



Glycation interferes with natural killer cell function

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ABSTRACT

One hallmark of molecular aging is glycation, better known as formation of so-called advanced glycation end products (AGEs), where reactive carbonyls react with amino-groups of proteins. AGEs accumulate over time and are responsible for various age-dependent diseases and impairments. Two very potent dicarbonyls to generate AGEs are glyoxal (GO) and methylglyoxal (MGO). The plasma level of such dicarbonyls is higher in aging and age-related diseases. Natural killer (NK) cells are cells of the innate immune system and provide a major defense against tumor cells and virus infected cells. They are able to kill modified or infected cells and produce different cytokines to modulate the function of other immune cells. Here we investigated the effect of GO- and MGO-induced glycation on the function of NK cells. Using the human NK cell line NK-92, we could demonstrate that both GO and MGO lead to glycation of cellular proteins, but that MGO interferes much stronger with NK cell function (cytotoxicity) than GO. In addition, glycation of NK cell targets, such as K562 tumor cells, also interferes with their lysis by NK cells. From this data we conclude that glycation acts negatively on NK cells function and reduces their cytotoxic potential towards tumor cells.

1. Introduction

Glycation is a process in which carbonyls, such as sugar molecules like glucose or fructose, react with amine residues of proteins or lipids without involvement of enzymes (Singh et al., 2014). Following further chemical, non-enzymatic reactions, so-called advanced glycation end products (AGEs) are formed, which lead to protein crosslinking and dysfunction. Not only sugars, but also other physiologically occurring metabolites are able to form AGEs. Highly reactive dicarbonyl metabolites can lead to glycation and are produced in the human body during lipid peroxidation, amino acid oxidation and as side products of metabolic pathways like glycolysis (Semchyshyn, 2014). Dicarbonyl metabolites are the most important intermediates in non-enzymatic protein modification (Henning and Glomb, 2016) and are much stronger glycating agents than sugars (Brings et al., 2017). Two prominent dicarbonyls existing in mammals are methylglyoxal (MGO) and glyoxal (GO) (Fig. 1). An increase in the steady state level of reactive carbonyl species is defined as carbonyl stress (Semchyshyn, 2014). Under physiological conditions dicarbonyls are detoxified via the glyoxalase system, which prevents AGE formation. During aging the glyoxalase activity is reduced (Xue et al., 2011). Aging is associated with an accumulation of AGEs which are thought to be responsible for

the pathogenesis of cardiovascular and metabolic diseases and impairments (Kilhovd et al., 2005; Mallipattu and Uribarri, 2014; Bennmann et al., 2014; Hussain et al., 2016; Scheffler et al., 2018). Supporting this idea is the observation that high levels of dicarbonyls can be found in the plasma of patients with age-related diseases like type II diabetes and obesity (Nakayama et al., 2008; Masania et al., 2016).

The immune system protects an organism against pathogens and cancer cells. With increasing age, the function of the immune system is slowly and constantly decreasing (Montecino-Rodriguez et al., 2013; Weiskopf et al., 2009). Carbonyl stress and protein glycation have been shown to have negative effects on the immune system. For example, MGO suppresses T cell and dendritic cell functions and is linked with reduced immune activity in diabetes (Price et al., 2010; Price and Knight, 2009). Additionally, AGE-modified matrix proteins interfere with migration and adhesion of T cells (Haucke et al., 2014). AGEs are also able to upregulate adhesion molecule and cytokine expression in monocytes (Takahashi et al., 2009). Other important immune effector cells are natural killer (NK) cells which are cells of the innate immune system and provide a defense against virus infected cells and tumor cells because of their ability to detect and kill transformed cells. Furthermore, they are able to modulate the function of other immune cells

Abbreviations: AGEs, advanced glycation end products; CFSE, carboxyfluorescein diacetate succinimidyl ester; CML, carboxymethyllysine; DMSO, dimethyl sulfoxide; GO, glyoxal; IL-2, interleukin-2; MGO, methylglyoxal; MTT, methylthiazolyldiphenyl-tetrazolium bromide; NK, natural killer

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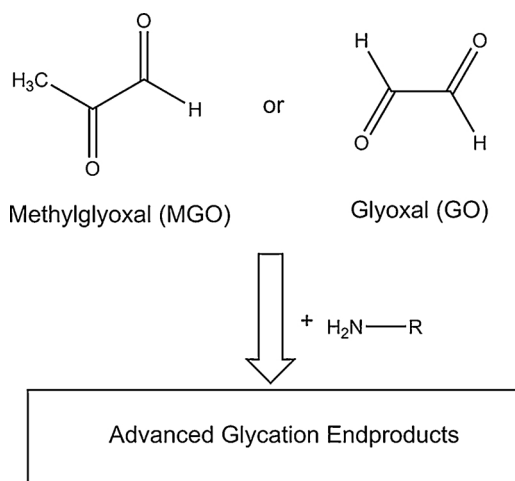


Fig. 1. Methylglyoxal and glyoxal.

Reaction of the two dicarbonyls methylglyoxal (MGO) and glyoxal (GO) with amine residues of proteins or lipids results in the formation of advanced glycation end products (AGEs).

through the release of immunoregulatory cytokines like interferon γ , tumor necrosis factor α or macrophage-colony stimulating factor (Farag and Caligiuri, 2006). The lytic activity of NK cells is regulated by different receptors that can be classified as either activating or inhibitory. Based on the expression of activating and inhibitory ligands, normal cells, that should not be attacked, can be discriminated from transformed cells, that need to be killed by NK cells (Pegram et al., 2011). Important for target cell recognition and lysis is the formation of an immunological synapse between the target cell and the NK cell. If a cell is detected as abnormal, the NK cell will release perforin- and granzyme-containing granules that will lead to the death of the target cell (Krzewski and Strominger, 2008). Because of their ability to detect transformed cells, NK cells play a major role in preventing tumor formation. Different human NK cell lines have been established to study the function of NK cells, such as the well characterized cell line NK-92, which shows a high cytotoxic activity towards tumor cells (Maki et al., 2001).

Changes in the phenotype of NK cells and a decline in their function have been observed during the aging process and with age-related diseases. (Pera et al., 2015; Piątkiewicz et al., 2013; Bähr et al., 2018; O'Shea et al., 2010; Rosenstock et al., 2017). Furthermore, aging and age-related diseases are linked with high levels of reactive carbonyls and an accumulation of AGEs. As NK cells are circulating in the bloodstream they might be influenced by these carbonyl compounds, however the role of carbonyl stress and glycation specifically on NK cell function has not been studied yet. Therefore, the aim of the present study is to investigate how carbonyl stress induced glycation of NK cell proteins affects their function. The highly reactive dicarbonyl compounds MGO and GO were used to treat NK cells and their impact on NK cell-dependent tumor lysis was investigated.

