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High and low molecular weight hyaluronic acid differentially influence macrophage activation

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Abstract

Macrophages exhibit phenotypic diversity permitting wide-ranging roles in maintaining physiologic homeostasis. Hyaluronic acid, a major glycosaminoglycan of the extracellular matrix, has been shown to have differential signaling based on its molecular weight. With this in mind, the main objective of this study was to elucidate the role of hyaluronic acid molecular weight on macrophage activation and reprogramming. Changes in macrophage activation were assessed by activation state selective marker measurement, specifically quantitative real time polymerase chain reaction, and cytokine enzyme-linked immunoassays, after macrophage treatment with differing molecular weights of hyaluronic acid under four conditions: the resting state, concurrent with classical activation, and following inflammation involving either classically or alternatively activated macrophages. Regardless of initial polarization state, low molecular weight hyaluronic acid induced a classically activated-like state, confirmed by up-regulation of pro-inflammatory genes, including *nos2*, *tnf*, *il12b*, and *cd80*, and enhanced secretion of nitric oxide and TNF-α. High molecular weight hyaluronic acid promoted an alternatively activated-like state, confirmed by up regulation of pro-resolving gene transcription, including *arg1*, *il10*, and *mrc1*, and enhanced arginase activity. Overall, our observations suggest that macrophages undergo phenotypic changes dependent on molecular weight of hyaluronan that correspond to either (1) pro-inflammatory response for low molecular weight HA or (2) pro-resolving response for high molecular weight HA. These observations bring significant further understanding of the influence of extracellular matrix polymers, hyaluronic acid in particular, on regulating the inflammatory response of

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Supporting Information

Additional experimental procedures and supporting data, specifically influence of endotoxin on macrophage activation, influence of HA concentration on macrophage activation, the levels of markers after 48 and 72 hours of activation, and data presented as the ratios of functional enzymes, *nos2* and *arg1*, interleukins, *il12b* and *il10*, and cell surface markers, *cd80* and *mrc1*. This information is available free of charge via the Internet at http://pubs.acs.org/.

macrophages. This knowledge can be used to guide the design of HA-containing biomaterials to better utilize the natural response to HAs.

Keywords

macrophage; hyaluronic acid; molecular weight; classically activated; alternatively activated; polarization

Introduction

Since its discovery,[1] hyaluronic acid (HA) has received great attention as a versatile and highly functional biopolymer.[2-6] HA is evolutionarily conserved from simple prokaryotes all the way to complex eukaryotes, which is an undeniable testament to its biologic relevance.[7] Structurally, hyaluronic acid is not inert. The native, high molecular weight, form can be broken down into smaller molecular weight fragments in response to glycosidase activity upregulated by environmental cues, such as pH and reactive oxygen species.[2, 8] The molecular weight variants have been used in a variety of biomedical applications eliciting varying biologic responses.[9-10] Clinically, HA has been used in several applications, including ophthalmology as a drug delivery system,[11-12] in osteoarthritis for viscosupplementation,[13-15] and as a dermal filler.[16-17]

Physiologic responses to HA are, in part, mediated through the immune system, resulting in either acute or chronic inflammation,[18] manifested by the production of specific inflammatory mediators.[19-24] Low molecular weight HA promotes the production of inflammatory mediators.[25-28] Similarly, high molecular weight HA inhibits production of pro-inflammatory mediators,[25, 29-31] suggesting differential macrophage activation by different molecular weight HAs. However, to our knowledge, there has been no work clearly elucidating the role of hyaluronic acid molecular weight in reprogramming of resting, classically activated, and alternatively activated macrophages. An understanding of macrophage reprogramming in response to HA of various molecular weights would establish a clear role for the biomaterial within the context of disease and for the production of biologically responsive biomaterial based systems.[32-33]

Nominally, there are two extremes of macrophage activation *in vitro* (Figure 1), which are, in part, mediated by T-helper cell responses to stimuli.[33-35] *In vivo*, the definitions of classically activated macrophages (cMφs) and alternatively activated macrophages (aMφs) are understood to be theoretical extremes and the complexity of *in vivo* signaling results in mixed macrophage populations exhibiting characteristics of both activation states.[33] Classically activated macrophages produce and release a robust milieu of inflammatory mediators,[32, 34-36] resulting in a destructive environment, evolutionarily designed to purge infecting pathogens or respond to danger signals.

On the opposing extreme are aMφs, which are involved in the resolution of inflammation, extracellular matrix reconstruction, and angiogenesis.[37-40] The classification as “alternatively” activated has become rather loose, and some use it to refer to any activation

state, which is not classical.[32, 37] We refer to alternative activation as described when IL-4 was found to induce a novel form of activation in macrophages.[37]

Although these are two extremes of the macrophage spectrum, the macrophage is multi-faceted in its polarization capability and often exists within a spectrum of these extremes (Figure 1).[32-33] Therefore, macrophage activation state can be characterized by the expression of both pro-inflammatory and pro-resolving markers. Herein, we have explored the effect of different hyaluronic acid molecular weights, low (≤ 5 kDa), intermediate (60-800 kDa), and high (> 800 kDa), on macrophage activation. Additionally, we have explored the role of initial macrophage polarization state in mediating response to these various molecular weights to assess reprogramming potential. We have analyzed the expression of inflammatory and pro-resolving markers to unravel and clarify the effects of HA molecular weight on macrophages and compared these results with the initial activation state (Figure S1). We hypothesized that macrophages, either activated or not, would differentially respond to HAs of different molecular weights.

Methods

Hyaluronic acids and hyaluronic acid digestion

Hyaluronic acid, molecular weights 5 kDa, 60 kDa, 800 kDa, and 3,000 kDa, was purchased from Lifecore Biomedical as lyophilized powder (Pharmaceutical Grade, endotoxin ≤ 0.01 EU/mg, Figure S2). Polydispersity (M_w/M_n) of each of these HAs was narrow, not exceeding 1.3 for any of the polymers. Hyaluronic acid digests were formed as previously described with modification.[41] Briefly, 50 mg of high molecular weight HA (3,000 kDa) was dissolved in 10 mL of phosphate buffered saline. Mammalian (bovine testicular) hyaluronidase (Sigma-Aldrich, St Louis, MO, USA) was added at a concentration of 10 U/mg of HA, and reacted at 37°C for two hours, after which the temperature was raised to 95°C for twenty minutes. The solution was dialyzed (MWCO 3,500 g/mol) against distilled deionized water for three days. The dialysate was collected and filtered through a 0.22 μm filter, then lyophilized for use. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy was performed using a Voyager DE PRO Mass Spectrometer (Applied Biosystems, Foster City, CA) equipped with a 337-nm pulsed (~ 5 nsec) nitrogen laser[42-43] revealed a polydisperse sample with average molecular weight of approximately 1.3 kDa (not shown), and molar concentration of the digests was determined using this average molecular weight. The limulus amebocyte lysate (LAL; Pierce) assay was used to confirm the absence of endotoxin in the digest preparation according to manufacturer's instructions.

A HA concentration of 1 μM was chosen for all experiments since therapeutic pharmaceutical formulations are administered at approximately this concentration.[44-46] Similarly, most investigative biomaterial based systems utilize similar concentrations, and many organs with high infiltration of macrophages during injury, such as extracellular matrix,[30] vitreous body,[47-48] pleural cavity,[49] lymphatic system,[30] and joint space[50], contain similar concentrations of HA.

Cell culture and treatments

Murine macrophages (J774A.1 ATCC; TIB-67)[51-52] were cultured in phenol-free Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gemini, Calabasas, CA, USA) at 37°C in 5% CO_2. Macrophages were harvested by scraping and used between passage 2 and 7. Macrophages were plated at a density of 5×10^5 cells/mL in a 24 well plate (500 μL/well) and allowed to adhere overnight before use.

