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Aldosterone, Methylglyoxal, and Glycated Albumin Interaction with Macrophage Cells Affects Their Viability, Activation, and Differentiation

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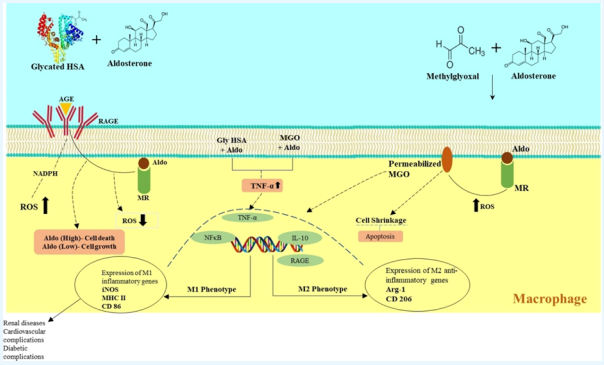
ABSTRACT: Background: The inflammatory response in diabetes is strongly correlated with increasing amounts of advanced glycation end products (AGEs), methylglyoxal (MGO), aldosterone (Aldo), and activation of macrophages. Aldo is known to be associated with increased pro-inflammatory responses in general, but its significance in inflammatory responses under glycated circumstances has yet to be understood. In the current work, the aim of our study was to study the macrophage immune response in the presence of AGEs, MGO, and Aldo to comprehend their combined impact on diabetes-associated complications. Methods and Results: The viability of macrophages upon treatment with glycated HSA (Gly-HSA) promoted cell growth as the concentration increased from 100 to 500 μg/mL, whereas MGO at a high concentration ($\geq$300 μM) significantly hampered cell growth. At lower concentrations (0.5−5 nM), Aldo strongly promoted cell growth, whereas at higher concentrations (50 nM), it was seen to inhibit growth when used for cell treatment for 24 h. Aldo had no effect on MGO-induced cell growth inhibition after 24 h of treatment. However, compared to MGO or Aldo treatment alone, an additional decrease in viability could be seen after 48 h of treatment with a combination of MGO and Aldo. Treatment with Aldo and MGO induced expression of TNF-α independently and when combined. However, when combined, Aldo and MGO significantly suppressed the expression of TGF-β. Aldo, Gly-HSA, and MGO strongly induced the transcription of NF-κB and RAGE mRNA and, as expected, also promoted the formation of reactive oxygen species. Also, by inducing iNOS and MHC-II and suppressing CD206 transcript expression, Gly-HSA strongly favored the differentiation of macrophages into M1 type (pro-inflammatory). On the other hand, the combination of Aldo and MGO strongly induced the expression of MHC-II, CD206, and ARG1 (M2 macrophage marker). These findings suggest that Gly-HSA, MGO, and Aldo differently influence macrophage survival, activation, and differentiation. Conclusions: Overall, this study gives an insight into the effects of glycated protein and MGO in the presence of Aldo on macrophage survival, activation, differentiation, and inflammatory response.



1. INTRODUCTION

Glycation is a ubiquitous nonenzymatic reaction between glucose and the amino group of various proteins, including the most abundant plasma protein−human serum albumin (HSA).[1] This process generates irreversible advanced glycation end products (AGEs). The formation of AGEs occurs in normal aging as well but to a greater extent in the case of hyperglycemia.[2] Increased levels of AGEs have been shown to cause high oxidative stress and inflammation.[3] Additionally, shown to be antigenic, AGEs can induce an immune response upon binding to and signaling through the receptor for advanced glycation end products (RAGE).[4] Hyperglycaemic conditions also induce increased production of a variety of toxic α-oxoaldehydes, methylglyoxal (MGO) being one of the critical oxoaldehydes. MGO is even more potent than glucose in glycating proteins and is also seen to modulate the immune response.[5,6]

Macrophages, classically activated, have a vital role in eliminating the microbes by phagocytosis and antigen presentation to T cells, which induces an inflammatory response by releasing cytokines.[7] However, in diabetes, macrophage accumulation and infiltration occur in different parts of the body like kidneys, lungs, adipose tissues, and

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coronary arteries and play a major role in local tissue injuries[8,9] causing a decline in its functions. It is known that macrophages take up circulating AGEs and induce the production of reactive oxygen species (ROS) and NF-κB signaling through activation of NADPH oxidase via RAGE.[10] Furthermore, AGE−RAGE interaction also induces the expression of TNF-α, a pro-inflammatory cytokine, and through positive feedback, promotes enhanced expression of RAGE and more of TNF-α. Overall, under hyperglycaemic conditions, macrophages, specifically the M1 type (pro-inflammatory), play a major part in oxidative stress and promote diabetes-associated complications.[11]

Aldosterone (Aldo) is a hormone produced by adrenal glands, and studies have shown that its effect on macrophages contributes to the development of diabetic complications.[12−14] In DN, Aldo contributes toward kidney damage by increasing oxidative stress and activating pro-inflammatory pathways via the renin−angiotensin aldosterone system (RAAS), which causes renal inflammation and fibrosis.[15,16] Our earlier studies have shown that the interaction between Aldo and glycated albumin (MGO-induced) adversely affects the renal cells through NRF-2 and Rac-1 signaling, which might lead to DN.[17,18] However, the impact of Aldo on the immunological behavior of macrophages when paired with glycated proteins and glycating agent MGO is still unknown. Hence, this study aims to investigate how various concentrations of Aldo, when coupled with glycated albumin or MGO, affect macrophage cell survival, activation (expression of inflammatory markers and oxidative burst), and differentiation into M1 (pro-inflammatory) or M2 type (anti-inflammatory). The study results provide important insights into the impact of Aldo on macrophages in the presence of glycated albumin and MGO.

2. MATERIALS AND METHODS

2.1. Reagents and Chemicals. MGO (Cat. No. M0252), HSA (Cat. No. A1653), and Aldo (Cat. No. A9477) were purchased from Sigma-Aldrich. MTT reagent, Dulbecco's modified Eagle medium (DMEM, Cat. No. AT007), Opti-MEM from Gibco (Cat. No. 31985062), fetal bovine serum (FBS, Cat. No. RM9955), antibiotic antimycotic solution (Cat. No. A002), a nitric oxide estimation kit (Cat. No. CCK061), and a reactive oxygen species assay kit (Cat. No. CCK078) were procured from HiMedia, India. ELISA kits for mouse IL-10 (Cat. No. 88−7105), TNF α (Cat. No. 88−7324), and human/mouse TGF-β (Cat. No. 88−8350) were purchased from Invitrogen. The iScript cDNA synthesis kit and SYBR Green were procured from BioRad.

2.2. In Vitro Glycation of HSA Using MGO and Evaluation of Glycation. For glycation, HSA (40 g/L) was incubated with 10 mM MGO in 1× phosphate-buffered saline (PBS) at 37 °C for 48 h under aseptic conditions.[19] MGO is a potent glycating agent, and at 10 mM concentration with 48 h incubation, optimum glycation of HSA can be achieved.[17,18] After the incubation, samples were dialyzed three times against 1× PBS for 48 h to eliminate unbound MGO and further filter sterilized using 0.22 μm syringe filters. All of the glycated samples were stored at −20 °C in sterile cryovials until further use. Using the Lowry assay, protein concentration was estimated.[20] Lastly, the status of glycation was determined by measuring pentosidine, tryptophan, and AGEs specific fluorescence at λ_{ex} 335 nm/λ_{em} 400 nm, λ_{ex} 370 nm/λ_{em} 440 nm, and λ_{ex} 280 nm/λ_{em} 360 nm, respectively.[21] Native HSA in PBS was used as a control.

