozumi, K., Kim, H. W., Nakagawa, O.*, et al.*, Novel mechanism for regulation of extracellular SOD transcription and activity by copper: role of antioxidant-1. *Free Radic Biol Med* 2009, *46*, 95-104.

[39] Krejsa, C. M., Franklin, C. C., White, C. C., Ledbetter, J. A.*, et al.*, Rapid activation of glutamate cysteine ligase following oxidative stress. *J Biol Chem* 2010, *285*, 16116-16124.

[40] Pham, K., Pal, R., Qu, Y., Liu, X.*, et al.*, Nuclear glutaredoxin 3 is critical for protection against oxidative stress-induced cell death. *Free Radic Biol Med* 2015, *85*, 197-206.

[41] Gao, C., Guo, H., Mi, Z., Grusby, M. J., Kuo, P. C., Osteopontin induces ubiquitin-dependent degradation of STAT1 in RAW264.7 murine macrophages. *J Immunol* 2007, *178*, 1870-1881.

[42] Weinberg, J. B., Misukonis, M. A., Shami, P. J., Mason, S. N.*, et al.*, Human mononuclear phagocyte inducible nitric oxide synthase (iNOS): analysis of iNOS mRNA, iNOS protein, biopterin, and nitric oxide production by blood monocytes and peritoneal macrophages. *Blood* 1995, *86*, 1184-1195.

[43] Candas, D., Li, J. J., MnSOD in oxidative stress response-potential regulation via mitochondrial protein influx. *Antioxid Redox Signal* 2014, *20*, 1599-1617.

[44] Adachi, T., Hara, H., Yamada, H., Yamazaki, N.*, et al.*, Heparin-stimulated expression of extracellular-superoxide dismutase in human fibroblasts. *Atherosclerosis* 2001, *159*, 307-312.

[45] Sun, Y., Oberley, L. W., Redox regulation of transcriptional activators. *Free Radic Biol Med* 1996, *21*, 335-348.

[46] Laurila, J. P., Laatikainen, L. E., Castellone, M. D., Laukkanen, M. O., SOD3 reduces inflammatory cell migration by regulating adhesion molecule and cytokine expression. *PLoS One* 2009, *4*, e5786.

[47] Manna, S. K., Zhang, H. J., Yan, T., Oberley, L. W., Aggarwal, B. B., Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. *J Biol Chem* 1998, *273*, 13245-13254.

[48] Witte, S., Villalba, M., Bi, K., Liu, Y.*, et al.*, Inhibition of the c-Jun N-terminal kinase/AP-1 and NF-kappaB pathways by PICOT, a novel protein kinase C-interacting protein with a thioredoxin homology domain. *J Biol Chem* 2000, *275*, 1902-1909.

[49] Glass, C. K., Saijo, K., Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells. *Nat Rev Immunol* 2010, *10*, 365-376.

[50] Krausgruber, T., Blazek, K., Smallie, T., Alzabin, S.*, et al.*, IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat Immunol* 2011, *12*, 231-238.

[51] Thourani, V. H., Brar, S. S., Kennedy, T. P., Thornton, L. R.*, et al.*, Nonanticoagulant heparin inhibits NF-kappaB activation and attenuates myocardial reperfusion injury. *Am J Physiol Heart Circ Physiol* 2000, *278*, H2084-2093.

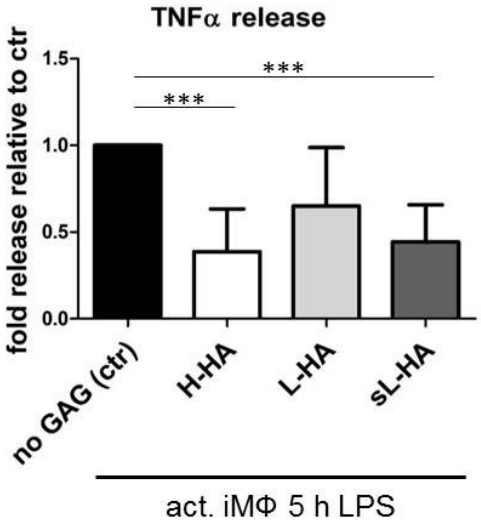
[52] Jomphe, C., Gabriac, M., Hale, T. M., Heroux, L.*, et al.*, Chondroitin sulfate inhibits the nuclear translocation of nuclear factor-kappaB in interleukin-1beta-stimulated chondrocytes. *Basic Clin Pharmacol Toxicol* 2008, *102*, 59-65.