Four states of macrophages were used in each experiment. Macrophages were cultured in the presence or absence of varying molecular weights (5-3,000 kDa, or the digest) of hyaluronic acid (1μM) (1) in their resting state, i.e. cultured in media with no activating agent (M), (2) with 100 ng/mL γ-irradiated LPS (M(LPS)) derived from *Escherichia coli* serotype 055:B5 (Sigma-Aldrich, St Louis, MO, USA), (3) with 20 ng/mL interferon-γ (Peprotech) and 100 ng/mL γ-irradiated LPS (M(IFN-γ+LPS)),[36] or (4) following activation with 20 ng/mL interleukin-4 (Peprotech) (M(IL-4))[37]. In each experiment scenario, the reference state—M, M(LPS), M(IFN-γ+LPS), or M(IL-4)—macrophages were cultured for the same time as the experimental groups without HAs added (Figure S1).

Nitric oxide (NO)

J774A.1 murine macrophages were plated with treatments as described above. Supernatant media was collected after 24-hour incubation period and nitrite measured using the Griess reagent (Promega, Madison, WI, USA) according to manufacturer's instructions.

Arginase activity

Arginase activity was assayed as previously described[53-54] Briefly, lysates of 10^5 macrophages cultured as described above were centrifuged at 12,000×*g* for 20 minutes and the supernatant was collected. Arginase in the supernatant was activated by heating the lysate for 10 minutes at 55°C in a 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MnCl_2$. L-arginine hydrolysis was then carried out with the addition of 25 μL of 0.5 M L-arginine (pH 9.7) to 25 μL of the activated lysate and incubated at 37°C for 60 minutes. The reaction was stopped with the addition of an acid mixture containing H_2SO_4, H_3PO_4, and H_2O (1:3:7). After the addition of 25 μL of a 9% solution of α-isonitrosopropiophenone and heating to 100°C for 45 minutes, the end product of the hydrolysis reaction, urea was detected colorimetrically on a Labsystems Multiskan plus spectrophotometric microplate reader (Thermo Scientific, Hanover Park, IL) at 540 nm.

TNF-α production

Tumor necrosis factor- α (TNF-α) secreted by macrophages was determined by the measuring the protein in cell-culture supernatants using a TNF-α enzyme-linked immunosorbent assay kit (R&D systems) according to the manufacturer's instructions.

Gene expression

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from J774A.1 murine macrophages. Integrity of RNA was determined via spectrophotometric analysis of

each sample at 260 and 280 nm. Samples with 260 to 280 ratios greater than 1.8 were reverse transcribed into cDNA (Applied Biosystems, Carlsbad, CA, USA). Real time PCR was carried out on an Applied Biosystems StepOnePlus™ PCR using SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). A melting curve analysis was performed after each run to confirm product specificity. All primers were designed to span exon-exon junctions (Table 1). Transcripts of β-glucuronidase (*gusb*) were quantified and used as endogenous control.[55] Relative quantities were estimated by the delta-delta-Ct method.[56] The expression of each gene was normalized to untreated cells as control.

Statistical Analyses

ANOVA was used to test all groups, and post-hoc Tukey analysis was utilized if ANOVA suggested significant differences between the groups with significant *p*-values less than 0.05. Statistical significant differences from the reference states (Figure S2) are presented with the average value for that reference state presented in the figures with a dotted line of appropriate color for that state (resting, LPS, LPS with IFN-γ, or IL-4). All experiments were independently replicated at least three times.

Results and Discussion

Resting Macrophage: Stimulation with Hyaluronic Acid

Our initial assessment of macrophage phenotype in response to hyaluronic acid was done on resting macrophages (Figure 2A). We observed that the transcription of *nos2* was significantly up-regulated upon treatment with all molecular weights of HA, except 800 kDa HA compared to resting macrophages (Figure 2B). Lower molecular weight HAs induced the greatest increase in *nos2* expression, with the digestion showing an over 20-fold increase from resting state (Figure 2B). Although lower molecular weights increased *nos2*, this was significantly less than M(LPS) and M(IFN-γ+LPS). Higher molecular weight HAs (800 and 3,000 kDa) exhibited *nos2* expression levels comparable to M(IL-4) ($p > 0.05$).

Arginase expression was significantly up-regulated in macrophages treated with 3,000 kDa HA compared to resting macrophages ($p < 0.0001$) (Figure 2C). This was not significantly different from expression detected in M(IL-4) and is suggestive of movement to an alternatively activated state. Differences in functional by-products for both of these enzymes were undetectable after treatment for 24 hours (data not shown).

Upon treatment with the digest, macrophages exhibited a 4-fold increase in the amount of *tnf* gene transcription compared to resting macrophages (Figure 2D). All other molecular weights tested were not statistically different from resting macrophages, indicating that only oligomeric HA modulates transcription of *tnf* (Figure 2D). TNF-α production was minimal in macrophages treated with the highest molecular weight HAs (800 and 3,000 kDa), and the secreted levels were comparable to M(IL-4) ($p > 0.05$) (Figure 2E).

Next, we examined the expression of *il12b* and *il10*. Macrophages treated with all HA molecular weights exhibited significantly higher transcription of *il12b* compared to both resting macrophages and M(IL-4)(Figure 2F). Resting macrophages treated with lower molecular weight HAs (5kDa and the digest) expressed *il12b* that exceeded that of M(LPS).

Macrophages treated with intermediate and high molecular weight HAs transcribed lower levels of *il12b* compared to M(LPS) and M(IFN-γ+LPS). Transcription of *il10* was significantly up-regulated in resting macrophages treated with all HAs compared to their native state. Macrophages treated with the lowest molecular weight HAs (digest, 5, and 60 kDa) showed no significant difference in *il10* expression compared to M(LPS) and M(IFN-γ +LPS) (Figure 2G), suggesting polarization that is similar to the classically activated state. HAs with molecular weight of 800 kDa or 3,000 kDa, though significantly deviating from M(IL-4), had highly enhanced transcription of *il10* (Figure 2G), suggesting a movement toward the alternatively activated state.

Finally, we examined the transcription of two phenotype specific surface markers: *cd80* and *mrc1*. High molecular weight HAs (800 and 3,000 kDa) reduced expression of *cd80* to levels comparable to M(IL-4) (Figure 2H). On the other hand, the digestion of HA enhanced the expression of *cd80* to levels associated with the classically activated state ($p > 0.05$; Figure S1J, 2H). Transcription of *mrc1* was minimally influenced by HA treatment (Figure 2I). Overall, it was clear that resting macrophage exposure to lower molecular weight HAs resulted in activation similar to cMφs while higher molecular weight HAs stimulated activation similar to aMφs.

Classically Activated Macrophages: Stimulation with LPS and HA simultaneously

In an effort to mimic an inflammatory response that may occur simultaneous to administration of HA, we examined simultaneous administration of HA and LPS (Figure 3A).[57] Delivery of lipopolysaccharide derived from E. coli cell wall was chosen to simulate this response *in vitro*.[58] LPS activates macrophages through a pathway, which is shared with other biomaterials, *in vitro* as well as *in vivo*, so we considered this an adequate *in vitro* method of simulating an acute classical inflammatory response.[57-61] Previous studies have established that high molecular weight hyaluronic acid has the ability to suppress inflammation due to LPS.[62] However, molecular weight dependent effects on specific phenotype markers have not been clarified.

Transcription of *nos2* was significantly increased upon treatment with all HA molecular weights except for 3,000 kDa HA (Figure 3B). This increase matched or exceeded transcription levels of *nos2* for M(LPS) and M(IFN-γ+LPS). Macrophages treated with the highest molecular weight HA (3,000 kDa) decreased *nos2* transcription to levels approaching levels of M(IL-4). Low (digest and 5 kDa) and intermediate molecular weight HAs (60 kDa) significantly enhanced the production of nitrite compared to untreated LPS stimulated macrophages (Figure 3C) while *arg1* expression steadily increased with increasing molecular weight HA.