2.3. Cell Culturing and Treatment. J774A.1 cells (passage no. 11), a murine macrophage cell line, procured from the National Centre for Cell Sciences (NCCS), Pune, India, were expanded and maintained in DMEM along with 10% FBS, L-glutamine, and sodium bicarbonate in the presence of an antibiotic solution containing amphotericin (2.5 μg/mL), gentamicin (50 μg/mL), penicillin (100 units/mL), and streptomycin (100 μg/mL). Initially, to determine the cytotoxicity of HSA, Gly-HSA, and MGO, the cells were incubated with varying concentrations of native HSA (100−500 μg/mL), Gly-HSA (100−500 μg/mL), and MGO (100−500 μM) for 24 h. At 80% confluency, additional treatments of the cultured cells were done in two different groups: (I) Gly-HSA (100 μg/mL) and Aldo (0.5, 5, 50 nM) and (II) MGO (100 μM) and Aldo (0.5, 5, 50 nM) for 24 or 48 h. Untreated cells (grown in regular complete media) were used as the control group. The plasma level of Aldo has been reported to be up to 0.41 nM in normal conditions, and in pathological conditions, the aldosterone levels go up to 8 nM (8322 pmol/L).[22] Therefore, we used normal levels to higher levels (0.5, 5, and 50 nM) of Aldo to evaluate its impact on macrophages. Similarly, normal endogenous levels of MGO range from 50 to 300 nM. However, hyperglycemic conditions cause a significant increase in MGO levels in diabetic individuals,[23] and to mimic similar conditions in our cell culture system, we used supraphysiological levels of MGO (10 mM) for glycation and 100−500 μM MGO for cellular treatments.[22]

2.4. Cell Survival and Growth Estimation. The effect of various treatment regimens on cell survival and growth was estimated by the MTT assay.[24] Cells seeded at 2 × 10^4 cells/well (in 200 μL of growth media) in a 96-well plate were kept under culture conditions overnight prior to treatment with various stimuli for 24 or 48 h. After the treatment period, collected culture supernatants were frozen (at −80 °C) until further use. Afterward, 100 μL of the MTT reagent (0.5 mg/mL) was added to the cell layer and incubated in a CO2 incubator for 4 h at 37 °C. Then, after removing the supernatant, formazan crystals were dissolved using 100 μL of DMSO. Using a microplate reader (Eon Biotek), absorbance was measured at 570 nm. Absorbance of wells containing only media was used as background. Percentage viability relative to untreated cells (control) was calculated using the following formula.

$$\%\text{viability} = [(\text{absorbance}_{(\text{test})} - \text{background}) / (\text{absorbance}_{(\text{control})} - \text{background})] \times 100$$

2.5. Estimation of Secretory Cytokines. Determination of secretory TNF-α, IL-10, and TGF-β in the culture supernatant (50 μL) of cells treated for 24 or 48 h was done using respective kits (mentioned above) following the manufacturer's manual.[24−27] The kit measures the amount of the target that gets bound between the matched antibody pairs. The ELISA plates were coated with a target-specific antibody for 24 h, after which the samples, standards, and controls that bind to the immobilized capture antibody were added. The addition of the detection antibody resulted in the formation of a sandwich, and a substrate solution reacted with the enzyme−antibody target combination to give a quantifiable signal. The signal intensity produced was directly proportional to the target complex present in the original sample. Three independent experiments were done in a set of triplicates,

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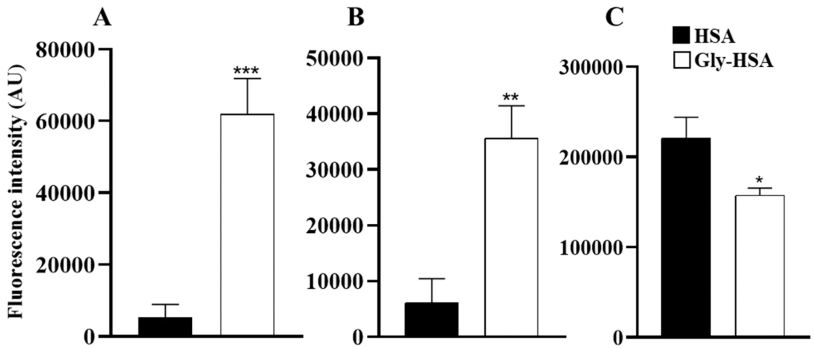

Figure 1. Glycation of HSA showed a change in its conformation. (A) Pentosidine-specific fluorescence was measured at λ_{ex}(335 nm)/λ_{em}(400 nm). (B) AGE-specific fluorescence was measured at λ_{ex}(370 nm)/λ_{em}(440 nm). (C) Tryptophan-specific fluorescence was measured at λ_{ex}(280 nm)/λ_{em}(360 nm). Data represents the results of one of the three independent experiments. Bars indicate the mean ± SD of fluorescence values obtained from one of the assays done in triplicates. *p < 0.05, **p < 0.01, and ***p < 0.001 were considered significantly different compared to HSA. AU: arbitrary units; Gly-HSA: glycated HSA.

and the concentration (pg/mL) of each cytokine in various cell culture supernatants was calculated from the standard graphs.

2.6. Oxidative Stress Marker Analysis. *2.6.1. Estimation of Reactive Oxygen Species.* For the estimation of reactive oxygen species, 1×10^4 cells/well were grown in growth media in a 96-well black plate and were given treatment with an appropriate stimulus for 24 h. The ROS levels were measured using the kit mentioned above. The ROS detection kit included an oxidative stress detection reagent. The level of ROS was measured using DCFDA dye. This fluorogenic dye diffused inside the cells and was deacetylated to a non-fluorescent compound by cellular esterases. This nonfluorescent dye was oxidized in the presence of ROS (hydroxyl, peroxyl, and other reactive oxygen species). It can be detected using a multimode microplate reader (BioTek Synergy H1) at an excitation wavelength of 488 nm and an emission wavelength of 528 nm. Results were reported in arbitrary units (AU). *tert*-Butyl hydrogen peroxide was used as an active oxygen-positive control in this assay.[28] Untreated cells were used as a negative control.

2.6.2. Estimation of Reactive Nitrogen Species. The presence of reactive nitrogen species in the culture supernatants of macrophages treated for 24 h was determined using a commercially available kit.[29] The kit used the enzyme nitrate reductase to convert nitrate (NO_3^-) to nitrite (NO_2^-). The nitrite formed reacted with the Griess reagent, which produced a blue-colored azo dye product that can be quantified using a spectrophotometer between 580 and 630 nm. The observed absorbance readings were directly proportional to the total nitric oxide concentration present in the sample. The results were calculated using a standard plot obtained for known concentrations (5 to 320 μM) of sodium nitrite. Untreated cells were used as a negative control.