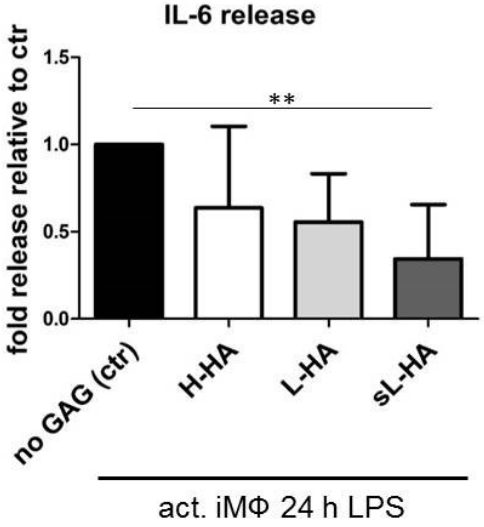
[53] Arend, W. P., Malyak, M., Guthridge, C. J., Gabay, C., Interleukin-1 receptor antagonist: role in biology. *Annu Rev Immunol* 1998, *16*, 27-55.

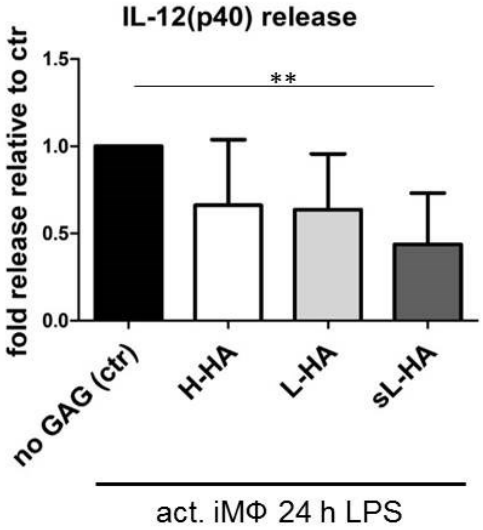
[54] Ripoll, V. M., Irvine, K. M., Ravasi, T., Sweet, M. J., Hume, D. A., Gpnmb is induced in macrophages by IFN-gamma and lipopolysaccharide and acts as a feedback regulator of proinflammatory responses. *J Immunol* 2007, *178*, 6557-6566.

Figure legends

Figure 1. Effects of HA derivatives on the cytokine response of iMΦ. Cytokine release from activated (act.) iMΦ (LPS stimulation for 5 h (TNFα) and 24 h (others)) cultured for six days with H-HA, L-HA, sL-HA or without GAGs (ctr) calculated as fold release relative to ctr, detected by ELISA. Bars represent calculated mean values ± SD of experiments with at least six donors. ANOVA with multiple comparisons versus control using Tukey's post-test: ***$p \leq 0.001$, **$p \leq 0.01$.