The highest levels of *arg1* transcription were detected in macrophages treated with 3,000 kDa HA, and these expression levels significantly exceeded the levels of M(IL-4) (Figure 3D). Treatment with low and intermediate HAs resulted in *arg1* levels that were not different from M(LPS) (Figure 3D). In macrophages treated with HA with molecular weight less than 3,000 kDa, arginase activity was comparable to M(LPS) (Figure 3E). These HAs also suppressed arginase activity to the same extent as found in both M(LPS) and M(IFN-γ +LPS). Macrophages treated with the highest molecular weight HA, 3,000 kDa, resulted in

significantly elevated urea production compared to macrophages treated with LPS (Figure 3E). However, this level of urea production was still significantly lower than M(IL-4) (Figure S1E).

Only the lowest molecular weight (digests) and the highest molecular weight HA (3,000 kDa) showed statistically different *tnf* transcription compared to M(LPS) (Figure 3F). The HA digest promoted expression of *tnf*, inducing a robust 24-fold increase in *tnf* expression compared to untreated cells and a 14-fold increase in expression compared to M(LPS) (Figure 3F). On the other hand, treatment with high molecular weight HA (3,000 kDa) decreased the expression *tnf* from M(LPS) by about 7-fold (Figure 3F). TNF-α protein secretion exhibited a similar trend to *tnf* gene expression. Macrophages treated with the HA digest exhibited TNF-α release that was 2.5 times greater than M(LPS) (Figure 3G). On the other hand, macrophages treated with 3,000 kDa HA decreased TNF-α secretion to approximately half that secreted by M(LPS).

Macrophages treated with all molecular weight HAs, except 3,000 kDa, resulted in significantly enhanced expression of *il12b* compared to M(LPS) (Figure 3H). Transcription of *il12b* did not suggest differential polarization in response to HAs; however, differences in pro-resolving *il10* were much more defined by molecular weight, with increasing molecular weight HAs enhancing the expression of this pro-resolving gene (Figure 3I). The greatest expression of il10 was observed following treatment with 3,000 kDa, suggesting that higher molecular weight HAs enhance pro-resolving function and activate macrophages similar to M(IL-4).

To further confirm this trend, we analyzed surface markers mRNA expression for *cd80* and *mrc1* in this population of macrophages. All HA molecular weights significantly increased cd80 transcription compared to M(LPS) and M(IFN-γ+LPS) (Figure 3J). However, *mrc1* expression, a marker of alternative activation, was also up-regulated in macrophages exposed to higher molecular weight HAs (Figure 3K) suggesting that these molecular weight HAs enhance expression of both classical- and alternative-associated surface receptors. Moreover, lower molecular weight HAs (digest and 5kDa) expressed each surface marker to and extent that matched or exceeded that of M(LPS) and M(IFN-γ+LPS).

Classically Activated Macrophage: Stimulation with HAs

Chronic diseases are often associated with persistently activated macrophages, whether in the alternative or classically activated state[32, 34-36, 39-40] but, cMφs are considered to have the most detrimental effects. Therefore, we explored the modification macrophage phenotype with HA in a cMφ population, produced in the presence of both IFN-γ and LPS to represent a more sustained and robust inflammatory response.[63] Modification of such a population would not only suggest therapeutic potential in reprogramming macrophage phenotype, but also, a potential role for HA in the propagation or alteration of macrophage function within the tissue.

Upon treatment with the HA digest, M(LPS) and M(IFN-γ+LPS) exhibited significantly enhanced transcription of *nos2*. Classically activated macrophages treated with 3,000 kDa HA attenuated *nos2* transcription, although this remained significantly higher than M(IL-4)

(Figure 4B). Classically activated macrophages treated with the lowest molecular weight HAs, the digests and 5 kDa, produced significantly more nitrite than M(IFN-γ+LPS) not treated with HAs (Figure 4C). On the other hand, cMφs treated with 800 to 3,000 kDa HA suppressed production of nitrite to levels measured in M(IL-4) (Figure 4C). Biologically significant decreases in nitric oxide produced by M(IFN-γ+LPS) were dependent on treatment of HA with molecular weight greater than 60 kDa, with higher molecular weight HAs having the greatest reduction in nitrite production.

Arginase was significantly up-regulated to extents similar to M(IL-4) in macrophages treated with all molecular weight HAs. The highest increase in *arg1* transcription was detected in macrophages treated with HA 3,000 kDa (Figure 4D), which was significantly higher than M(IL-4). The ability of HA 3,000 kDa to significantly reduce *nos2* expression and enhance *arg1* expression was consistent with an alternatively activated phenotype. Urea production was significantly elevated, by approximately 3 fold, in macrophages treated with 3,000 kDa HA compared to untreated M(IFN-γ+LPS) (Figure 4E) while M(IFN-γ +LPS)treated with the digest and 5, 60 and 800 kDa HAs produced urea to the same extent as M(IFN-γ+LPS) not treated with the HAs (Figure 4E).

TNF-α expression and production demonstrated that treatment of the macrophages with HA molecular weights less than 5 kDa resulted in augmented transcription of *tnf*, 10 to 25-fold over untreated macrophages (Figure 4F). Classically activated macrophages treated with 5 through 3,000 kDa HAs significantly reduced the expression of *tnf* (Figure 4F) compared to the untreated M(IFN-γ+LPS); however, only 800 and 3,000 kDa HAs diminished transcription to levels observed with the M(IL-4) (Figure S1F). Production of TNF- α followed similar pattern to mRNA transcription. Classically activated macrophages treated with the digestion of HA produce up to 3.5 times more TNF-α compared to untreated M(IFN-γ+LPS) (Figure 4G). Higher molecular weight HA, 3,000 kDa, suppressed production of TNF-α to levels observed for M(IL-4).

Transcription of *il12b* was significantly reduced in M(IFN-γ+LPS) treated with higher molecular weight HAs, with the greatest reduction, approximately 5-fold decrease from the level of cMφs upon treatment with 3,000 kDa HA (Figure 4H). Unlike *il12b* transcription, transcription of *il10* mRNA was elevated with increasing molecular weight (Figure 4I). The highest molecular weight HA, 3,000 kDa, promoted *il10* transcription, which significantly exceeded that of M(IL-4), once again suggesting that treatment with high molecular weight HA resulted in activation similar to aMφs (Figure 4I).

Classically activated macrophages treated with HA digests enhanced *cd80* transcription to levels significantly greater than M(IFN-γ+LPS) (Figure 4J). All other molecular weight HAs did not result in activation that significantly deviated from the *cd80* levels of M(IFN-γ+LPS) (Figure 4J). Transcription of *mrc1* remained at a basal level in M(IFN-γ+LPS) (Figure 4K) and was only significantly elevated after treatment with HA 3,000 kDa and not significantly different from levels of M(IL-4) ($p > 0.05$).

Alternatively Activated Macrophages: Stimulation with HA

Although cMφs are considered destructive in many diseases, aMφs are also associated with several chronic diseases.[39-40] Therefore, we examined the effect of HA molecular weight on macrophages of this population. Macrophages were alternatively activated with overnight treatment with IL-4.[37]

When M(IL-4) were treated with HAs, molecular weights lower than 800 kDa significantly increased transcription of *nos2* compared to the untreated M(IL-4) (Figure 5B). The digest and 5 kDa HAs promoted a 10 to 16 fold increase in *nos2* transcription compared to M(IL-4). All HA treatments resulted in significantly lower *nos2* transcription compared to M(LPS) and M(IFN-γ+LPS) ($p < 0.05$; Figure 5B). Although *nos2* gene expression was significantly increased from baseline levels of M(IL-4) for all macrophage treatments, only the smallest of the HA fragments, the digest, was able to elicit significantly increased nitrite production ($p < 0.0001$) compared to M(IL-4) (Figure 5C).