2.7. Measurement of Cellular Antioxidant Markers. *2.7.1. Reduced Glutathione (GSH).* Cultured macrophage (24 h) cell lysates (50 μL) were mixed with 500 μL of 5% EDTA buffer (5% trichloroacetic acid in 1 mM EDTA). The solution was centrifuged for 10 min at 3000 rpm, the collected supernatant (100 μL) was added to 0.01% DTNB 5,5′-dithiobis(2-nitrobenzoic acid) followed by 10 min incubation at room temperature, and the absorbance was measured at 412 nm.[30] GSH content was calculated using the following formula.

$$[(\text{test OD} - \text{background OD})/\text{molar extinction coefficient}]$$

$$\text{molar extinction coefficient} = 13.6\ \text{mM}^{-1}\ \text{cm}^{-1}$$

2.7.2. Superoxide Dismutase (SOD). Cultured macrophage cell lysate (50 μL) was added to a reaction mixture containing Na_2CO_3 (50 mM), Triton X (10%), NBT (16 mM), and hydroxylamine hydrochloride (0.1 M) ref. The enzyme activity was measured by taking absorbance at 560 nm for a 5-min interval of 1 min.[31]

2.8. Analysis of Gene Expression by RT-qPCR. RNA isolation from cells treated for 24 h was done using TRIZOL.[32] The RNA was stored at −80 °C after quantification using a multimode microplate reader (BioTek Synergy H1) until further use. cDNA synthesis was done using the iScript cDNA synthesis kit (BioRad) according to the manual provided by the manufacturer. Reaction parameters: priming for 5 min at 25 °C, cDNA synthesis for 20 min at 46 °C, reverse transcriptase inactivation at 95 °C for 1 min, and hold at 4 °C for infinity. Quantitative RT-PCR was performed after mixing the SYBR green PCR master mix (BioRad) with cDNA and gene-specific primers for NF-κB, RAGE, iNOS, MHC-II, CD86, CD206, ARG1, and GAPDH (Supporting Table 1) and running the reaction in CFX 96 RT-PCR systems (BioRad, California).[33] The results were expressed as a fold increase relative to GAPDH.

2.9. Statistical Analysis. All of the bar graphs are expressed as mean ± standard error of the mean (SEM). Statistical significance between different groups was calculated using ANOVA. The values *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 were considered significant to control. The values $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001, and $^{\#\#\#\#}p$ < 0.0001 were considered significant as compared to Gly-HSA or MGO.

3. RESULTS

3.1. Glycation of HSA Showed a Change in Its Conformation. In order to confirm the formation of glycated HSA, the AGE-specific fluorescence was recorded after the process of glycation. Additionally, pentosidine-specific fluorescence was also measured, as it forms a fluorescent cross-link between the protein's arginine and lysine residues and is therefore used as a biomarker for AGEs. After incubation with MGO, HSA showed significantly higher AGEs and pentosidine-specific fluorescence in comparison to native HSA,

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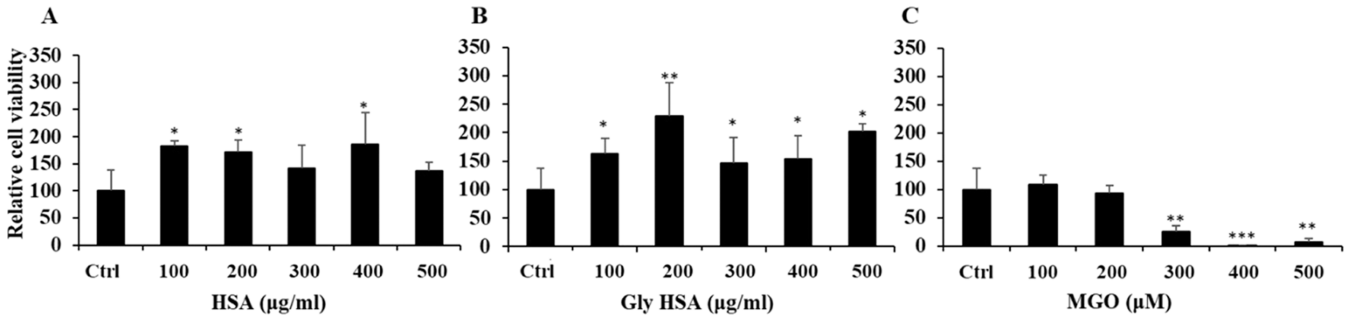

Figure 2. Higher concentrations of MGO causes cell cytotoxicity. Cultured J774A.1 macrophages were treated for 24 h with (A) native HSA (100−500 μg/mL) or (B) Gly-HSA (100−500 μg/mL) or (C) MGO (100−500 μM) and then subjected to the MTT assay. Graphs show % cell viability relative to untreated cells. Data represent the results of one of the three independent experiments. Bars indicate the mean ± SD of the values obtained from the MTT assay done in triplicates. *p < 0.05, **p < 0.01, and ***p < 0.001 were considered significantly different compared to the control (untreated) group.

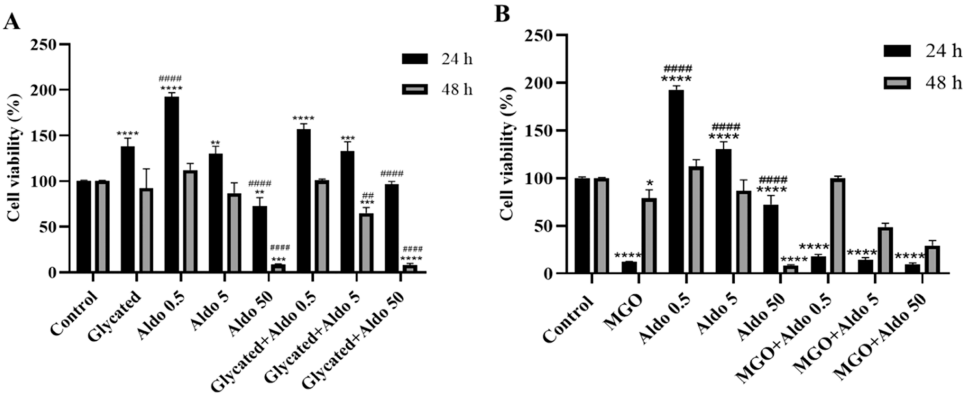

Figure 3. MGO with Aldo affects the cell viability of macrophages. Cultured J774A.1 macrophages were treated for 24 and 48 h with 100 μg/mL Gly-HSA (A) or 250 μM MGO (B) and/or with Aldo (0.5, 5, 50 nM) (A, B) and were then subjected to the MTT assay. The bar graph shows % cell viability compared to untreated cells. The data represent the results of one of the three independent experiments. Bar indicates mean ± SD. The experiments were carried out in triplicates. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 were considered significantly different from control. $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, and $^{\#\#\#\#}p$ < 0.0001 were considered significantly different as compared to Gly-HSA or MGO.

confirming the formation of glycated HSA (Figure 1A,B).[34] Conformational changes leading to the alteration in the surroundings of tryptophan residues in the protein are reflected by lowering the tryptophan-specific fluorescence intensity.[35] As shown in Figure 1C, the intensity of tryptophan fluorescence for glycated HSA was significantly less than that for HSA, indicating a conformational change in HSA upon glycation.