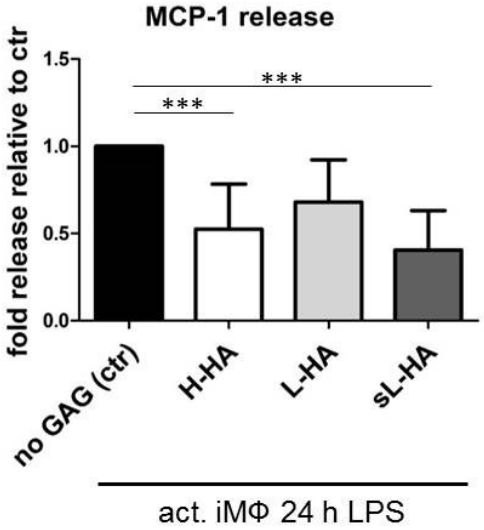
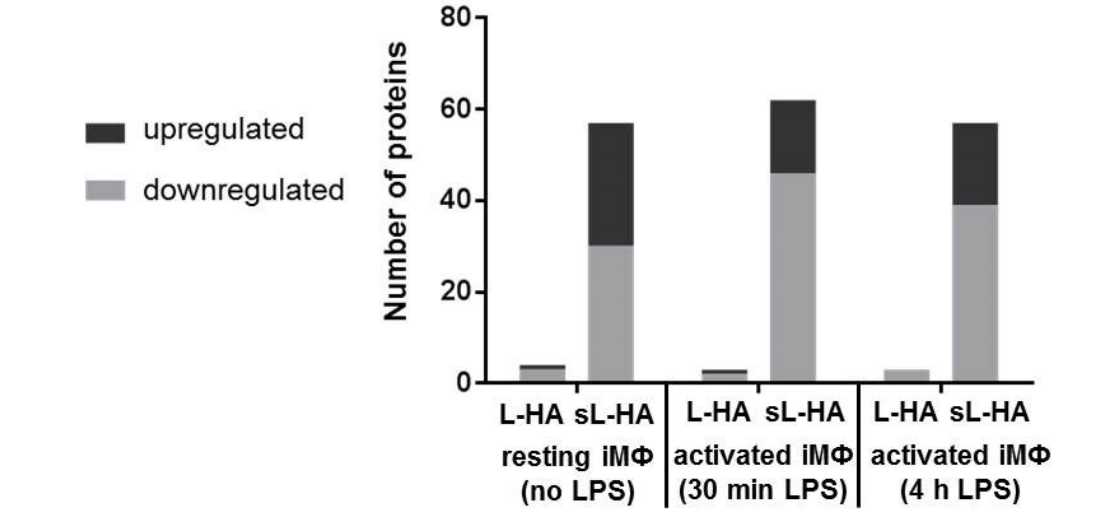


Figure 2. Global proteomic analysis. iMΦ cultured for six days with or without GAGs (L-HA or sL-HA). GAG effects in resting (no LPS) and activated iMΦ (30 min or 4 h LPS) are analyzed. **A)** Number of proteins significantly regulated by more than 20% in GAG-treated iMΦ compared to iMΦ without GAGs. **B)** Regulated biological pathways predicted by IPA of regulated proteins (p-value < 0.1). A biological pathway is deemed significant if the p-value < 0.01 (*). The z-score describes the regulation of the pathway: positive values (orange) indicate activated pathways, negative values (green) indicate inhibited pathways. A white entry indicates that no z-score could be calculated. **A+B)** Data are based on at least three biological replicates.

A



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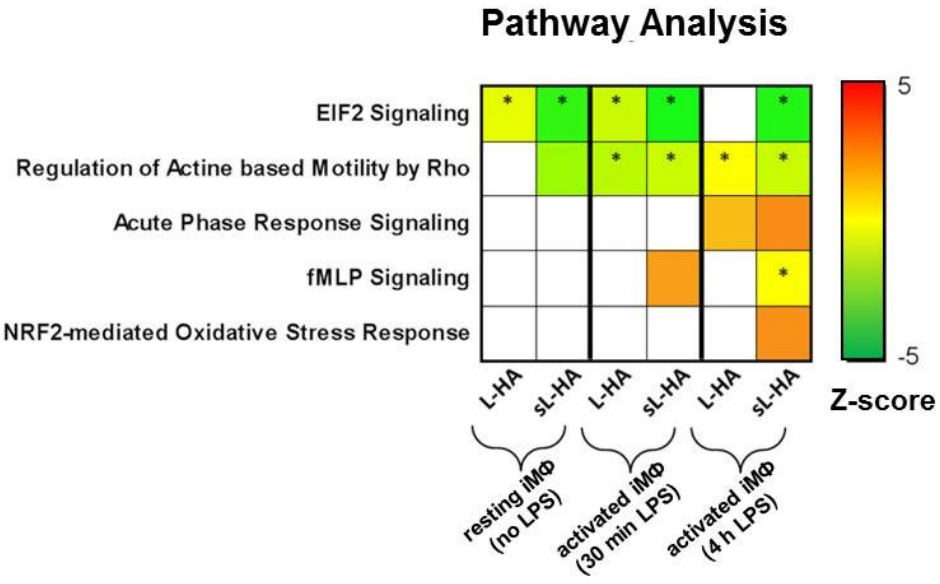