Arginase (*arg1*) expression and activity (urea production) was significantly reduced following lower molecular weight HA, 60 kDa or lower, exposure (Figure 5D-E). Higher HA molecular weights (800 and 3,000 kDa) maintained *arg1* transcription at levels at untreated M(IL-4) levels. Additionally, urea production by macrophages treated with higher HA molecular weights was no different from M(IL-4), whereas lower molecular weights HAs significantly reduced urea production (Figure 5E). This suggests that 800 kDa and 3,000 kDa HAs may maintain alternative activation, but that lower molecular weights drive macrophage polarization away from the alternative state.

Classically activated macrophages exhibited *tnf* transcription and production that was amplified upon treatment with low molecular weight HAs. This observation holds true for M(IL-4). Alternatively activated macrophages treated with low molecular weight HAs exhibited 5 to 6 fold increased *tnf* transcription (Figure 5F) compared to M(IL-4) not treated with HA. TNF-α production was similar to baseline levels in M(IL-4) treated with 60 to 3,000 kDa HAs. Only the lowest molecular weight HAs (digest and 5 kDa) elicited a significantly enhanced secretion of TNF-α compared to untreated M(IL-4) (Figure 5G).

Alternatively activated macrophages treated with 800 kDa HAs did not exhibit changes in *il12b* transcription compared to untreated M(IL-4) ($p > 0.05$; Figure 5H). All other molecular weight HAs significantly enhanced *il12b* transcription. On the other hand, *il10* expression was significantly reduced to levels similar to M(LPS) and M(IFN-γ+LPS) in M(IL-4) treated with HAs 60 kDa and lower (Figure 5I). Macrophages treated with 800 to 3,000 kDa HAs showed no differences in *il10* from the alternatively activated state.

Alternatively activated macrophages treated with all HAs resulted in elevated *cd80* transcription compared to untreated M(IL-4). Low and intermediate molecular weight HAs (less than or equal to 60 kDa) up-regulated *cd80* by approximately 10 fold (Figure 5J). Conversely, treatment with higher molecular weight HAs resulted in significantly lower enhancement of *cd80* transcription, approximately 5-fold higher compared to M(IL-4) (Figure 5J). Levels of alternative activation marker, *mrc1*, were maintained nearly constant

upon treatment with all HAs and did not significantly deviate from levels associated with M(IL-4) (Figure 5K).

Again, lower molecular weight HAs drove activation away from aMφs, and toward cMφs, while higher molecular weight HAs drove activation toward aMφs, and away from cMφs. In addition, the relative expression of classical and alternative activation markers (Figure S4-S6) further confirmed the cMφ and aMφ phenotypes for all cases, but it is does not appear possible to differentiate resting macrophages from aMφs using the relative expression of cMφ and aMφ markers.

Overall observations

The work presented in this study demonstrates that HA has molecular weight dependent effects on macrophage gene expression, enzyme activity, and cytokine production under a variety of conditions. Based on the interpretation and analysis of several key genes and their products, hyaluronic acid has the ability to modulate and reprogram macrophage phenotype within the wide spectrum of activation states.

We have specifically chosen to look at several key genes and products involved in regulating macrophage response and shown to shape macrophage phenotype. Arginase, urea, *il10*, and *mrc1* are associated with the aMφ.[37-40] Conversely, *nos2*, nitric oxide, TNF-α, *il12b*, and *cd80* are associated with the cMφ.[36, 64-65] Although the two phenotype paradigm is a simple framework to understand macrophage function, in truth, macrophages reside within a wide spectrum of these activation states and are likely to express all of these genes and markers at once and may express both aMφ and cMφ markers simultaneously and their relative expression of several markers (Figures S4-S7) yields information that describes their current position within this complex spectrum of function.[32-35]

Most macrophage reprogramming approaches have focused on the aMφ as a desired response, since these macrophages have the ability to decrease inflammation, induce vascularization, promote matrix deposition, and support constructive tissue remodeling.[38-39] On the other hand, the cMφ is often thought of as a mediator of immunopathology and damage. However, the cMφ is actually very important in host defense against pathogens. Proper clearance of pathogen cannot be done without this activation and is hindered in other activation states.[35, 39-40] Classically activated macrophages also secrete tumoricidal cytokines, and spacio-temporal control of this activation may be desirable in the tumor environment.[35] Though both macrophage responses are desired in certain situations, it should be noted that abnormal polarization of the macrophage in either direction leads to detrimental function. Our findings suggest that endogenous HA or therapeutically applied HA, *i.e.* as a biomaterial or drug delivery system, can alter macrophage polarization in a molecular weight-dependent manner.

Our original hypothesis was that macrophage reprogramming depends, in part, upon the initial polarization state. We chose to examine the response of macrophages to HA in four initial polarization states: the resting state and models of classical and alternative activation. In each of these cases, hyaluronic acid has the ability to modulate response dependent on its molecular weight. Based on our results in all four initial polarization states, we can conclude

that HA with molecular weights in the megadalton range tend to decrease the expression of markers normally associated with the classical state and enhance or sustain those associated with an alternative activation state. Though the magnitude of response may be different in each case, modulation of the mediators by hyaluronic acid follows a similar trend independent of initial macrophage activation state. This suggests that treatment with these molecular weights may have true reprogramming potential, promoting an alternatively activated-like state. Our results are in concordance with findings that show that administration of exogenous high molecular weight HA promotes resolution phase of wound healing and that removal of this polymer is detrimental to the process.[66-68] Our findings also suggest that administration of high molecular weight hyaluronic acid may be therapeutically used to control the phenotype and subsequent activity of macrophages, which are bound in an inflammatory state.

Similarly, we can conclude that HA oligomers smaller than 12 disaccharides in length likely promote a classically activated-like state since they sustain or enhance expression of pro-inflammatory genes and cytokines and reduce those associated with the resolution of inflammation, i.e. the alternative activation state. This too, is independent of initial macrophage activation state. Interestingly, under conditions of classical activation, macrophage treatment with low molecular weight HAs actually augments classically activated markers. Once inflamed, either via classically activating cytokines or other activators, macrophages produce reactive oxygen species and increase the expression of hyaluronidase.[8, 27] Both of these are involved in the depolymerization of HAs.[8] We observed that cleaved HA products have the highest pro-inflammatory activity. Therefore, treatment of macrophages with HAs under inflammatory conditions may actually promote cleavage of the polymer into even smaller chains, inducing an even greater inflammatory response, thus propagating the classically activated state. Cleavage fo HAs was not examined in this work, and the investigation of the effect of hyaluronidase should be examined in the future. Regardless of the potential for cleavage under inflammatory stress, we observed that treatment with higher molecular weight HAs induced up-regulation of alternatively activated markers, whereas low molecular weight HAs induced the up-regulation of classically activated markers.

Overall, a trend for most genes, enzymes, and cytokines shows that macrophages treated with higher molecular weights exhibit reduced expression of classically activated genes and mediators and augmented expression of alternatively activated markers. The opposite is true for macrophages treated with low molecular weight HAs. In these cases, classical markers are enhanced. The macrophage response to intermediate molecular weights is much more difficult to decipher. Macrophage response upon treatment with these molecular weights is very much dependent on their initial state, suggesting that response to these molecular weights results in (1) a single population of macrophages which simultaneously express both classically activated as well as alternatively activated markers, (2) mixed populations of activated macrophages, or (3) these molecular weights do not have preference for binding to specific receptors that have distinct signaling toward a particular activation state. The intermediate molecular weights may have similar affinities to several receptors, some of which promote classical activation and others that promote alternative activation concomitantly.