3.2. Higher Concentrations of MGO Causes Cell Cytotoxicity. Previous studies have shown that AGEs as well as MGO do influence cell viability.[36] Hence, first, the impact of various concentrations of these two molecules on cell viability was assessed by treating the cultured J774A.1 cells with different concentrations of native HSA, Gly-HSA, and MGO for 24 h. The cell viability did not alter with increasing concentrations of native and glycated HSA (Figure 2A,B). However, MGO above a concentration of 200 μM was seen to adversely affect the cell viability (Figure 2C). Since, at 300 μM, MGO reduced the viability of macrophages from 100% (for 200 μM MGO) to approximately 25%, a mean concentration of 250 μM was decided to be used for further experiments. Therefore, in terms of cell survival, no significant difference between treatment with 100 and 500 μg/mL native HSA or glycated HSA was observed. The lowest (100 μg/mL) concentration was chosen to be used for the rest of the experiments.

3.3. MGO with Aldo Affects the Viability of Macrophages. In diabetic conditions, along with AGEs, Aldo levels also increase.[37] Hence, it is important to study the combined effect of AGEs and Aldo under conditions mimicking diabetic environments on the viability of immune cells to see whether Aldo has any additional effect on the viability of macrophages. We observed that increasing concentration of Aldo alone and when combined with Gly-HSA induced severe cell death, especially after 48 h of treatment, indicating that Aldo does alter the macrophage viability at a higher concentration (Figure 3A). Besides AGEs and Aldo, elevated production of MGO during hyperglycemic conditions is known to induce oxidative stress.[38] However, the effect of MGO on the survival of macrophages in the presence of varying concentrations of Aldo is not known. Hence, we further analyzed the viability of MGO-treated J774A.1 cells in the presence of various concentrations of Aldo. In contrast to Aldo-treated cells, where reduced viability is observed after 48 h compared to 24 h, MGO-treated cells showed much less viability at 24 h compared to 48 h (Figure 3B). In general, MGO-treated cells showed a severely reduced viability after 24 h. Compared to treatment with MGO or Aldo alone, combined treatment with MGO and Aldo further reduced the cell viability. For the combination treatment also, with increasing concentration of Aldo, a decreasing trend in the cell viability was observed (Figure 3B). We also studied the cellular morphology after 24

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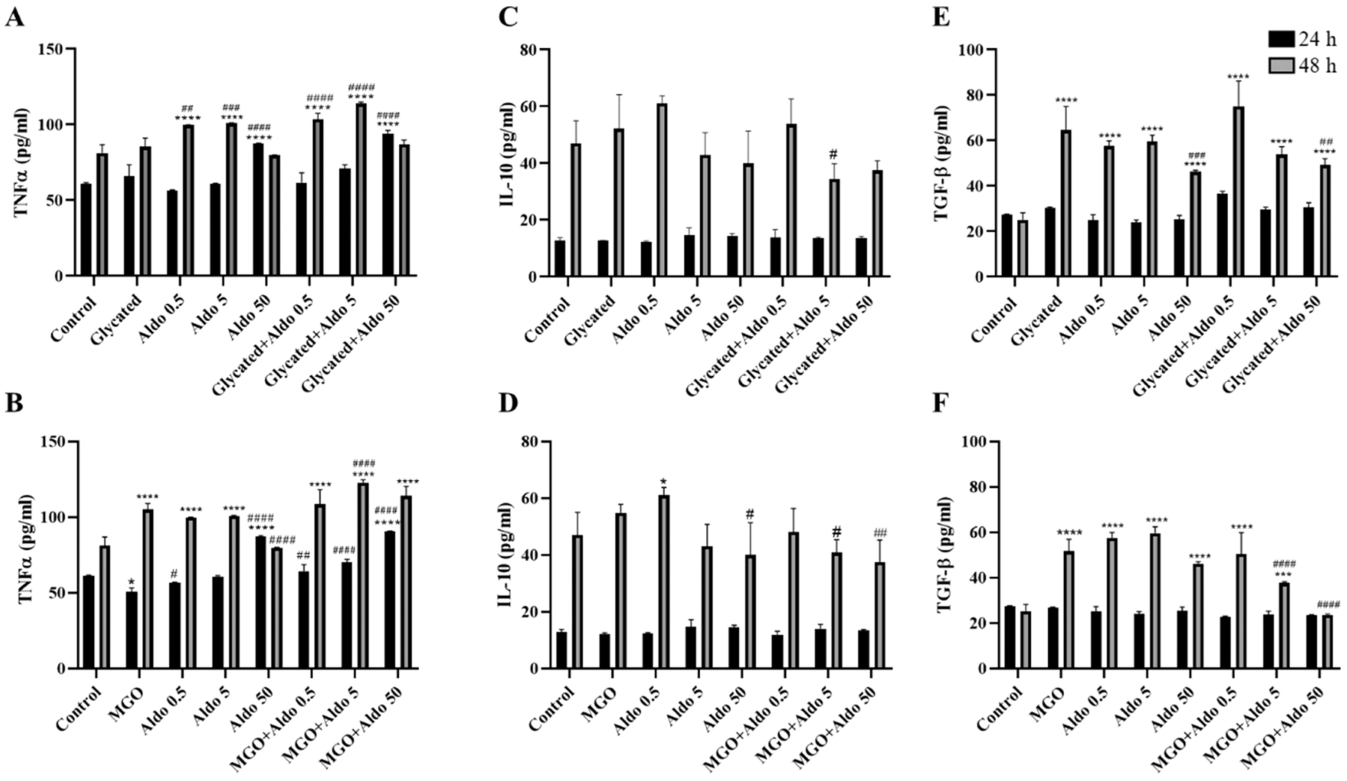

Figure 4. MGO as well as Aldo induces TNF-α production by cultured macrophages. Supernatants of cultured J774A.1 macrophages treated with Gly-HSA (100 μg/mL) or MGO (250 μM) in combination with Aldo (0.5, 5, and 50 nM) for 24 or 48 h were subjected to TNF-α ELISA (A, B), IL-10 ELISA (C, D), and TGF-β ELISA (E, F). Values on the y-axis represent the concentration (pg/mL) of the indicated cytokine. The data represent the results of one of the three independent experiments. Each bar graph represents the mean ± SD of concentration values obtained from ELISA done in triplicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ were considered significantly different as compared to control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ were considered significantly different in comparison to Gly-HSA or MGO.

h of treatment with Gly-HSA, MGO, and Aldo, and the results showed shrinkage in Aldo- and MGO-treated cells; these morphological changes can be correlated to the fact that MGO is associated with the increased oxidative stress, which can damage several cellular components including proteins, lipids, and DNA, and this damage eventually causes disturbances in the cellular structure as well as function (Supporting Figure S1).

3.4. Aldo Induces the Expression of TNF-α and TGF-β by Macrophages. To assess whether Gly-HSA and MGO treatment in the presence of Aldo induces a macrophage-mediated inflammatory response, the supernatant of cultured cells treated with Aldo in the presence and absence of Gly-HSA or MGO was assessed for the levels of secretory TNF-α (pro-inflammatory) or IL-10 and TGF-β (anti-inflammatory) cytokines.