2. Materials and methods

2.1. Cell line and cell culture

The NK cell line NK-92 and the myelogenous leukemia cell line K562 were kindly provided by Roland Jacobs (Hannover Medical School, Germany). The cells were cultured in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS) (GE Healthcare, Little Chalfont, UK), 100 U·ml⁻¹ penicillin and 100 mg·ml⁻¹ streptomycin (Thermo Fisher Scientific, Waltham, USA) and 1 mM sodium pyruvate (Thermo Fisher Scientific) in humidified 5% CO₂ atmosphere at 37 °C. NK-92 cells were additionally supplemented

with 200 U/ml Interleukin-2 (IL-2) (Novartis Pharma GmbH, Zwickau, Germany). All assays using NK cells were performed with IL-2 supplementation.

2.2. Glycation of the cells

Cells were glycated using MGO and GO (both from Sigma Aldrich, St. Louis, USA). NK-92 or K562 at a density of 0.3×10^6 cells/ml were incubated in low serum medium (1% FCS, + 200 U/ml IL-2 for NK cells) with 0.3 mM, 0.6 mM, 1 mM or 2 mM of MGO or GO for 4 h in humidified 5% CO₂ atmosphere at 37 °C. Afterwards the cells were washed with PBS and proteins were isolated or the cells were used for the different assays in normal medium (10% FCS).

2.3. Protein isolation and western blot

After glycation the NK cells were washed with PBS and cell pellets were solubilized at 4 °C for 2 h in buffer containing 150 mM NaCl, 50 mM Tris, 1% Triton X, 100 mM phenyl-methylsulfonyl fluoride and a protease inhibitor cocktail (Sigma-Aldrich) at pH 8. After centrifugation at 16,000 g, supernatant was collected. Proteins were separated by SDS-PAGE on a 10% gel and transferred to a nitrocellulose membrane. Protein bands were visualized using ponceau red staining solution containing 0.1% Ponceau S (Carl Roth, Karlsruhe, Germany), 3% trichloroacetic acid and 3% sulfosalicylic acid. Glycation was detected using monoclonal anti-CML antibody (CML-26, ab125145) (Abcam, Cambridge, U.K.) at 0.05 µg/ml followed by incubation with a peroxidase conjugated secondary antibody. Antibody binding was visualized using Luminata Forte (Merck Millipore, Billerica, USA) as substrate and a molecular Imager® Gel Doc™ XR System (Bio-Rad, Hercules, USA). Anti-Actin Ab5 antibody (612,657) (BD Biosciences, San Diego, USA) at a concentration of 0.05 µg/ml served as loading control.

2.4. Cytotoxicity assay

Cytotoxic function of NK-92 cells was analyzed by flow cytometry with the use of K562 cells as target cells similar described by Duan et al. (2011). The K562 cells were washed with PBS and labeled with 0.5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) (BD Biosciences) in PBS for 5 min. The reaction was quenched by adding serum containing medium and after an additional washing step with medium, the cells were used for the assay. 20,000 labeled target cells were mixed with NK-92 cells at an Effector:Target (E:T) Ratio of 10:1 in a V-bottom plate in normal medium (10% FCS) without phenol red in a total volume of 200 µl. The cells were centrifuged for 1 min at 135g and incubated at 37 °C for 4 h. After the incubation, all cells were stained with 5 µl 7-AAD Viability Staining Solution (BioLegend, San Diego, USA) and analyzed using a BD Accuri C6 flow cytometer (BD Biosciences). CFSE was measured in the FL-1 channel (excitation 488 nm, 530/30 nm band pass filter) and 7 AAD was measured in the FL-3 channel (excitation 488 nm, 670 nm long pass filter). At least 4000 CFSE⁺ cells were recorded and the proportion of dead target cells (CFSE⁺/7 AAD⁺) was measured. Spontaneous cell death was analyzed by incubating target cells without NK cells and the NK cell-dependent cytotoxicity was calculated as:

$$\frac{(\%(\text{dead target cells}) - \%(\text{spontaneous cell death}))}{(100\% - \%(\text{spontaneous cell death}))}$$

Cells without MGO or GO treatment served as control and their cytotoxicity were set to 100%

2.5. Conjugate forming assay

For measuring the conjugate formation of NK cells and target cells an assay similar to Burshtyn and Davidson (2010) was used. K562 cells

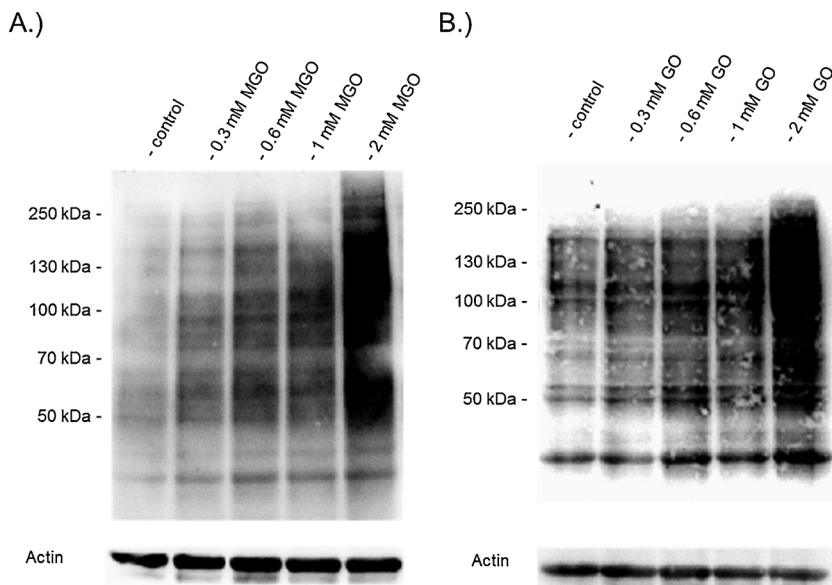


Fig. 2. Detection of glycation by western Blot.

NK-92 cells were incubated with different concentrations of MGO (A.) and GO (B.) for 4 h in low serum medium (1% FCS). Cells were washed and proteins were isolated, separated by SDS-PAGE and transferred to a nitrocellulose membrane. Glycation was detected using monoclonal CML-26 antibody. Representative Blots from at least 3 experiments are shown.

were stained with CFSE, as described for the cytotoxicity assay. NK-92 cells were stained similar to the target cells but with 1 μ M Cell Proliferation Dye eFluor™ 670 (Thermo Fisher Scientific). 40,000 target cells were mixed with NK cells at E:T ratio of 2:1 in medium without phenol red in a reaction tube. The cells were centrifuged for 1 min at 100g and incubated at 37 °C for 20 min. Afterwards the mixture was analyzed by flow cytometry. CFSE was measured in the FL-1 channel (excitation 488 nm 530/30 nm band pass filter) and eFluor™ 670 was measured in the FL-4 channel (excitation 633 nm 675/25 nm band pass filter). Percentage of CFSE⁺/eFluor™ 670⁺ events (conjugates of NK cells and target cells) related to the total number of events was calculated. Cells without MGO or GO treatment served as control and their conjugate formation were set to 100%.