Although HA is notoriously promiscuous in its binding to several different receptors and associated proteins, signaling through one of or a combination of mechanisms may be involved in the macrophage reprogramming we observe. The cluster determinant 44 (CD44) receptor is the best-characterized receptor of HA. Stimulation of CD44 with HA has been shown to play a role in cell adhesion, cell-substrate interactions, metastasis, and inflammation.[69-71] Pro-inflammatory stimuli, such as IFN-γ, LPS, and TNF-α, induce a high-affinity HA binding state of CD44 by increasing expression of the receptor and inducing certain post-translational modifications, such as reducing chondroitin sulfation and N-glycosylation of CD44.[72-75] Priming of macrophages with IFN-γ has been shown to enhance CD44 signaling.[76] In contrast, IL-4 induces post-translational modifications of CD44 receptor, which inhibit HA binding.[72, 74-75] This observation may explain why classically activated macrophages exhibit augmented response to HAs compared to those alternatively activated. On the other hand, binding of high molecular weight HA to CD44 may be inducing its suppressive and alternatively activating effects through up-regulation of transcription factors.[77-79] Additionally, multi-ligand CD44 occupancy, i.e. from high molecular weight hyaluronan, enhances macrophage phagocytosis and clearance of apoptotic cells, a function associated with alternatively activated, pro-resolving, macrophages.[80-81] Even with signaling through this one receptor, there are multiple potential mechanisms that need to be examined in addition to other HA receptors that must be considered.

HA fragments have also been shown to signal through toll-like receptor 4 (TLR-4).[28, 82-83] This receptor is essential in recognizing pathogen associated molecular patterns, such as motifs in exterior carbohydrates, including as mannose or LPS[84] leading to subsequent up-regulation of the expression of several pro-inflammatory genes associated with cMφs.[84] Low molecular weight HAs may be signaling through toll-like receptors to produce the cytokines and up-regulate markers associated with classical activation. It does not appear that high molecular weight HAs signal through TLR-4, and the mechanism of discriminating between low and high molecular weight HA is not known.

Macrophages activated with multiple toll-like receptor ligands tend to show either a sustained or additive classical activation state.[85] Our observations fall in line with these discoveries. In our models, macrophages treated simultaneously with low molecular weight HAs and TLR ligands (LPS) exhibit a sustained and sometimes augmented expression and production of inflammatory genes and mediators, which may further suggest that low molecular weight HAs are inducing inflammatory response through toll-like receptors. In contrast to the activating potential of low molecular weight HAs, high molecular weight HAs have been shown to reduce TLR-4 expression in LPS stimulated cells, which may account for its anti-inflammatory activities, as well as its ability to modify macrophage response to a more alternatively activated-like state.[86]

Other receptors that may be involved in the differential response to HA include the receptor for hyaluronan-mediated motility (RHAMM). RHAMM is involved in cytoskeletal rearrangement, and recent evidence has suggested that cytoskeleton arrangement and cell shape has a role in macrophage polarization.[87] This may be partially mediated by the different molecular weights of HA and their interactions with RHAMM. In endothelial cells,

for instance, low molecular weight hyaluronan, but not high molecular weight HAs, were shown to induce endothelial cell proliferation and migration in a RHAMM-dependent manner.[88] Complete understanding of hyaluronan binding to RHAMM and subsequent signaling in other cell types has been complicated by the fact that RHAMM does not have a typical trans-membrane domain and is found both intracellularly as well as on the cell surface.[89-91] Several HA receptors exist as splice-variants,[92] directly interact with one-another,[93-94] and bind HA by different mechanisms,[92] further obscuring true mechanistic understanding of HA signaling.

Physico-chemical characteristics of HA, dependent on the molecular weight, also convolutes true mechanistic elucidation. Different molecular weights of HA will have varying viscosities and structural conformations in solution.[95-97] For instance, short HA chains exhibit non-Gaussian behavior. Flexibility and viscosity of hyaluronan chains increases starting at molecular weights on the order of 10^4 daltons and reaches a maxima, corresponding to a Gaussian coil on the order of 10^5 daltons.[97] At equimolar concentrations of the HAs, there would be about a 10^{12} difference in specific viscosity between the highest and lowest molecular weight HA solutions,[98-99] which would suggest that viscosity of the HA solutions could dictate the differential phenotypes. However, if viscosity of high (3,000 kDa) and low molecular weight (5 kDa) is adjusted to a similar order of magnitude by modifying the concentration, a similar divergent biologic trend between low and high molecular weight HAs was observed (Figure S3) suggesting that viscosity is not a primary force for polarization following treatment with the HAs. Although also a consequence of dose-response, viscosity's role in modulating the magnitude of polarization cannot be ignored, as more viscous solutions of either molecular weight elicit exaggerated response (Figure S3). Therefore, although we have categorized the effects by molecular weight, our studies do not exclude the possibility that any one of these characteristics or a combination of them may contribute to the differences we have observed.

Our analysis of hyaluronic acid molecular weights in response to several different activation states offers us preliminary understanding of the role of this polymer in solution. However, our study has analyzed a simplified *in vitro* model of an immortalized macrophage line analyzed with a limited number of markers. *In vivo*, there are many different types of macrophages derived from monocytes in the circulating blood or from different tissues, which could interact with the HAs very differently. Additionally, settings in an *in vivo* environment will include the interaction of other cell types, components of the extracellular matrix, and proteins with these macrophages, which could modify the response that we see under controlled conditions. Although our cell line has been analyzed and confirmed in numerous other studies, there is still the potential that the responses of the immortalized cell line will not correlate to those from a primary cell line, and so further work elucidating the response in these cells as well as a more complex *in vivo* setting are necessary to truly elucidate the power of varying the molecular weight of HA on macrophage response.

Despite these limitations, our study is the first of its kind to show that molecular weight can modify macrophage phenotype, and that this modification is independent of initial activation state of the macrophage. While it has been recognized that HA plays a role in inflammation, this was thought to be through its scaffolding capabilities allowing cell-matrix

interactions.[100] Our study provides evidence that HA in solution can modulate the macrophage response, dependent on its molecular weight, inducing or suppressing the expression and production of phenotype-specific genes and products. This understanding may help in the use of hyaluronic acid to control macrophage phenotype, the development of other agents to modify macrophage response, supplement an understanding of the role of extracellular matrix and macrophage response, and improve biomaterial-based systems.

Conclusions

An understanding of macrophage phenotype reprogramming in the context of extracellular matrix biopolymers, such as hyaluronic acid, may begin to clarify improvements in biomaterial based systems and methods of modifying macrophage response to these systems. Our results indicate that hyaluronic acid has molecular weight dependent effects on modulating macrophage phenotype. In solution, the high molecular weight form of the polymer promotes alternative macrophage activation, even when macrophages are challenged with a classically activating stimulus. Conversely, macrophages exposed to low molecular weight HAs are encouraged to produce pro-inflammatory mediators associated with the classically activated state. The most pronounced effects are seen with the lowest and highest molecular weight forms of the polymer. Further mechanistic understanding of HA's ability to induce macrophage reprogramming is warranted, but several existing studies exhibit results that suggest a high-likelihood of the role of CD44, TLR-4, and RHAMM. We conclude that hyaluronic acid has molecular weight dependent effects on macrophage phenotype that traverse multiple activation states of the macrophage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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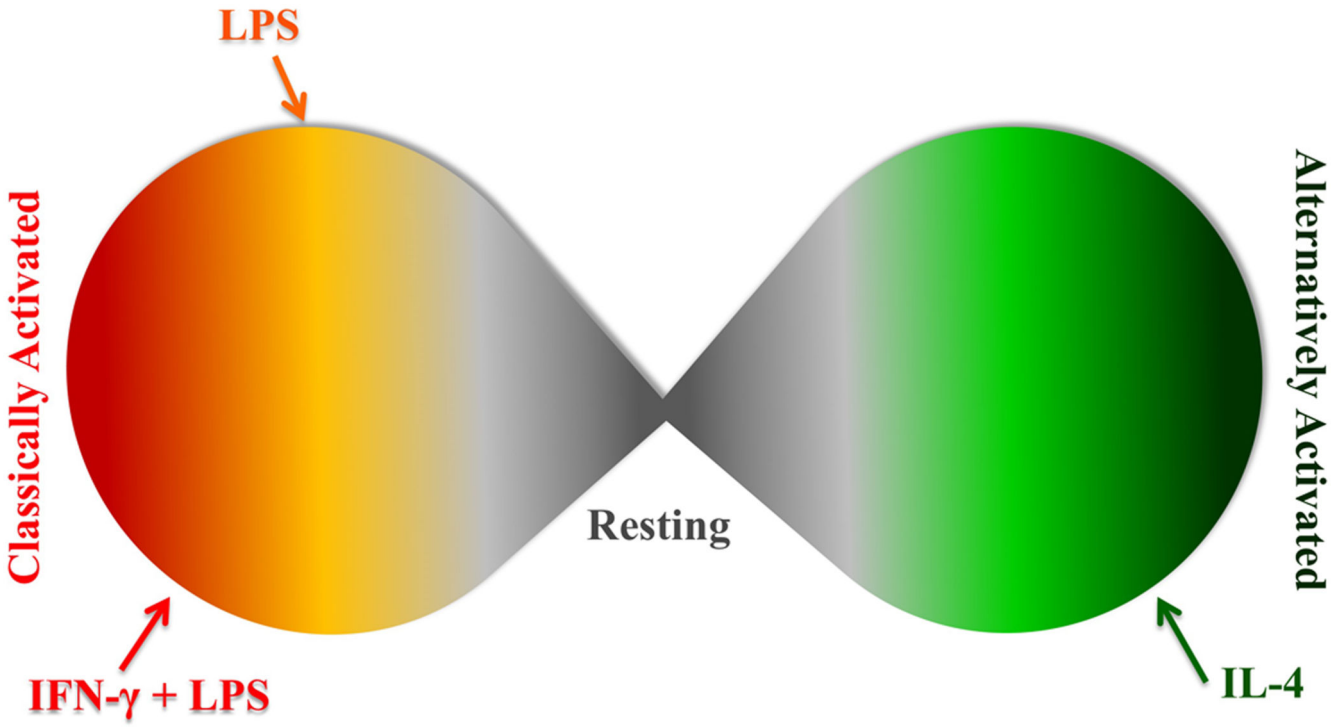