It was observed that the level of TNF-α in the culture supernatant of the cells treated for 24 h with 50 nM of Aldo increased by 1.4-fold in comparison to the control. Interestingly, cells treated with 0.5 or 5 nM Aldo showed a significant increase in the levels of secretory TNF-α only after 48 h of treatment. Furthermore, in comparison to Gly-HSA or 5 nM Aldo alone, TNF-α levels in the cultures treated with the combination of both were higher by 33 and 13%, respectively (Figure 4A). Treatment with MGO also enhanced TNF-α levels by 1.3-fold in comparison to the control, and its combination with Aldo increased the cytokine concentration by 17% in comparison to MGO or Aldo alone (Figure 4B).

After 24 h of treatment, no difference in the levels of IL-10 was observed between the treated cells and the control. However, supernatants of the control as well as the treated cultures showed higher levels of IL-10 in comparison to those of the same collected at 24 h. In comparison to treatment with Gly-HSA or MGO alone, their combination with higher levels of Aldo showed decreased production of IL-10 (Figure 4C,D).

In comparison to the control, barring the culture treated with the combination of MGO and 50 nM Aldo, all of the treated cultures showed significantly increased levels of TGF-β after 48 h of treatment. Interestingly, when cells were treated with Gly-HSA or MGO in combination with 50 nM Aldo, the secretion of TGF-β significantly reduced in comparison to treatment with Gly-HSA or MGO alone (Figure 4E,F). These data suggest that high concentrations of Aldo in the presence of Glycated HSA or MGO induce macrophages to secrete TNF-α, whereas they suppress the production of IL-10 and TGF-β.

3.5. Gene Expression Analysis of NF-κB and RAGE. Further, a gene expression analysis of NF-κB and RAGE by real-time PCR was done. In line with the ELISA results, which showed an increased expression of TNF-α upon treatment with Gly-HSA or MGO with Aldo, the expression levels of NF-κB, a transcription factor regulating the expression of genes related to inflammatory response, were also observed to increase significantly as compared to the control (Figure 5A,B). Glycated HSA and MGO showed higher expression levels of NF-κB as compared to the control, and Aldo did not show any increase in the expression levels when combined with

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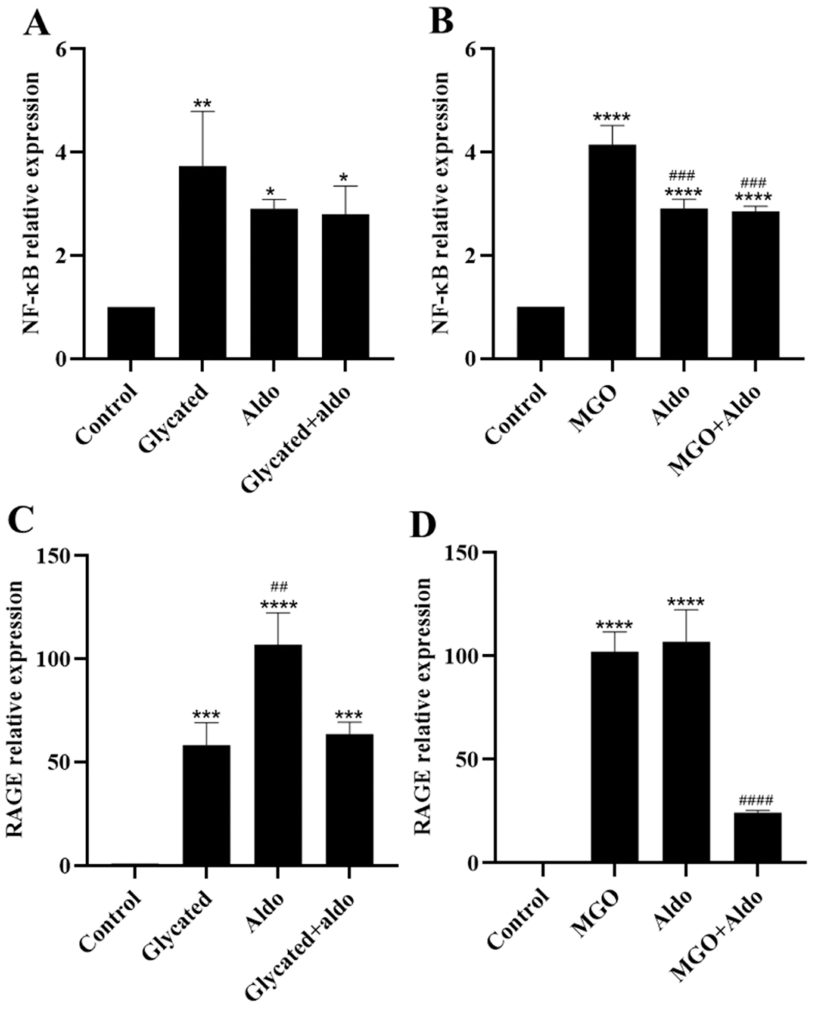

Figure 5. Aldo strongly induces RAGE expression at the transcription levels. The expression levels of genes for NF-κB (A, B) and RAGE (C, D) after 24 h treatment of cultured J774A.1 cells with Gly-HSA (100 μg/mL) or MGO (250 μM) in combination with Aldo (50 nM) were determined by real-time qPCR. The graphs represent relative gene expression in comparison to the control. Each bar graph represents the mean ± SD of one of the three independent experiments carried out in triplicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ were considered significantly different as compared to control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ were considered significantly different in comparison to Gly-HSA or MGO.

them (Figure 5A,B). Additionally, treatment with Aldo and Gly-HSA also upregulated the expression of RAGE, though 50 nM Aldo alone induced considerably higher expression of RAGE than Gly-HSA or the combination treatments (Figure 5C). Furthermore, in comparison to Aldo or MGO treatment alone, treatment with the combination of the two decreased the expression levels of RAGE from 100- to 20-fold (Figure 5D). These results indicated that Aldo has no additional impact on NF-κB expression in glycated macrophages.

3.6. Aldo Generates Higher Levels of ROS in MGO-Treated Murine J774A.1 Macrophages. Generation of reactive oxygen and nitrogen species (ROS and RNS) is one of the hallmarks of the inflammatory response by macrophages. Assessment of ROS levels after 24 h of treatment showed that though Gly-HSA and MGO significantly increased the levels of ROS by approximately 2.5- and 5-fold, respectively, Aldo did not have similar effects when combined with Gly-HSA, but in combination with MGO, high levels of ROS were observed (Figure 6A,B). Compared to the control, no significant change in the levels of RNS was observed among the differently treated cells (Figure 6C,D).

3.7. Aldo and Gly-HSA Significantly Reduce the Antioxidant Enzyme Content in the Macrophages. Antioxidants, such as GSH, protect tissues from injuries due to oxidative stress caused by reactive oxygen and nitrogen radicals generated during various metabolic processes.[39] Since cells treated with glycated HSA and MGO showed high levels of ROS, it was necessary to see whether the treated macrophages produced any antioxidant defense marker to help resist oxidative stress. Gly-HSA and MGO alone or in combination with Aldo led to a significant decrease in the levels of GSH (Figure 7A,B). In the presence of Aldo, MGO further reduced the levels of GSH in comparison to MGO treatment alone (Figure 7A,B).