2.6. Metabolic activity

30,000 NK cells were incubated in V-bottomplates in a total volume of 100 μ l with different concentrations of MGO or GO in NK cell medium with low serum concentration (1% FCS) for 4 h at 37 °C. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich) was dissolved in RPMI 1640 (Lonza, Basel, Switzerland) without phenol red. Cells were washed with PBS and incubated for 4 h at 37 °C in normal NK cell medium (10% FCS) without phenol red supplemented with 0.5 mg/ml MTT. After incubation the medium was discarded, formazan crystals were dissolved in 150 μ l dimethyl sulfoxide (DMSO) and the solution was transferred to a normal 96-well flat-bottom plate. Absorption was measured at 570 nm using a CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany) and the background absorption at 630 nm was subtracted. Cells without MGO or GO treatment served as control and were set to 100%.

2.7. Apoptosis assay

Cells were glyated as described in 2.2, washed with PBS and apoptosis assay using APC Annexin V Apoptosis Detection Kit with 7-AAD (Biolegend) was performed according to the protocol. In brief, 0.5 $\times$ 10⁶ cells were resuspended in 100 μ l Annexin V binding buffer, stained with 7 AAD and Annexin V for 15 min and were analyzed using a BD Accuri C6 flow cytometer (BD Biosciences). Annexin V APC was measured in the FL-4 channel (excitation 633 nm, 675/25 nm band pass filter) and 7 AAD was measured in the FL-3 channel (excitation 488 nm, 670 nm long pass filter). The percentage of Annexin V⁺/7 AAD⁺ cells was used to determine the number of living cells.

2.8. Bright-field images

NK-92 cells were glyated for 4 h as previously described, washed with PBS and transferred to normal medium (10% FCS). After 24 h bright-field images were taken using an Axiovert 100 microscope (Carl Zeiss, Oberkochen, Germany).

2.9. Statistical analysis

Data is presented as scatter blots including mean $\pm$ standard deviation (SD). For statistical analysis, one-way ANOVA with post-hoc Tukey-Test was performed using OriginPro 2017 software (OriginLab Corporation, Northampton, USA). A difference between a treatment and the control at $p < 0.05$ were considered as statistically significant and P-values of the Tukey-Test (treatment compared to control) are displayed on each graph.

3. Results

3.1. MGO or GO treatment leads to glycation of NK cells

In the first series of experiments we analyzed glyated proteins of NK-92 cells cultured in the absence or presence of the two dicarbonyls MGO and GO. Therefore, NK-92 cells were incubated with increasing concentrations (0, 0.3, 0.6, 1 and 2 mM) of MGO or GO in low serum medium (1% FCS) for 4 h to generate AGE modifications. Proteins were isolated and separated by SDS-PAGE. The composition of the protein bands did not change with treatment which could be seen by ponceau red staining of the membranes (Suppl. Fig. 1). Glycation was detected using an anti-CML antibody which not only detects the carboxymethyllysine (CML) modification, but also AGEs derived by MGO, which has been shown previously (Bennmann et al., 2014; Hussain et al., 2016). Treatment with MGO (Fig. 2A) led to glycation of the cells, which could be detected as a broad smear in the western blot analysis. A concentration-dependent increase in the signal was visible with the strongest signal at 2 mM. Similar results were obtained using GO as glycating agent (Fig. 2B).

3.2. Metabolic activity is only reduced at high MGO concentrations

To test whether treatment with MGO or GO changes the viability of the cells, apoptosis assays were performed using Annexin V and 7 AAD staining and no changes could be observed (Suppl. Fig. 2). We next

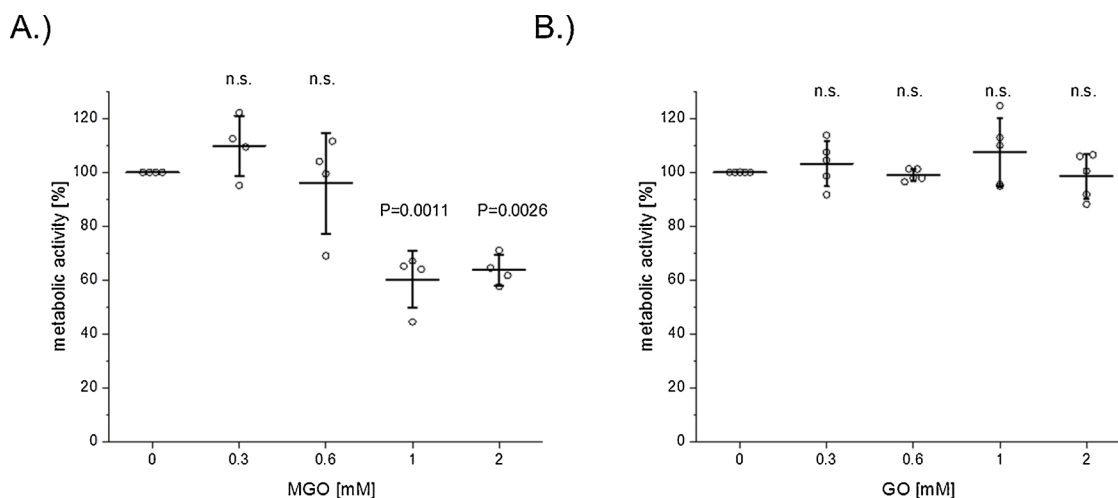


Fig. 3. MTT Assay.

NK-92 cells were treated with MGO (A.) or GO (B.) for 4 h in low serum medium (1% FCS) and MTT assay was performed. Cells without MGO or GO treatment were set to 100%. Data represents average mean $\pm$ SD of 4 (A.) and 5 (B.) independent experiments. Each dot represents data from one independent experiment.