Figure 3. Recognition and uptake of sL-HA by iMΦ. **A)** CD44 surface expression on resting iMΦ before and after culture with sL-HA determined by flow cytometry. **B)** Molecular modeling of sL-HA interacting with CD44. Docking results showing CD44 in grey and the HA463' (dp6) binding poses in green sticks. CD44 HA binding domain is highlighted in yellow and basic residues are numbered. **C)** sL-HA binding / uptake by resting iMΦ in the presence and absence of CD44 functional blocking antibody assessed by flow cytometry. **D)** Co-localization of sL-HA (labeled with ATTO565) with Lox1 and CD36 visualized by immunofluorescence microscopy. Resting iMΦ were incubated with antibodies targeting Lox1 and CD36 for 30 min before addition of sL-HA-ATTO565. Scale: 10 µm. **E)** Molecular modeling of sL-HA interaction with Lox1. Docking results showing Lox1 in grey and HA463' (dp6) in green sticks. Basic residues involved in OxLDL recognition are highlighted in orange. **F)** Molecular modeling of sL-HA interaction with CD36. Docking results showing CD36 in grey and HA463' (dp6) in green sticks. CD36 functional domain is shown in yellow with the residues critical for OxLDL recognition in orange.

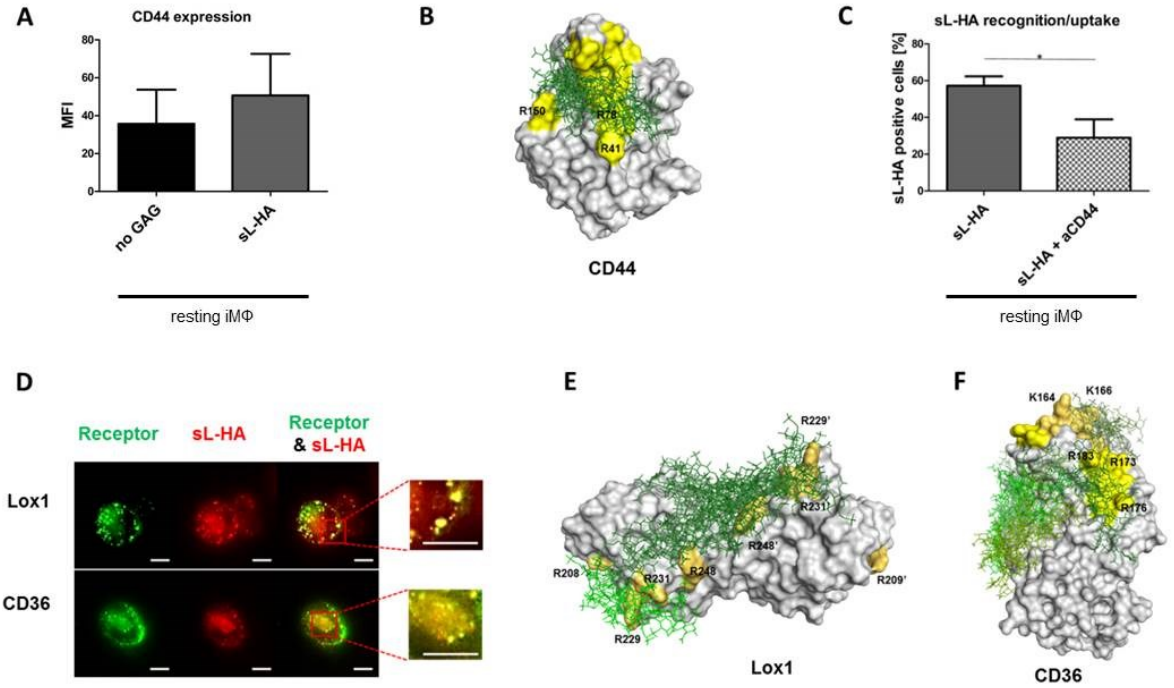


Figure 4. Oxidative stress response and anti-inflammatory regulation in iMΦ by sL-HA. Resting iMΦ were cultured with L-HA, sL-HA or without GAG as indicated and either immediately analyzed (no LPS) or after activation with LPS for 30 min or 4 h. **A)** The log_2 [ratio(HA vs. no GAG)] of the proteins belonging to oxidative and inflammation pathways are shown. Green: upregulated by > 20 %, yellow: regulated by 10-20 %, grey: not regulated (< 10 %). Values in bold represent significantly regulated proteins, underlined values represent almost significant proteins ($0.05 < p < 0.1$). All values are based on at least three biological replicates. ND: not detected. **B)** RNA expression of SOD1, SOD2 and SOD3 in resting iMΦ after culture for 24 h or six days presented as fold induction to control. Bars represent calculated mean values ± SD of experiments with at least six donors. ANOVA with