3.8. Gly-HSA But Not Aldo Strongly Induced the Expression of iNOS and MHC-II. To determine whether the treated macrophages were activated classically (M1 macrophages) or alternatively (M2 macrophages), a gene expression analysis of macrophage activation markers was assessed using qPCR. It is known that iNOS, MHC-II, and CD86 are markers for M1 macrophages, whereas ARG1 and CD206 are markers for M2 macrophages.[40] To determine the effect of these

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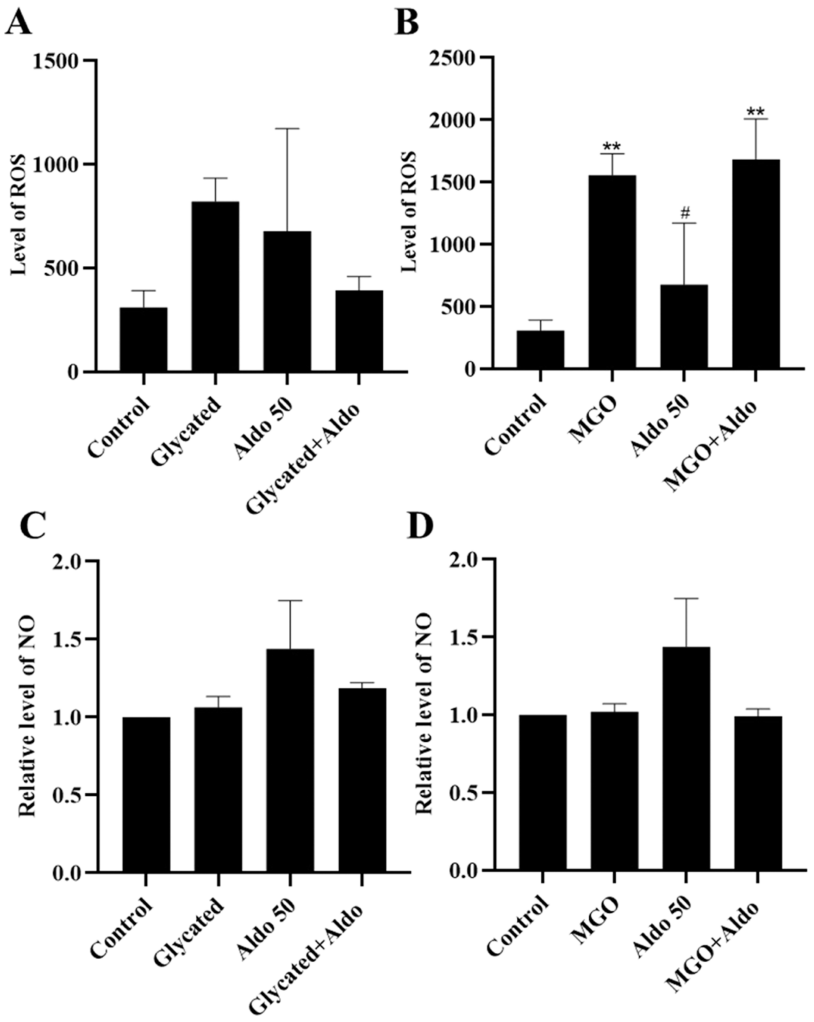

Figure 6. MGO alone and in combination with Aldo increases ROS production in macrophages. Relative levels of ROS (A, B) and NO (C, D) generation by J774A.1 cells stimulated with Gly-HSA (100 μg/mL) or MGO (250 μM) in combination with Aldo (50 nM) for 24 h. The graphs represent relative ROS or NO levels with respect to the control. Each bar graph represents the mean ± SD of one of the three independent experiments carried out in triplicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ were considered significantly different as compared to the control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#}p < 0.0001$ were considered significantly different in comparison to Gly-HSA or MGO.

markers on treated macrophages, mRNA levels of iNOS, MHC-II, CD86, CD206, and ARG1 were assessed by qPCR. As compared with MGO, Gly-HSA-treated cells showed significantly higher iNOS and MHC-II expression (Figure 8A−D). Like the ROS generation (Figure 6A,B), Aldo did not have any significant impact on the induction of iNOS as well (Figure 8A,B). However, MHC-II expression levels were strongly enhanced under the combined effect of Aldo and MGO as compared to Aldo alone (Figure 8C,D). The costimulatory molecule CD86, which is constitutively expressed by macrophages and plays an important role in helper T cell activation, showed an increase in expression under the combined effect of Gly-HSA and Aldo in comparison to Gly-HSA or MGO or Aldo treatment alone (Figure 8E,F). CD206 and ARG1 are expressed on alternatively activated macrophages (M2 type) and have an anti-inflammatory response to promote wound healing and tissue repair.[7] MGO when combined with Aldo induced significantly higher expression levels of CD206 and ARG1 as compared to single treatments (Figure 8G−J).

4. DISCUSSION

It has been extensively reported that the increased development of AGEs causes diabetes-related complications under hyperglycemic conditions.[41] On the other hand, Aldo is also recognized for its vital role in kidney fibrosis, inflammation, and, to a certain extent, triggering an immunological response in diabetic conditions.[42] However, the adjacent participation of Aldo and AGEs in aggravating vascular complications is not known. Macrophages are known to play a key role in tissue damage and repair through the M1 pro-inflammatory and M2 anti-inflammatory phenotypes, respectively, though these two distinctly polarized states of macrophages appear to be an oversimplification because these cells display remarkable plasticity and represent a wide spectrum of immunophenotypes and overlapping properties.[43] Increased differentiation of macrophages into the M1 phenotype and its accumulation is closely linked to the development of renal impairment in DN.[44] However, it is still debatable whether macrophage recruitment is the cause or consequence of chronic kidney diseases. To ascertain their combined function in developing diabetes-related complications, the current study set out to

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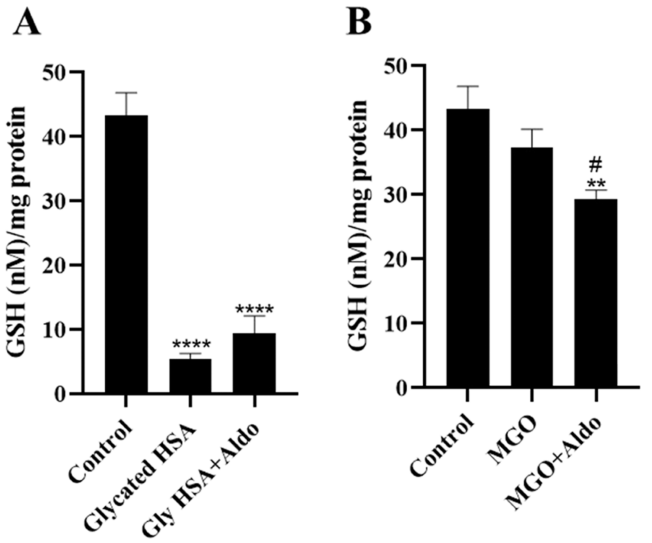

Figure 7. Glycated HSA and MGO both weaken the cellular antioxidant defense system in the presence of Aldo. The concentration of GSH (nM/mg protein) in the lysate of J774A.1 cells stimulated with (A) Gly-HSA (100 μg/mL) or (B) MGO (250 μM) in combination with Aldo (50 nM) for 24 h. Each bar graph represents the mean ± SD of one of the three independent experiments carried out in triplicates. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 were considered significantly different as compared to the control. #p < 0.05, ##p < 0.01, ###p < 0.001, and ####p < 0.0001 were considered significantly different in comparison to Gly-HSA or MGO.

investigate the mutual consequences generated by glycation and excessive Aldo levels on macrophages.