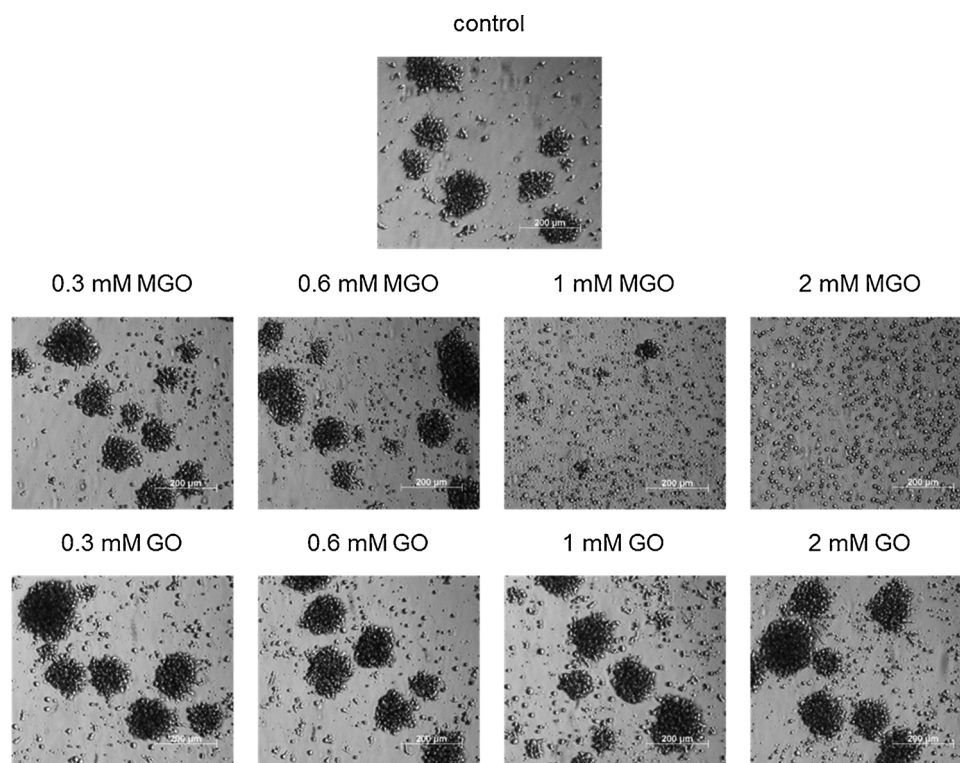


Fig. 4. Aggregation of NK-92 cells.

NK-92 cells were treated with MGO or GO for 4 h in low serum medium (1% FCS), washed with PBS and resuspended in normal Medium (10% FCS). The cells were incubated for 24 h and bright-field pictures were taken.

investigated whether glycation was affecting the metabolic activity of the NK-92 cells. An MTT assay was performed after incubating the NK-92 cells with MGO or GO for 4 h. Concentrations up to 0.6 mM MGO did not significantly alter the metabolic activity of the NK-92 cells (Fig. 3A). At 1 mM and 2 mM MGO, the metabolic activity was reduced by about 40%. Treatment with GO, even at high concentrations up to 2 mM, did not reduce the metabolic activity of the NK-92 cells (Fig. 3B). Additionally, the cells were glycated for 4 h, washed with PBS, transferred to normal medium with 10% FCS and pictures were taken after 24 h (Fig. 4). The NK-92 cell line usually grows in aggregates. No differences in growth patterns could be observed with treatments up to 0.6 mM, however, treatment with 1 mM MGO led to a marked reduction in

aggregate formation, and a treatment with 2 mM MGO completely prevented the formation of aggregates. In contrast, incubation with GO did not change aggregate formation of the cells even at high concentrations of 2 mM.

3.3. Glycation interferes with cytotoxic function of NK cells

The first step of the cell-mediated cytotoxicity by NK cells is the attachment to the target cell. Therefore, a conjugate forming assay was used to determine whether or how glycation by MGO or GO interferes with the ability of the NK cells to form conjugates with the myelogenous leukemia cell line K562. Because of the low expression of

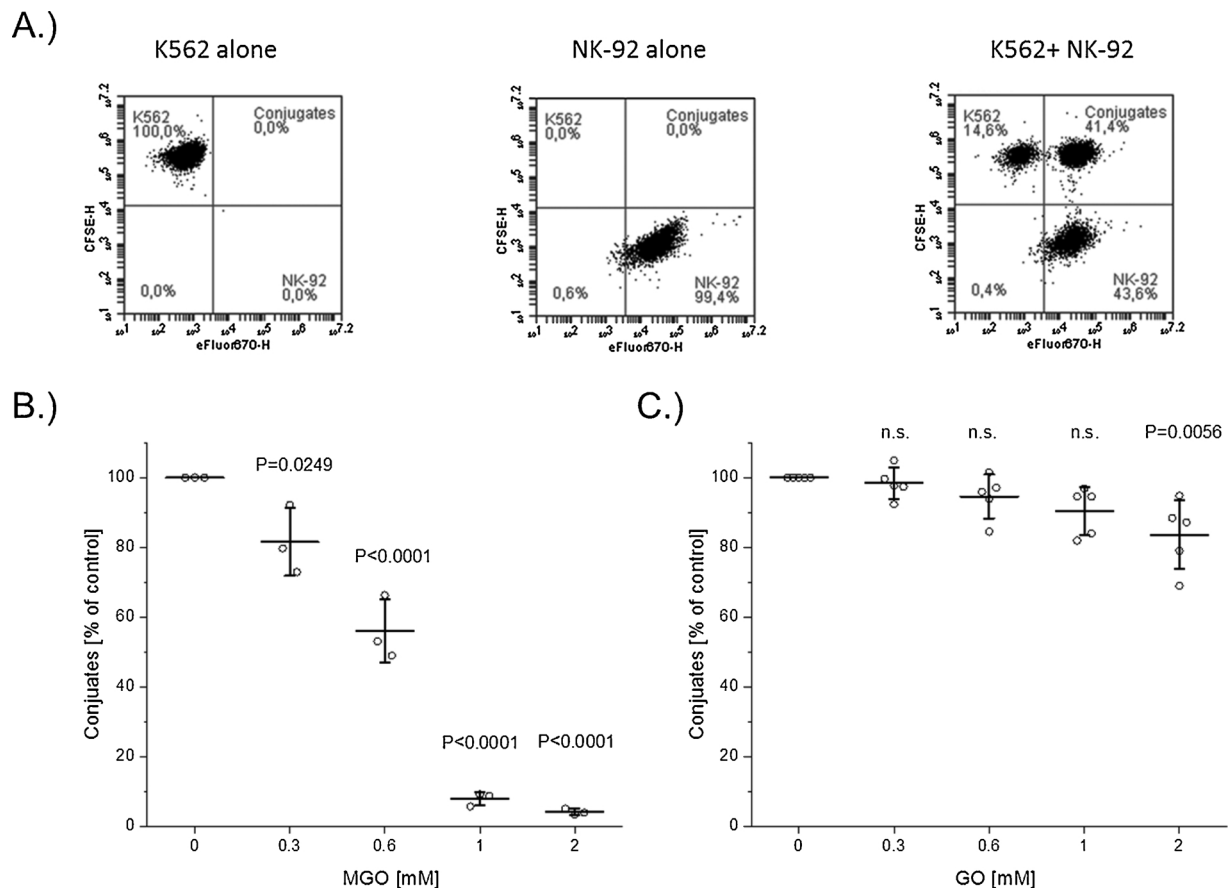


Fig. 5. Conjugate forming assay.

NK-92 cells were labeled with eFluor 670 and K562 cells were labeled with CFSE. K562 cells and NK cells were mixed at E:T ratio of 2:1 and conjugates were measured using flow cytometry. Tubes with K562 alone and NK-92 alone served as control to set the gates (A.). NK-92 cells were treated with MGO (B.) and GO (C.) for 4 h in low serum medium (1% FCS) and conjugate forming assay was performed. Data represents average mean $\pm$ SD of 3 (B.) and 5 (C.) independent experiments. Each dot represents data from one independent experiment.

inhibitory ligands, these cells can be easily lysed by NK cells and are a common model to study NK cell function (Lisovsky et al., 2015). NK-92 cells and K562 cells alone were used as control to set the gates (Fig. 5A). Treatment with 0.3 and 0.6 mM MGO led to a reduction of conjugate formation by about 20% and 40% respectively (Fig. 5B). High concentrations of 1 mM and 2 mM MGO inhibited the conjugate formation nearly completely. Glycation with GO led to a small reduction of conjugate formation (Fig. 5C). This effect was much weaker compared to the treatment with MGO with about 15% reduction at 2 mM GO.