multiple comparisons versus control using Tukey's post-test: ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. **C)** Protein abundance of SOD2 in resting iMΦ after 24 h or 6 d incubation analyzed by Western blotting of whole lysates. Representative blots are shown. **D**) Intracellular ROS levels determined using CellROX® and analyzed by flow cytometry in resting iMΦ after culture with GAGs for 1 h, 6 h, 1 d or 6 d, respectively. iMΦ stimulated with PMA served as positive ctr for ROS induction. Bars represent mean values ± SD of experiments with six donors. **E)** Viability of resting iMΦ (differentiated from monocytes over 6 days) after culture for further six days with HA derivatives by XTT cell viability assay. Medium was used as Blank value. Bars represent calculated mean values ± SD of experiments with at least three donors. **F)** Activation of transcription factors pSTAT1, pNFκB and IRF5 in activated iMΦ analyzed by Western blotting of whole lysates (10 min LPS, pNFκB) or nuclear fraction (4 h LPS, pSTAT1 and IRF5: use of same blot). Representative blots are shown.

A

Protein Name	Gene	Uniprot ID	resting iMΦ (no LPS) log2 ratio (L-HA / no GAG)	resting iMΦ (no LPS) log2 ratio (sL-HA / no GAG)	activated iMΦ (30 min LPS) log2 ratio (L-HA / no GAG)	activated iMΦ (30 min LPS) log2 ratio (sL-HA / no GAG)	activated iMΦ (4 h LPS) log2 ratio (L-HA / no GAG)	activated iMΦ (4 h LPS) log2 ratio (sL-HA / no GAG)
Neutrophil cytosol factor 1, p47phox	NCF1	P14598	0.19	**0.45**	0.03	**0.30**	0.01	**0.35**
Neutrophil cytosol factor 2 , p67phox	NCF2	P19878	-0.08	0.16	-0.03	**0.14**	0.00	0.09
Superoxide dismutase [Mn], mitochondrial	SOD2	P04179	0.22	**0.80**	**0.21**	**0.86**	0.16	**0.87**
Copper transport protein ATOX1	ATOX1	O00244	nd	nd	0.01	**0.14**	0.07	0.36
Glutaredoxin-3	GLRX3	O76003	nd	nd	0.03	**0.17**	nd	nd
Glutamate--cysteine ligase catalytic subunit	GCLC	P48506	0.00	**0.15**	0.02	0.06	-0.07	0.01
Osteopontin	SPP1	P10451	0.25	**0.33**	nd	nd	nd	nd
Interleukin-1 receptor antagonist protein	IL1RN	P18510	nd	nd	0.03	0.24	0.13	**0.44**
Transmembrane glycoprotein NMB	GPNMB	Q14956	-0.03	0.20	-0.11	**0.38**	-0.04	**0.37**