Our cell viability results showed that Aldo impacts macrophage viability in a concentration-dependent manner, promoting and inhibiting cell growth at 0.5−5 and 50 nM concentrations, respectively. MGO is a small organic compound that can permeate the cell membrane and is known to cause cell damage by reacting with cellular macromolecules. In our experiments, when added to the cell culture for 24 h, MGO slowed the growth of macrophages.

Glycation and AGEs enhance M1 macrophage polarization and upregulation of pro-inflammatory cytokines via the MAPK pathway.[45] RAGE and the mineralocorticoid receptor (MR) are expressed by macrophages, and studies have shown that AGEs and Aldo alone can trigger macrophages to generate TNF-α.[46] Our findings show that though at a lower concentration, Aldo weakly induces the production of TNF-α, at an extremely high concentration (50 nM), it strongly promotes the early production of this cytokine by macrophages. Glycating agent MGO seemed to induce more TNF-α production than Gly-HSA. Therefore, it is arguable that macrophages exposed to direct glycation produce a more inflammatory milieu than glycated proteins.

IL-10 is a very effective anti-inflammatory cytokine, as it negatively regulates the pro-inflammatory cytokines, interferes with immune functions, and prevents immune response.[47] Our study showed no significant change in the secretion of IL-10 upon 24 h of treatment. However, compared to 24 h, all of the 48 h cultures showed a significant increase in the production of IL-10. The murine macrophage J774A.1 has been reported to secrete IL-10 in the presence of apoptotic cells and also when cultured on various plastic surfaces.[48,49] This can be the reason for the enhanced production of this cytokine even by the control. Interestingly, a decrease in IL-10 production by

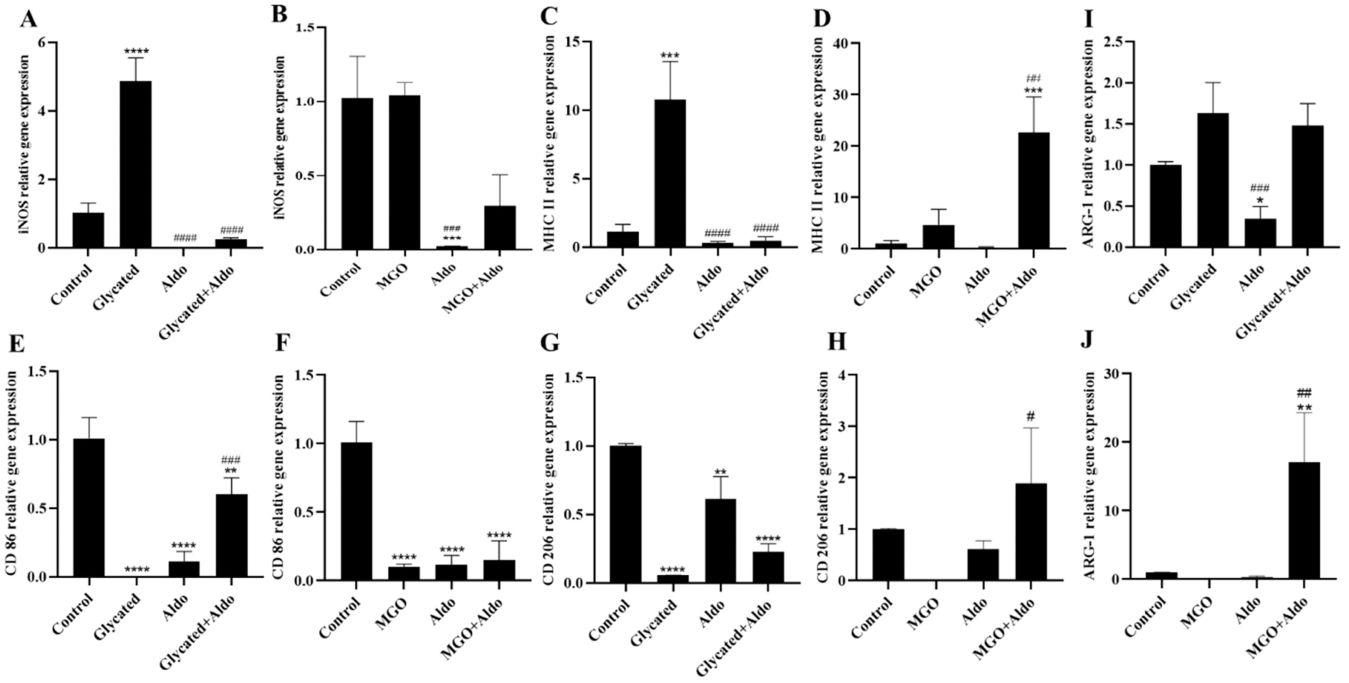

Figure 8. Glycated HSA enhances the expression of M1 macrophage inflammatory markers. Expression levels of transcripts for iNOS (A, B), MHC-II (C, D), CD86 (E, F), CD206 (G, H), and ARG1 (I, J) after 24 h treatment of cultured J774A.1 cells with Gly-HSA (100 μg/mL) or MGO (250 μM) in combination with Aldo (50 nM) were determined by real-time qPCR. The graphs represent relative gene expression in comparison to the control. Each bar graph represents the mean ± SD of one of the three independent experiments carried out in triplicates. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 were considered significantly different as compared to control. #p < 0.05, ##p < 0.01, ###p < 0.001, and ####p < 0.0001 were considered significantly different in comparison to Gly-HSA or MGO.

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cultures treated with Gly-HSA or MGO in the presence of higher concentrations of Aldo indicates the skewing of the macrophages toward the inflammatory response by these stimuli.

TGF-β is another important anti-inflammatory cytokine known to promote wound healing, angiogenesis, and immunosuppression. It is the primary factor that carries out fibrosis in most forms of chronic kidney diseases.[42] Assessment of TGF-β showed that in both treatment groups, TGF-β secretion significantly increased after 48 h of treatment as compared to the control. Therefore, this indicates that an anti-inflammatory response is also initiated by the stimulation of macrophages with AGEs and MGO after 48 h of treatment. Interestingly, with the increasing concentration of Aldo, a downward trend in the levels of TGF-β was observed, which showed how high levels of Aldo reduce the anti-inflammatory activities of macrophages. Also, a combined treatment with MGO and 50 nM Aldo significantly decreased TGF-β levels. Hence, we conclude that increasing concentrations of Aldo accelerate the production of TNF-α and downregulate TGF-β, whereas the presence of MGO together with Aldo further adds to TGF-β downregulation.

Our results showed a subtle modulation in the expression levels of TGF-β and TNF-α in macrophages following aldosterone treatment. While the differences in expression were modest, their significance should not be overlooked, as even minor alterations in these key immune regulatory factors can exert profound effects on cellular responses. These changes observed could stem from various factors including the dosage and duration of Aldo exposure, potential cellular heterogeneity within the macrophage population, or specific experimental conditions. It is important to acknowledge the limitations of our study, such as the need for further exploration to elucidate the precise mechanisms underlying these observations. The context of Aldo's role in immune modulation and its potential interactions with other signaling pathways could be considered.