To study the cytotoxicity of NK cells against tumor cells, a flow cytometry-based cytotoxicity assay was performed. CFSE labeled K562 cells were incubated with NK cells for 4 h and stained with 7 AAD afterwards to detect dead target cells (Fig. 6A). Cytotoxicity was reduced by about 40% at 0.6 mM MGO and was reduced by over 85% at the higher concentrations of 1 mM or 2 mM (Fig. 6B). GO had a weaker effect on cytotoxicity compared with MGO treatment with about 25% reduction at 2 mM GO (Fig. 6C).

3.4. Glycation of the tumor cells reduces their susceptibility to NK cells lysis

To further investigate the role of cell-cell contact, tumor cells were glycated using MGO and GO. Similar to the NK cells, K562 cells were incubated with MGO or GO for 4 h and glycation was proven by the western blot analysis (Suppl. Fig. 3).

Glycation of K562 cells with 1 mM and 2 mM MGO resulted in a reduced conjugate formation by about 25% and 30%, respectively (Fig. 7A). Incubation with 2 mM GO also reduced the conjugate formation by about 25% (Fig. 7B). We could also observe that glycation of

the target cells made them more resistant to NK cell-mediated lysis. An impaired NK cell-mediated cytotoxicity could be detected when the tumor cells were glycated with MGO concentrations from 0.6 mM to 2 mM (Fig. 7C). High concentrations of GO also reduced the NK cell-mediated cytotoxicity (Fig. 7D). A reduction of about 15% could be measured at a GO concentration of 2 mM. Both MGO and GO showed a similar reduction of conjugate formation and cytotoxicity by the NK cells.

4. Discussion

Dicarbonyl stress and glycation can have a negative effect on the immune system. We decided to use the well-characterized NK cell line NK-92 instead of using primary cells because primary cells differ in their cytotoxic potential and receptor expression due to a high heterogeneity of different donors (e.g. age, sex, body weight) (Viel et al., 2017; Almeida-Oliveira et al., 2011; Al-Attar et al., 2016). The K562 cell line was chosen as a target cell line because it is easily lysed by NK cells and is a well-established model to study NK cell function (Lisovsky et al., 2015). Using NK-92 cells we were able to show that incubation with the dicarbonyls MGO or GO resulted in detectable glycation of NK cell proteins. Only high MGO concentrations of 1 or 2 mM reduced the metabolic activity of NK cells whereas physiological occurring concentrations of less than 1 mM had no effect on the metabolic activity of NK-92 cells. Treatment with MGO or GO led to reduced conjugate formation and cytotoxicity towards tumor cells. Furthermore treatment with MGO and GO even at high concentrations of 1 or 2 mM did not induce apoptosis which could be shown by 7 AAD and Annexin V

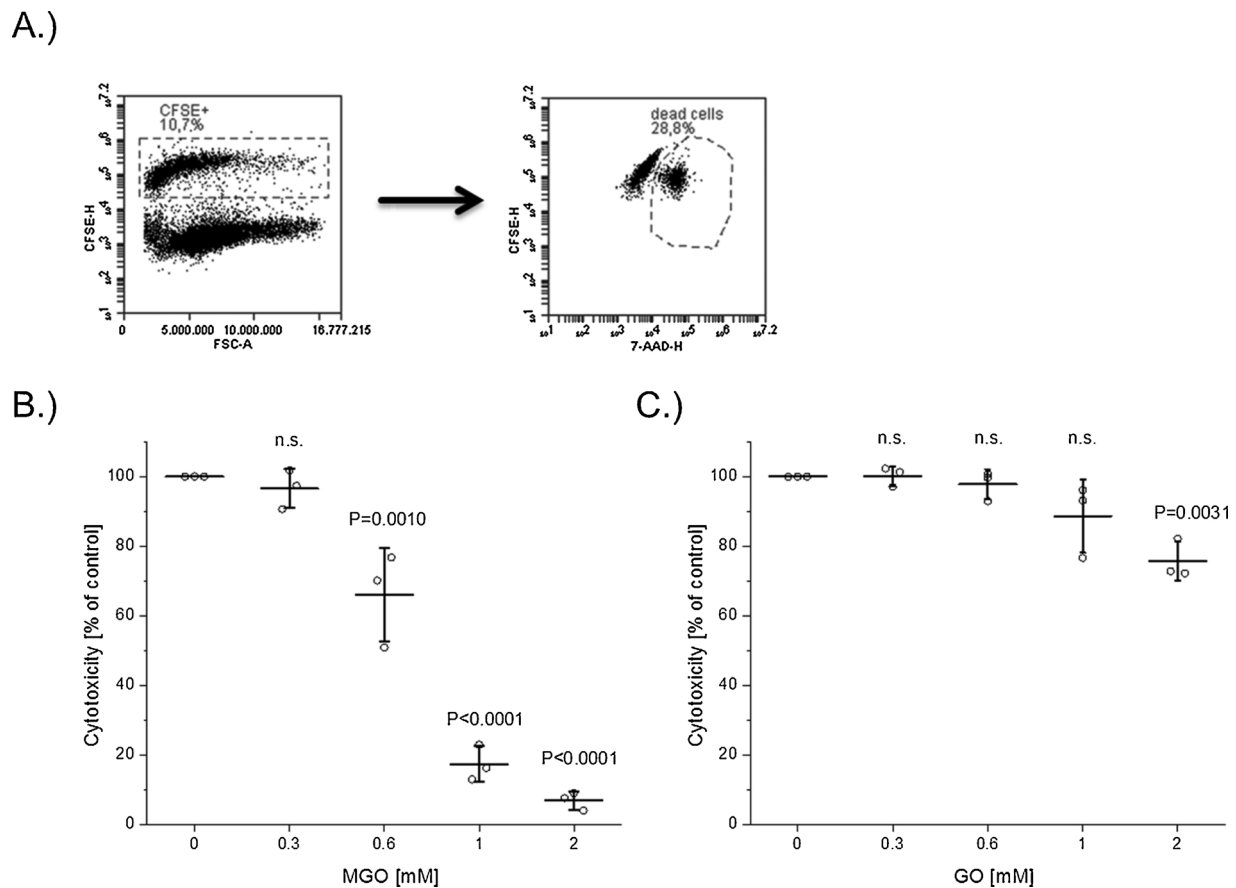


Fig. 6. Cytotoxicity Assay.