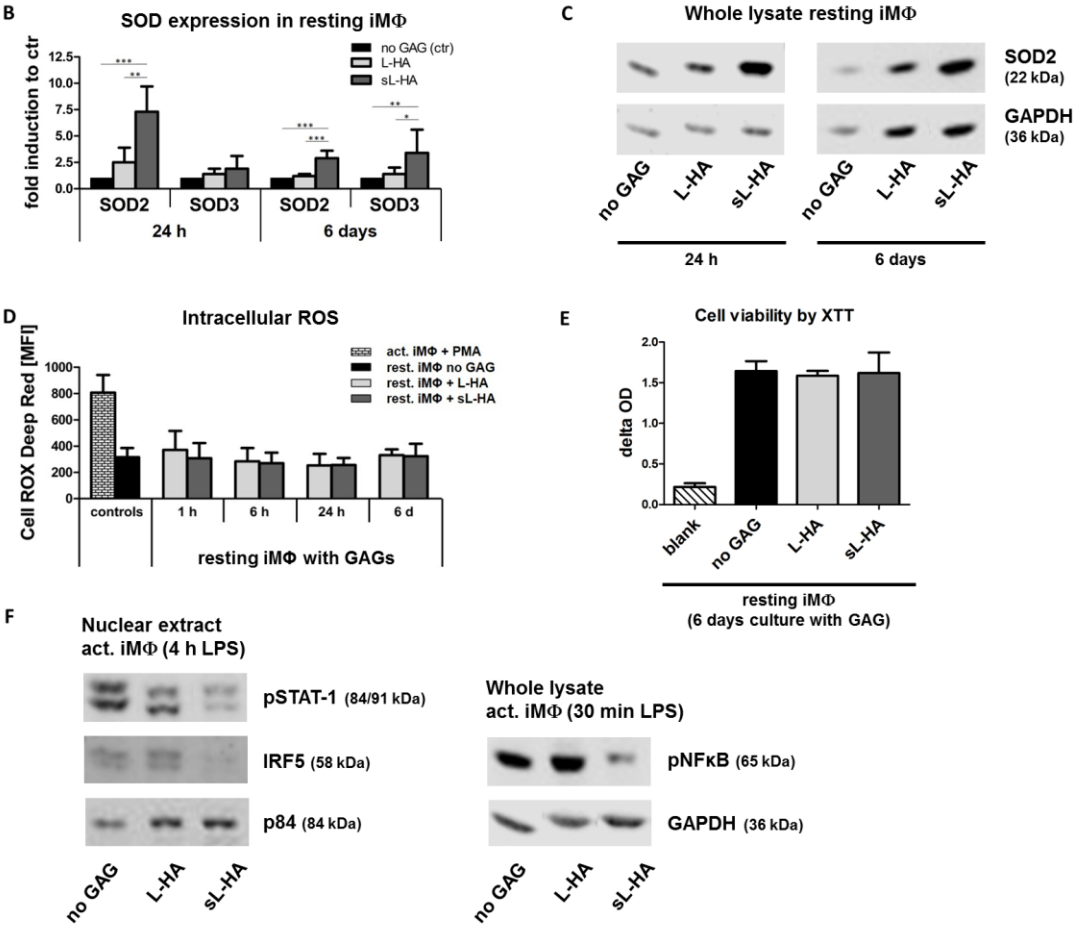
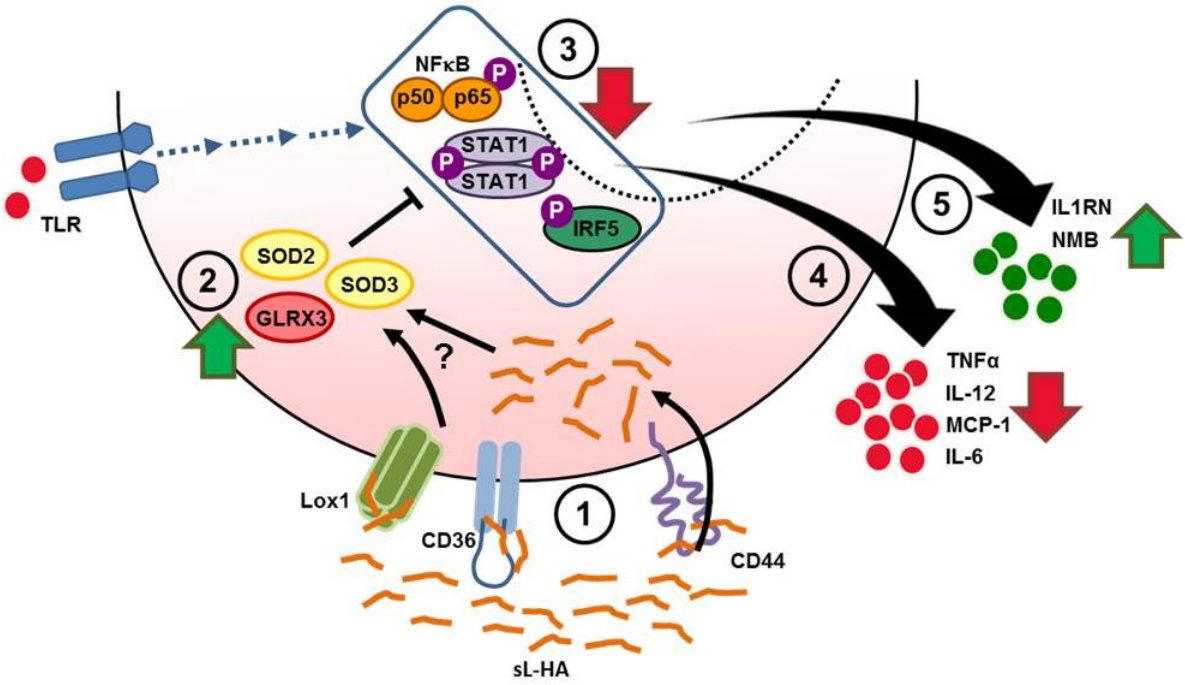