Figure 1. Macrophage activation is a continuum

Classically and alternatively activated macrophages are two extremes of a continuous spectrum. Many stimuli have the ability to polarize macrophages and even stimuli that give generally same phenotype (LPS or IFN-γ with LPS) do not result in the same activated state.

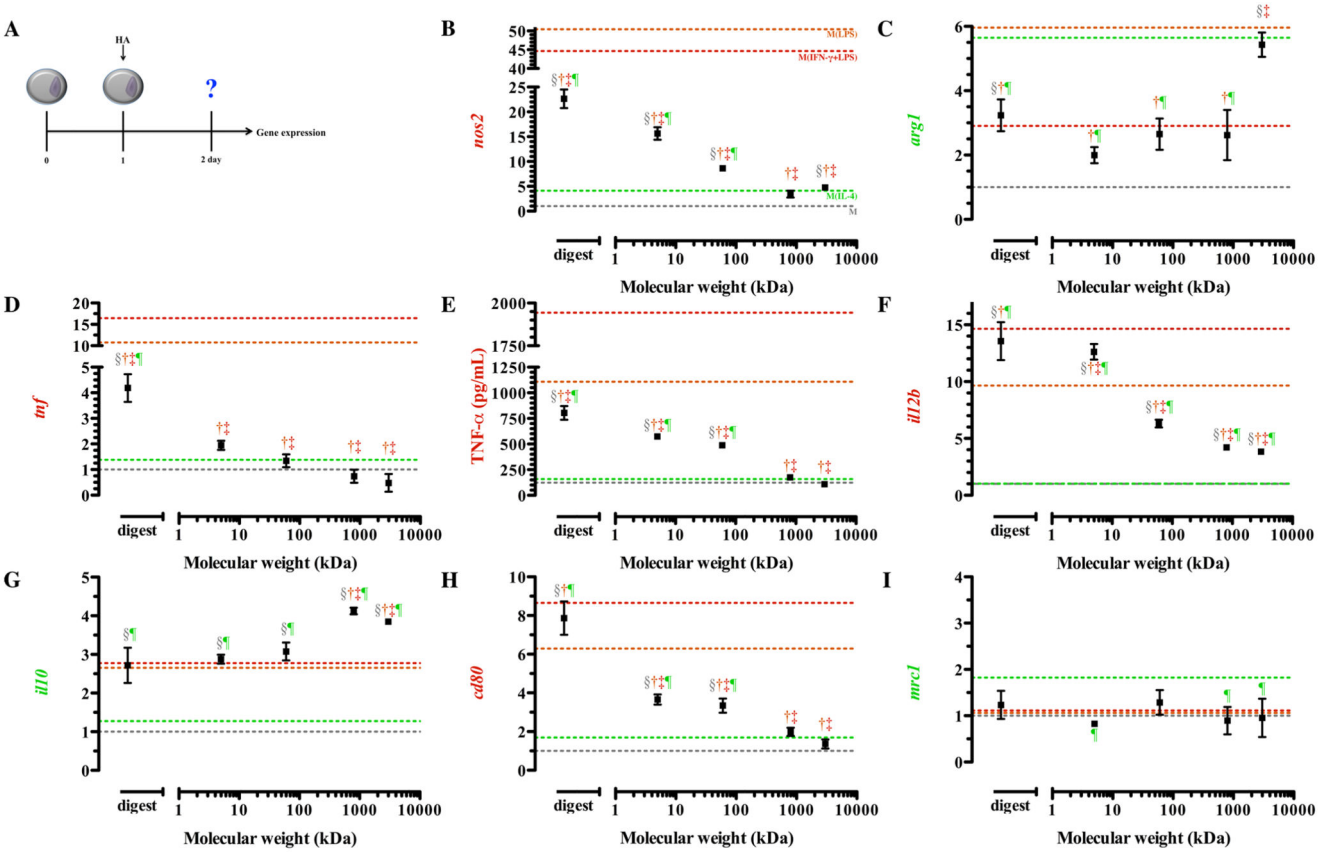

Figure 2. Gene expression of resting macrophages in response to hyaluronic acid

Resting macrophages were treated with 1μM HAs for 24 hours before phenotypic assessment was made. The schematic depicts the time course of the experiments (A). Low molecular weight HAs induced transcription of *nos2* (B), decreased *arg1* transcription (C), and enhanced *tnf* transcription (D) and TNF-a (E). High molecular weight HAs stimulated minimal *il12b* (F) transcription and enhanced *il10* (G) transcription. Low molecular weight HAs enhanced transcription of *cd80* (H) but did not change *mrc1* (I) transcription in resting macrophages. Points represent mean plus or minus (±) standard deviation of three independent experiments. The symbols indicate significant difference (*p*-value less than 0.05) from the reference states, which are represented by corresponding colored dotted lines: resting macrophages (§; M), classically activated macrophages, stimulated with LPS (†; M(LPS)) or LPS and IFN-γ (‡; M(IFN-γ+LPS)), or alternative activated macrophages, stimulated with IL-4 (¶; M(IL-4)).