CFSE-labelled K562 cells were mixed with NK-92 cell at E:T ratio of 10:1. After 4 h incubation, 7 AAD was added to each sample. Because of the CFSE labeling, target cells can be distinguished from NK cells and dead target cells can be identified as 7 AAD positive (A.). NK-92 cells are treated with different concentrations of MGO (B.) and GO (C.) for 4 h in low serum medium (1% FCS) and cytotoxicity assay was performed. Data represents average mean $\pm$ SD of 3 independent experiments. Each dot represents data from one independent experiment.

staining. From this data it can be concluded that functional decline of NK cells are not due to cell death. As mentioned previously, killing of the target cells requires cell-cell contacts and the formation of a lytic synapse between the NK cell and the target cell (Krzewski and Strominger, 2008). These contacts might be inhibited by glycation of the NK cells and could explain the reduced conjugate formation and cytotoxicity of glycated NK cells. Moreover, glycation of cell surface receptors might reduce their ability to activate the NK cell. Both MGO and GO inhibited the conjugate formation as well as the cytotoxicity of NK-92 cells. In diabetic patients, the plasma concentrations of MGO and GO are abnormally increased (Kong et al., 2014; Lapolla et al., 2005). Dicarbonyls are highly reactive, however its actual plasma concentration is relatively low, since most of the produced MGO or GO (90–99%) is bound to macromolecules (Allaman et al., 2015). To reach a high rate of glycation, the experiments in this study were performed within a short incubation time of 4 h with concentrations up to 2 mM. Similar concentrations and incubation times have been used before to study the influence of glycation on a neuronal-like cell line (Bennmann et al., 2014; Frank et al., 2019).

Comparing the treatment of NK cells with MGO and GO revealed a much stronger effect of MGO on NK cell function. Similar physiological differences between MGO and GO treatment have also been observed in other studies (Santos et al., 2017; Knip et al., 2006; Akhand et al., 2001). The reaction of MGO and GO with amino acids results in different AGE modifications. For example, GO lead to the formation of carboxymethyllysine (CML) and glyoxal-derived lysine dimer (GOLD), while Carboxyethyllysine (CEL) and Argpyrimide are MGO-derived AGEs (Nowotny et al., 2015). So the different AGEs could have different

effects on the function of the NK cells, which might explain the differences observed between the two treatments. In the western blot analysis we could show a concentration dependent increase in the signal with MGO and a similar result for the treatment with GO. However, we cannot conclude from these results that both compounds produce similar amounts of AGEs. The antibody used in this study was raised against CML, but is also able to detect AGEs derived by MGO, which has been shown previously (Bennmann et al., 2014; Hussain et al., 2016), but it still produces a stronger signal for GO modified proteins. MGO is known to be generally more reactive than GO and has a higher glycation potency (Argirova and Breipohl, 2002; Sadowska-Bartos et al., 2014). This might be the reason for the reduction of metabolic activity at high MGO concentrations and could also explain the stronger effect of MGO on the NK cell function. Even though the intensity of the two treatments differs, the present study shows an overall negative effect of MGO and GO on human NK cell function. Because high levels of MGO and GO are found in the plasma of diabetic and obese patients, these findings might help to explain the reduced NK cell function in these diseases. Nevertheless, in our experiments MGO was more harmful compare to GO.

Furthermore, we were able to show that glycation of the target cells led to a slight reduction of conjugate formation and NK cell-dependent lysis. Compared with the incubation of the NK cells with MGO, which showed a strong effect, an incubation of the target cells with MGO had a much weaker effect. Glycation of the NK cells could result in modifications of proteins that are involved in adhesion, target cell recognition and degranulation, which will make the NK cells less active. If the target cells are glycated, interaction between NK cells and tumor

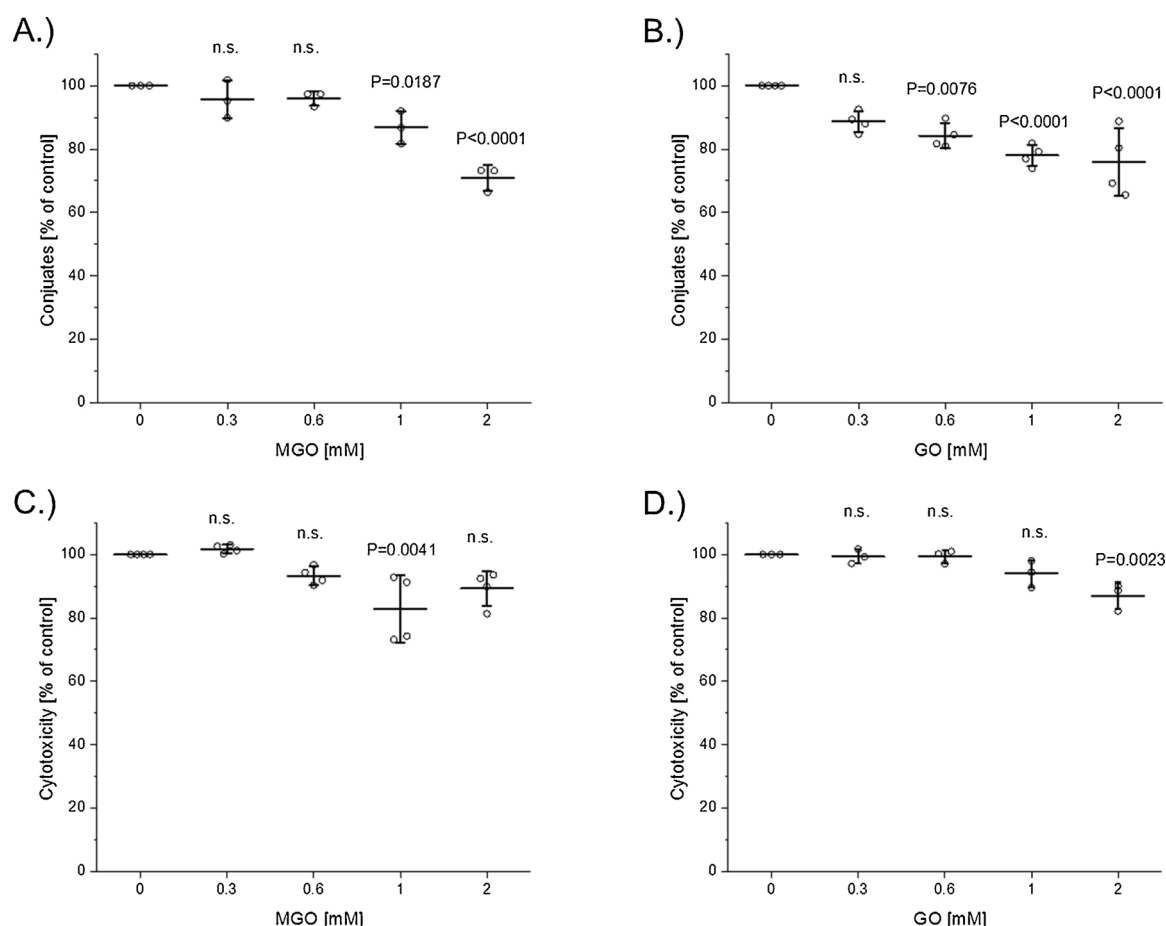


Fig. 7. Glycation of the tumor cells.