Figure 5. Supposed mechanisms contributing to the anti-inflammatory activity of sL-HA on iMΦ. 1) Rapid uptake of sL-HA involving the recognition via CD44 and the scavenger receptors CD36 and Lox1. **2)** Upregulation of antioxidants SOD2, SOD3 and GLRX3 is mediated by an unknown mechanism. **3)** Redox-dependent regulations inhibit TLR-induced activation of transcription factors pNFkB, pSTAT1, IRF5 resulting in **4)** reduced expression and release of pro-inflammatory cytokines (TNFα, IL-12(p40), MCP-1, IL-6). **5)** Upregulated anti-inflammatory proteins (IL1RN, NMB) counter-regulate autocrine/paracrine-mediated inflammatory activation of iMΦ.



Supporting files

Supporting information. Supplementary figure and materials/methods

Supporting data1. All Quantitative Proteomics

Supporting data2. Quantitative Proteomics_resting iMΦ

Supporting data3. Quantitative Proteomics_activated iMΦ(30min_LPS)

Supporting data4. Quantitative Proteomics_ activated iMΦ(4h_LPS)

Table 1: Characteristics of HA derivatives. DS: degree of sulfation per disaccharide; M_w: molecular weight, PD: polydispersity.

GAG	DS	M_w [kDa]	PD	Molarity [µM]	Final concentration applied to iMΦ [µg/ml]
H-HA	0	1175	4.8	1.43	1670
L-HA	0	48	2.3	1.43	68.6
sL-HA	3.4	53	1.7	1.43	75.8