RAGE is expressed on the membrane of many immune cells including the macrophages, and as a consequence, it has major involvement in inflammation.[50] RAGE activation by its ligand leads to NF-κB activation and further induces a pro-inflammatory response.[51] In our study, Gly-HSA-, MGO-, and Aldo-treated cells showed increased gene expression for NF-κB as well as RAGE, indicating that the increased inflammation due to glycation takes place through the NF-κB pathway under the combined influence of AGEs and high Aldo levels.

An increase in the release of pro-inflammatory cytokines is often associated with increased reactive oxygen species (ROS) generation. The relationship between the AGE−RAGE interaction and increased ROS generation in macrophages is already well established. Our aim was to determine how Aldo affects the production of ROS when combined with AGEs. Surprisingly, the addition of Aldo led to a significant reduction in Gly-HSA-induced ROS generation. MGO induced high levels of ROS generation, and in contrast to Gly-HSA, ROS levels remained high even after the addition of Aldo. These results suggest that Aldo might be a weak inducer of ROS at the concentration (50 nM) tested by us and can even suppress its production induced by Gly-HSA but not by MGO. Thus, our study showed that oxidative stress induced by glycating agent MGO appears to be more intense in nature. The antioxidant defense system was also seen to be hampered, as MGO in the presence of Aldo showed reduced levels of GSH. This indicates a difference between Gly-HSA- and MGO-mediated induction of oxidative stress. It is possible that MGO pushes the differentiation of macrophages into the M1 direction and drives the inflammatory response forward.

AGEs are known to enhance the secretion of pro-inflammatory cytokines, upregulate the expression of M1 macrophage markers, and downregulate the expression of M2 macrophage markers.[45] Our data confirmed the effect of AGEs in the form of Gly-HSA that led to the upregulation of M1 macrophage activation markers in J774A.1 murine macrophages. The Gly-HSA treatment significantly upregulated the expression of iNOS and MHC-II, which are associated with M1 macrophages.[52] Aldosterone has also been shown to induce inflammatory M1 macrophage subtype via the MR.[53] However, in our study, compared to control, Aldo did not induce higher levels of MHC-II. It is possible that at very high (50 nM) concentrations (used in this study), Aldo suppresses the expression of MHC-II. Interestingly, when combined with MGO, Aldo significantly enhanced MHC-II expression.[54] A well-defined and reliable marker to differentiate between the two activation states of macrophages is differential NO metabolism. Classically activated macrophages (M1) express high levels of iNOS, which participates in the synthesis of NO from arginine. On the other hand, alternatively activated macrophages (M2) have increased levels of ARG1, which competes for the arginine substrate and reduces NO generation.[55] In our work, we observed that only AGE treatment strongly induced iNOS expression. However, the culture supernatants of variously treated cells did not show significantly higher levels of NO than the control. Consistent with the NO levels in culture supernatants, Aldo- and MGO-treated cells did not show any increase in the levels of iNOS transcripts. Interestingly, MGO, when combined with Aldo, showed increased levels of CD206 and ARG1 expression, indicating that at high concentrations, Aldo together with MGO drives the differentiation of macrophages toward the M2 type. Intriguingly, Aldo and MGO alone did not induce the expression of M1- or M2-specific markers, suggesting that these two molecules retain the macrophages in some other undefined state at the concentrations used in this work. We could have provided a more comprehensive view if we had used already polarized macrophages to study the effects of Aldo and glycation on them.

Overall, by exposing the macrophages to Gly-HSA or MGO in the presence of physiological and extremely high concentrations of Aldo, the current study has demonstrated the ways in which Aldo can influence the survival and activation of macrophages in hyperglycemic conditions. Several investigations have demonstrated that glycated albumin causes inflammation by driving the polarization of macrophages toward the M1 subtype, which is associated with the production of pro-inflammatory cytokines and ROS generation.[56] Extending the previous studies, the current work shows that together with glycated albumin and MGO, though elevated levels of Aldo adversely affect macrophage survival, it not only accelerates the production of TNF- α but also suppresses the production of anti-inflammatory cytokines. Thus, by strongly inducing the secretion of pro-inflammatory cytokine and suppressing the regulatory cytokines, Aldo further aggravates the inflammatory responses by macrophages, which could be of a lesser degree in the presence of AGEs or MGO alone. All three stimuli used in this study increased the expression of NF-κB and RAGE. This suggests that like AGEs

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and MGO, Aldo also induces the production of inflammatory mediators TNF-α and ROS via the NF-κB pathway. Surprisingly, when the expression of M1- or M2-specific markers was examined, macrophages treated with various stimuli did not show a clear alignment with either of these two polarized states, indicating that each stimulus had its own effect and may have led to macrophage polarization to some undefined intermediate immunophenotypic stage.

As an in vitro study, the current work has limitations in accurately predicting the macrophage behavior in the presence of various concentrations of Aldo, AGEs, and MGO in vivo. Additionally, depending on the stimuli and current state, macrophages can exhibit a wide spectrum of overlapping immunophenotypes, and it is difficult to classify them in one or the other polarized type.[43] Because no defined population of prepolarized macrophages was used in this study, it is not possible to determine the effect of the stimuli used on the change in the properties of M1 or M2 macrophages. However, by utilizing this study as a foundation, we can further investigate the impact of available drugs and existing therapies against the combined action of these three components. New insights into these interactions can be obtained through future studies on macrophages isolated from diabetic murine models or human diabetic patients. Research in this direction can lead to the development of effective therapy regimens that would reduce the occurrence of several diabetes-associated complications.

5. CONCLUSIONS

Our study revealed that high levels of Aldo under glycated conditions certainly had an additional effect on the macrophage inflammatory responses, as it enhanced the production of pro-inflammatory cytokine TNF-α, increased the MHC-II expression, and decreased the expression of anti-inflammatory cytokines as well as hampered the cellular antioxidant defense system, as it reduced GSH levels. These data thus highlight the potential role of Aldo along with glycation in affecting macrophage behavior. Therefore, treatment strategies that not only target blood glucose levels but also maintain or regulate a normal level of aldosterone might be useful in maintaining a proper M1/M2 macrophage ratio and avoiding inflammation or vascular dysfunction in diabetic patients.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09420.

Primer sequences used for RT-qPCR; and MGO- and Aldo-induced morphological changes in the J774A.1 cells (PDF)

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Author Contributions

B.R. and R.T. conceived the study, designed the research, and edited the manuscript. S.Z. performed the experiments, did data analysis, and drafted the manuscript. A.M., N.B., and P.K. contributed to the experiments and manuscript reading and editing. M.S.K. and I.A. contributed to critical discussion, manuscript reading, and editing.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AGEs- advanced glycation end products; Aldo- aldosterone; ARG1- arginase-1; DMSO- dimethyl sulfoxide; DN- diabetic nephropathy; ELISA- enzyme-linked immunosorbent assay; HSA- human serum albumin; iNOS- inducible nitric oxide synthase; MGO- methylglyoxal; MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF-κB- nuclear factor κB; PBS- phosphate-buffered saline; RAGE- receptor for advanced glycation end products; RNS- reactive nitrogen species; ROS- reactive oxygen species; RT-PCR- reverse transcription-polymerase chain reaction; TNF-α- tumor necrosis factor-α

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