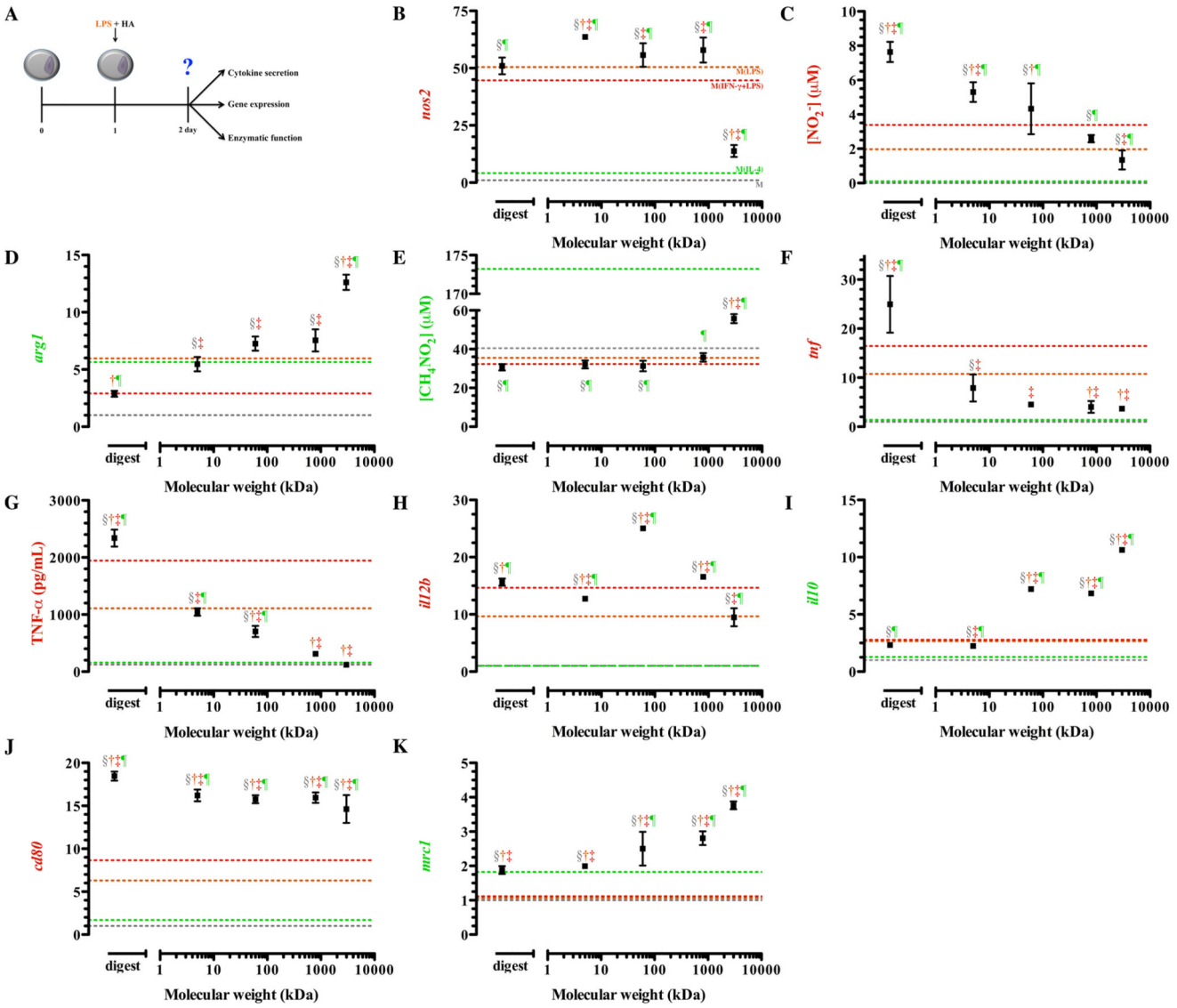

Figure 3. Gene expression, cytokine secretion, and enzymatic function of classically activated macrophages (M(LPS) treated simultaneously with HAs

After plating, macrophages were simultaneously treated with LPS and HAs. The schematic depicts the time course of the experiments (A). Expression of *nos2* (B) and nitrite production (C) was elevated in groups treated with low molecular weight HAs. Treatment of macrophages with LPS and high molecular weight HAs enhanced *arg1* (D) transcription and production of urea (E). Expression (F) and secretion (G) of tumor necrosis factor alpha was lowest in groups treated with high molecular weight HAs. Expression of *il12b* (H) and *cd80* (J) was elevated in groups treated with low molecular weight HAs. Transcription of il10 (I) and *mrc1* (K) steadily increased with increasing molecular weight. Points represent mean plus or minus (±) standard deviation of at least three independent experiments. The symbols indicate significant difference (*p*-value less than 0.05) from the reference states, which are represented by corresponding colored dotted lines: resting macrophages (§; M), classically

activated macrophages, stimulated with LPS (†; M(LPS)) or LPS and IFN-γ (‡; M(IFN-γ +LPS)), or alternative activated macrophages, stimulated with IL-4 (¶; M(IL-4)).

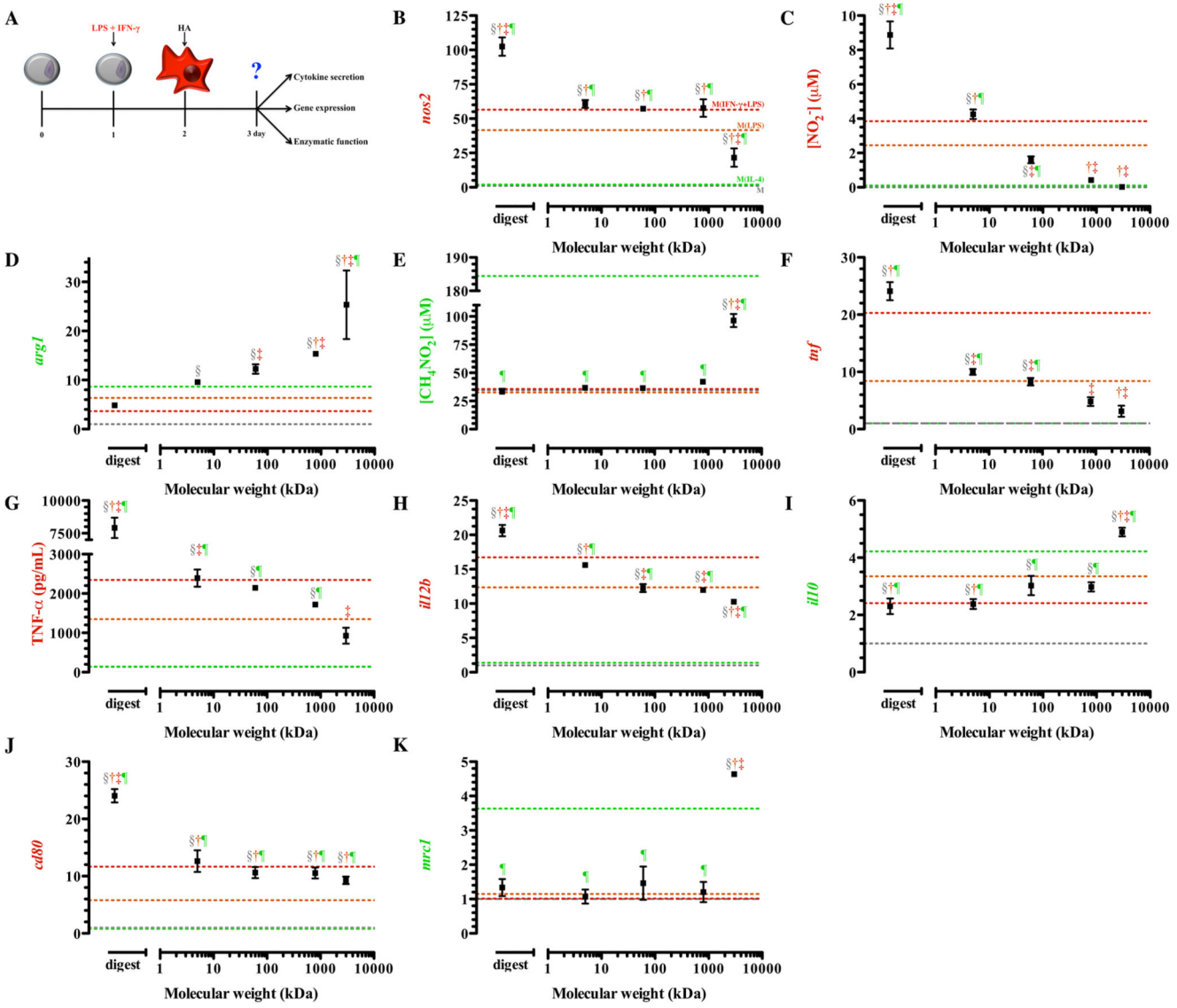

Figure 4. Gene expression, cytokine secretion, and enzymatic function of classically activated macrophages (M(IFN-γ+LPS)) treated with HAs