CFSE-labelled K562 cells were treated with different concentrations of MGO and GO for 4 h in low serum medium (1% FCS) and conjugate forming assay (A. and B.) as well as cytotoxicity assay (C. and D.) were performed. Data represents average mean $\pm$ SD of 3 (A. and D.) and 4 (B. and C.) independent experiments. Each dot represents data from one independent experiment.

cells might be decreased because of the glycation of cell surface proteins. In this case the NK cells are not directly influenced by glycation and are therefore still highly active. Moreover, the K562 cell line is expressing a low amount of inhibitory receptors, which make them an easy target for NK cells (Duan et al., 2011; Lisovsky et al., 2015). Therefore, the NK cells are still able to kill the glycosylated target cells, but the efficiency is slightly reduced. Both MGO and GO were affecting the susceptibility of K562 cells to NK cell lysis. Therefore, glycation of the tumor cell might have a protective effect against NK cells. Chiavarina et al. showed a high level of glycosylated proteins in colon cancer tissue, due to a high metabolism rate and a low glyoxalase 1 activity, that was beneficial for tumor growth (Chiavarina et al., 2017). The findings of this study could provide another explanation why glycation might be a benefit for tumor cells.

Anti-cancer effects of MGO have been discussed because of the ability of MGO to induce apoptosis in several cancer cell lines (Talukdar et al., 2009). Taking the results from this study, these anti-cancer effects may have to be critically discussed because MGO will also have strong negative effects on the function of NK cells, which are important suppressors of tumor growth.

In summary, this study showed that MGO and GO had a negative effect on NK cell function. Conjugate formation and NK cell-dependent cytotoxicity were reduced when NK-92 cells were glycosylated. MGO had a much stronger effect compared with GO. Glycation of the tumor cells also resulted in slightly lower conjugate formation and lysis, suggesting that glycation might be beneficial for the tumor.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.mad.2019.01.006>.

References

- Akhand, A.A., Hossain, K., Kato, M., Miyata, T., Du, J., Suzuki, H., Kurokawa, K., Nakashima, I., 2001. Glyoxal and methylglyoxal induce lysozyme and methylglyoxal induce aggregation and inactivation of ERK in human endothelial cells. *Free Radic. Biol. Med.* 31, 1228–1235.
- Al-Attar, A., Presnell, S.R., Peterson, C.A., Thomas, D.T., Lutz, C.T., 2016. The effect of sex on immune cells in healthy aging: elderly women have more robust natural killer lymphocytes than do elderly men. *Mech. Ageing Dev.* 156, 25–33.
- Allaman, I., Belanger, M., Magistretti, P.J., 2015. Methylglyoxal, the dark side of glycolysis. *Front. Neurosci.* 9, 23.
- Almeida-Oliveira, A., Smith-Carvalho, M., Porto, L.C., Cardoso-Oliveira, J., Ribeiro, Ad.S., Falcão, R.R., Abdelhay, E., Bouzas, L.F., Thuler, L.C.S., Ornellas, M.H., Diamond, H.R., 2011. Age-related changes in natural killer cell receptors from childhood through old age. *Hum. Immunol.* 72, 319–329.

- Argirova, M., Breipohl, W., 2002. Comparison between modifications of lens proteins resulted from glycation with methylglyoxal, glyoxal, ascorbic acid, and fructose. *J. Biochem. Mol. Toxicol.* 16, 140–145.
- Bähr, I., Jahn, J., Zipprich, A., Pahlow, I., Spielmann, J., Kielstein, H., 2018. Impaired natural killer cell subset phenotypes in human obesity. *Immunol. Res.* 66 (2), 234–244.
- Bennmann, D., Horstkorte, R., Hofmann, B., Jacobs, K., Navarrete-Santos, A., Simm, A., Bork, K., Gnanapragassam, V.S., 2014. Advanced glycation endproducts interfere with adhesion and neurite outgrowth. *PLoS One* 9, e112115.
- Brings, S., Fleming, T., Freichel, M., Muckenthaler, M.U., Herzig, S., Nawroth, P.P., 2017. Dicarboxyls and advanced glycation end-products in the development of diabetic complications and targets for intervention. *Int. J. Mol. Sci.* 18.
- Burshdyn, D.N., Davidson, C., 2010. Natural killer cell conjugate assay using two-color flow cytometry. In: Campbell, K.S. (Ed.), *Natural Killer Cell Protocols: Cellular and Molecular Methods*. Humana Press, Totowa, NJ.
- Chiavarina, B., Nokin, M.-J., Bellier, J., Durieux, F., Bletard, N., Sherer, F., Lovinfosse, P., Peulen, O., Verset, L., Dehon, R., Demetter, P., Turtot, A., Uchida, K., Goldman, S., Hustinx, R., Delvenne, P., Castronovo, V., Bellahcène, A., 2017. Methylglyoxal-mediated stress correlates with high metabolic activity and promotes tumor growth in colorectal cancer. *Int. J. Mol. Sci.* 18.
- Duan, X.-M., Huang, J., Ma, H.-X., Wang, X.-Q., Che, Y.-Y., Tan, Y., Wang, Y.-P., 2011. A flow cytometric assay for simultaneously measuring the proliferation and cytotoxicity of cytokine induced killer cells in combination with carboxyfluorescein succinimidyl ester (CFSE) labeling. *Afr. J. Biotechnol.* 10, 14598–14607.
- Farag, S.S., Caligiuri, M.A., 2006. Human natural killer cell development and biology. *Blood Rev.* 20, 123–137.
- Frank, F., Bezold, V., Bork, K., Rosenstock, P., Scheffler, J., Horstkorte, R., 2019. Advanced glycation endproducts and polysialylation affect the turnover of the neural cell adhesion molecule (NCAM) and the receptor for advanced glycation endproducts (RAGE). *Biol. Chem.* 400, 219–226.
- Haucke, E., Navarrete-Santos, A., Simm, A., Silber, R.-E., Hofmann, B., 2014. Glycation of extracellular matrix proteins impairs migration of immune cells. *Wound Repair Regen.* 22, 239–245.
- Henning, C., Glomb, M.A., 2016. Pathways of the Maillard reaction under physiological conditions. *Glycoconj. J.* 33, 499–512.
- Hussain, M., Bork, K., Gnanapragassam, V.S., Bennmann, D., Jacobs, K., Navarrete-Santos, A., Hofmann, B., Simm, A., Danker, K., Horstkorte, R., 2016. Novel insights in the dysfunction of human blood-brain barrier after glycation. *Mech. Ageing Dev.* 155, 48–54.
- Kilhovd, B.K., Juutilainen, A., Lehto, S., Rönnemaa, T., Torjesen, P.A., Birkeland, K.I., Berg, T.J., Hanssen, K.F., Laakso, M., 2005. High serum levels of advanced glycation end products predict increased coronary heart disease mortality in nondiabetic women but not in nondiabetic men: A population-based 18-year follow-up study. *Arterioscler. Thromb. Vasc. Biol.* 25, 815–820.
- Knip, E.M., Roehlecke, C., Ozkucur, N., Steinberg, A., Reber, F., Knels, L., Funk, R.H.W., 2006. Inhibition of apoptosis and reduction of intracellular pH decrease in retinal neural cell cultures by a blocker of carbonic anhydrase. *Invest. Ophthalmol. Vis. Sci.* 47, 1185–1192.
- Kong, X., Ma, M.-Z., Huang, K., Qin, L., Zhang, H.-M., Yang, Z., Li, X.-Y., Su, Q., 2014. Increased plasma levels of the methylglyoxal in patients with newly diagnosed type 2 diabetes. *J. Diabetes* 6, 535–540.
- Krzewski, K., Strominger, J.L., 2008. The killer's kiss: the many functions of NK cell immunological synapses. *Curr. Opin. Cell Biol.* 20, 597–605.
- Lapolla, A., Reitano, R., Seraglia, R., Sartore, G., Ragazzi, E., Traldi, P., 2005. Evaluation of advanced glycation end products and carbonyl compounds in patients with different conditions of oxidative stress. *Mol. Nutr. Food Res.* 49, 685–690.
- Lisovsky, I., Isitman, G., Bruneau, J., Bernard, N.F., 2015. Functional analysis of NK cell subsets activated by 721.221 and K562 HLA-null cells. *J. Leukoc. Biol.* 97, 761–767.
- Maki, G., Klingemann, H.G., Martinson, J.A., Tam, Y.K., 2001. Factors regulating the cytotoxic activity of the human natural killer cell line, NK-92. *J. Hematother. Stem Cell Res.* 10, 369–383.
- Mallipattu, S.K., Uribarri, J., 2014. Advanced glycation end product accumulation: a new enemy to target in chronic kidney disease? *Curr. Opin. Nephrol. Hypertens.* 23, 547–554.
- Masania, J., Malczewska-Malec, M., Razny, U., Goralska, J., Zdzienicka, A., Kiec-Wilk, B., Gruca, A., Stancel-Mozwillo, J., Dembinska-Kiec, A., Rabbani, N., Thornalley, P.J., 2016. Dicarboxyl stress in clinical obesity. *Glycoconj. J.* 33, 581–589.
- Montecino-Rodriguez, E., Berent-Maoz, B., Dorshkind, K., 2013. Causes, consequences, and reversal of immune system aging. *J. Clin. Invest.* 123, 958–965.
- Nakayama, K., Nakayama, M., Iwabuchi, M., Terawaki, H., Sato, T., Kohno, M., Ito, S., 2008. Plasma α -oxoaldehyde levels in diabetic and nondiabetic chronic kidney disease patients. *AJN* 28, 871–878.
- Nowotny, K., Jung, T., Höhn, A., Weber, D., Grune, T., 2015. Advanced glycation end products and oxidative stress in type 2 diabetes mellitus. *Biomolecules* 5, 194–222.
- O'Shea, D., Cawood, T.J., O'Farrelly, C., Lynch, L., 2010. Natural killer cells in obesity: impaired function and increased susceptibility to the effects of cigarette smoke. *PLoS One* 5, e8660.
- Pegram, H.J., Andrews, D.M., Smyth, M.J., Darcy, P.K., Kershaw, M.H., 2011. Activating and inhibitory receptors of natural killer cells. *Immunol. Cell Biol.* 89, 216–224.
- Pera, A., Campos, C., Lopez, N., Hassouneh, F., Alonso, C., Tarazona, R., Solana, R., 2015. Immunosenescence: implications for response to infection and vaccination in older people. *Maturitas* 82, 50–55.
- Piátkiewicz, P., Miłek, T., Bernat-Karpińska, M., Ohams, M., Czech, A., Ciostek, P., 2013. The dysfunction of NK cells in patients with type 2 diabetes and Colon Cancer. *Arch. Immunol. Ther. Exp. (Warsz.)* 61, 245–253.
- Price, C.L., Knight, S.C., 2009. Methylglyoxal: Possible link between hyperglycaemia and immune suppression? *Trends Endocrinol. Metab.* 20, 312–317.
- Price, C.L., Hassi, H.O.S.A., English, N.R., Blakemore, A.I.F., Stagg, A.J., Knight, S.C., 2010. Methylglyoxal modulates immune responses: relevance to diabetes. *J. Cell. Mol. Med.* 14, 1806–1815.
- Rosenstock, P., Horstkorte, R., Gnanapragassam, V.S., Harth, J., Kielstein, H., 2017. Siglec-7 expression is reduced on a natural killer (NK) cell subset of obese humans. *Immunol. Res.* 4, 579.
- Sadowska-Bartoszyk, I., Galiniak, S., Bartosz, G., 2014. Kinetics of glycoxidation of bovine serum albumin by methylglyoxal and glyoxal and its prevention by various compounds. *Molecules* 19, 4880–4896.
- Navarrete Santos, A., Jacobs, K., Simm, A., Glaubitz, N., Horstkorte, R., Hofmann, B., 2017. Dicarboxyls induce senescence of human vascular endothelial cells. *Mech. Ageing Dev.* 166, 24–32.
- Scheffler, J., Bork, K., Bezold, V., Rosenstock, P., Gnanapragassam, V.S., Horstkorte, R., 2018. Ascorbic acid leads to glycation and interferes with neurite outgrowth. *Exp. Gerontol.*
- Semchysyn, H.M., 2014. Reactive carbonyl species in vivo: generation and dual biological effects. *Sci. World J.* 417842 2014.
- Singh, V.P., Bali, A., Singh, N., Jaggi, A.S., 2014. Advanced glycation end products and diabetic complications. *Korean J. Physiol. Pharmacol.* 18, 1–14.
- Takahashi, H.K., Mori, S., Wake, H., Liu, K., Yoshino, T., Ohashi, K., Tanaka, N., Shikata, K., Makino, H., Nishibori, M., 2009. Advanced glycation end products subselectively induce adhesion molecule expression and cytokine production in human peripheral blood mononuclear cells. *J. Pharmacol. Exp. Ther.* 330, 89–98.
- Talukdar, D., Chaudhuri, B.S., Ray, M., Ray, S., 2009. Critical evaluation of toxic versus beneficial effects of methylglyoxal. *Biochem. Mosc.* 74, 1059–1069.
- Viel, S., Besson, L., Charrier, E., Marçais, A., Disse, E., Biennu, J., Walzer, T., Dumontet, C., 2017. Alteration of Natural Killer cell phenotype and function in obese individuals. *Clin. Immunol.* 177, 12–17.
- Weiskopf, D., Weinberger, B., Grubeck-Loebenstein, B., 2009. The aging of the immune system. *Transpl. Int.* 22, 1041–1050.
- Xue, M., Rabbani, N., Thornalley, P.J., 2011. Glyoxalase in ageing. *Semin. Cell Dev. Biol.* 22, 293–301.