After plating, macrophages were classically activated with interferon-γ and LPS for 24 hours, after which media was replaced with media containing HAs for treatment (A). Expression of *nos2* (B) and production of nitrite (C) was elevated in groups treated with low molecular weight HAs, whereas *arg1* (D) and urea production (E) were elevated in groups treated with high molecular weight HAs. (B). Expression (F) and secretion (G) of tumor necrosis factor alpha was significantly reduced in groups treated with high molecular weight HAs. Expression of *il12b* (H) was elevated in groups treated with lower molecular weight HAs. High molecular weight HAs potentiated *il10* expression (I), but did not change *cd80* (J) or *mrc1* (K) expression. Points represent mean plus or minus (±) standard deviation of at least three independent experiments. The symbols indicate significant difference (*p*-value less than 0.05) from the reference states, which are represented by corresponding colored

dotted lines: resting macrophages (§; M), classically activated macrophages, stimulated with LPS (†; M(LPS)) or LPS and IFN-γ (‡; M(IFN-γ+LPS)), or alternative activated macrophages, stimulated with IL-4 (¶; M(IL-4)).

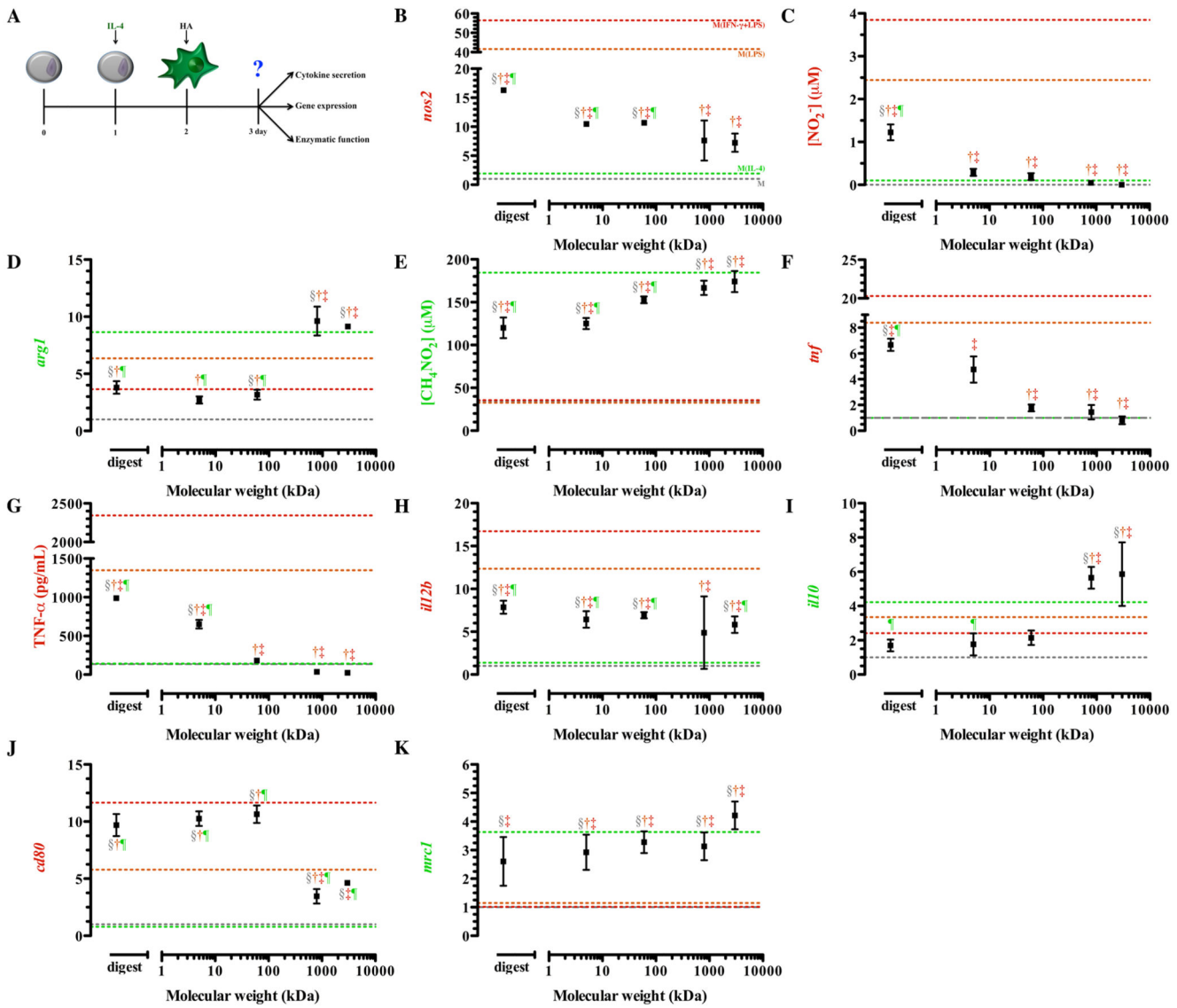

Figure 5. Gene expression, cytokine secretion, and enzymatic function of alternatively activated macrophages (M(IL-4)) treated with HAs

After plating, macrophages were alternatively activated with IL-4 for 24 hours, after which they were washed and media was replaced with media containing HAs for treatment (A). Expression of *nos2* was elevated in all treatment groups (B), but nitrite was only elevated in groups treated with the HA digest (C). Transcription of *arg1* was enhanced by high molecular weight HAs (D). Treatment with high molecular weight HAs maintained production of urea (E). Expression (F) and secretion (G) of tumor necrosis factor alpha was significantly elevated in groups treated with low molecular weight HAs. Elevated transcription of *il12b* (H), reduced transcription of *il10* (I), enhanced transcription of *cd80* (J), and diminished *mrc1* transcription (K) was detected in groups treated with low molecular weight HAs. Points represent mean plus or minus (±) standard deviation of at least three independent experiments. The symbols indicate significant difference (*p*-value less than 0.05) from the reference states, which are represented by corresponding colored

dotted lines: resting macrophages (§; M), classically activated macrophages, stimulated with LPS (†; M(LPS)) or LPS and IFN-γ (‡; M(IFN-γ+LPS)), or alternative activated macrophages, stimulated with IL-4 (¶; M(IL-4)).

Table 1

Genes and primers used for quantitative real-time PCR.

Gene	Protein	Acronym	Primers (5′→3′)	Accession number
gusb	glucuronidase, beta	GUSB	FW: GCAAGACATCGGGCTGGTGA REV: TGGCACTGGGAACCTGAAGT	NM_010368.1
nos2	nitric oxide synthase 2, inducible	iNOS	FW: AGCCCCGCTACTACTCCATC REV: GCCACTGACACTTCGCACAA	NM_010927.3
tnf	tumor necrosis factor	TNF-α	FW: AACTTCGGGGTGATCGGTCC REV: TGGTTTGTGAGTGTGAGGGTCT	NM_001278601.1
il12b	interleukin 12b	IL-12	FW: GAACTGGCGTTGGAAGCACG REV: GGCGGGTCTGGTTTGATGAT	NM_008352.2
il10	interleukin 10	IL-10	FW: CTCCTAGAGCTGCGGACTGC REV: GGCAACCCAAGTAACCCTTAAAGT	NM_010548.2
arg1	arginase	Arg1	FW: AAGACAGGGCTCCTTTCAGGAC REV: TCCCGTTGAGTTCCGAAGCA	NM_007482.3
cd80	cluster of differentiation-80	CD80	FW: GAAAAACCCCCAGAAGACCCTC REV: TGACAACGATGACGACGACTGT	NM_009855.2
mrc1	mannose receptor, C type 1	Mrc1	FW: CAGACAGGAGGACTGCGTGG REV: TGCCGTTTCCAGCCTTTCCG	NM_008625.2