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Methylglyoxal modulates immune responses: relevance to diabetes

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Abstract

Increased methylglyoxal (MG) concentrations and formation of advanced glycation end-products (AGEs) are major pathways of glycaemic damage in diabetes, leading to vascular and neuronal complications. Diabetes patients also suffer increased susceptibility to many common infections, the underlying causes of which remain elusive. We hypothesized that immune glycation damage may account for this increased susceptibility. We previously showed that the reaction mixture (RM) for MG glycation of peptide blocks up regulation of CD83 in myeloid cells and inhibits primary stimulation of T cells. Here, we continue to investigate immune glycation damage, assessing surface and intracellular cytokine protein expression by flow cytometry, T-cell proliferation using a carboxyfluorescein succinimidyl ester assay, and mRNA levels by RT-PCR. We show that the immunomodulatory component of this RM was MG itself, with MG alone causing equivalent block of CD83 and loss of primary stimulation. Block of CD83 expression could be reversed by MG scavenger N-acetyl cysteine. Further, MG within RM inhibited stimulated production of interleukin (IL)-10 protein from myeloid cells plus interferon (IFN)-γ and tumour necrosis factor (TNF)-α from T cells. Loss of IL-10 and IFN-γ was confirmed by RT-PCR analysis of mRNA, while TNF-α message was raised. Loss of TNF-α protein was also shown by ELISA of culture supernatants. In addition, MG reduced major histocompatibility complex (MHC) class I expression on the surface of myeloid cells and increased their propensity to apoptose. We conclude that MG is a potent suppressor of myeloid and T-cell immune function and may be a major player in diabetes-associated susceptibility to infection.

Keywords: methylglyoxal • advanced glycation • dendritic cells • T cells • diabetes

Introduction

Methylglyoxal (MG) is a reactive dicarbonyl compound formed as a natural by-product of metabolism, including glucose and fatty acid pathways, found both intra- and extracellularly *in vivo*. Enzyme defence mechanisms, especially the glyoxalase I system, usually detoxify MG before it can damage cellular systems; however, in diabetes mellitus, glyoxalase expression is inhibited and glucose flux is increased, leading to raised MG concentrations [1]. MG is associated with oxidative stress, production of reactive oxygen species (ROS) and apoptosis in cellular systems [2]. It is also a potent precursor of advanced glycation end-products (AGEs) and forms adducts on free amine and thiol groups, preferentially attacking arginine, but also lysine, and cysteine residues of proteins and deoxyguanosine moieties in DNA. In forming AGEs, MG can alter the structure and function of its target molecules and also provide ligands for the receptor for AGEs (RAGE), which drives tissue damage by perpetuating a pro-inflammatory and pro-adhesive milieu [3]. Denaturing of proteins and RAGE-mediated inflammation contribute greatly to the vascular and renal complications of diabetes.

In addition, diabetes patients suffer lower resistance to many infections, including lower respiratory tract, urinary tract, skin and mucous membrane infections [4], from a variety of bacteria, yeasts and viruses. The cause of this increased infection was thought to be high glucose providing a more favourable culture condition for pathogens. Alternatively, inherent abnormalities in immune function associated with autoimmune disease have been postulated [5], but no conclusive links have been provided.

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Dendritic cells (DC) are professional antigen-presenting cells (APCs) [6], unique in their potency in stimulating primary immune responses with establishment of memory [7]. Immature DC in the periphery classically capture bacterial and other pathogenic antigen through their expression of pattern recognition receptors. These DC also process and present antigens, *via* major histocompatibility complex (MHC) apparatus, to T cells in secondary lymphoid organs [8]. This process results in proliferation of T-helper (Th) cells, which can have effector or suppressor actions, depending upon the stimulation received. Cytokines produced by DC influence both T-cell differentiation and function. DC also drive regulation of central and peripheral tolerance to self-antigen.

We have previously shown that a reaction mixture (RM) for MG-glycated peptide blocks up regulation of the maturation marker CD83 on DC and reduces their ability to stimulate T cells in allogeneic culture [9]. We now show that these effects are caused by MG itself and further characterize immunomodulation by this glycation precursor.

Materials and methods

Preparation of AGE peptide

The RM was prepared as described previously [9]. Briefly, angiotensin I peptide (2.4 mM) was incubated with MG (0.25 M) and 0.02 M sodium phosphate buffer (SPB) in a glass vial (37°C, 24 hrs) and stored at 4°C.

Analysis of reaction mixtures

The RM was analysed on Micromass TofSpec 2E MALDI-TOF mass spectrometer, as previously described [9]. The RM contained starting peptide, peptide–MG–hydroimidazolone adduct (+54 mu) plus one by-product of unknown identity, 45 mu below starting peptide. A low level of endotoxin was periodically confirmed using LAL Endosafe™ kit (Wilmington, Charles River, MA, USA).

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from human venous blood, following informed consent, as described previously [9]. Briefly, the blood was diluted with culture medium and spun over Ficoll Paque gradient (Amersham Biosciences, Chalfont St. Giles, UK). Mononuclear cells aspirated from the gradient interfaces were washed twice in culture medium, counted (trypan blue exclusion) and suspended at 3.5 million/ml of culture medium containing 10% foetal calf serum (FCS), unless otherwise stated.

Culture of cells

Freshly isolated PBMC were cultured with SPB (2 μl/ml), RM (2 μl/ml) or 500 μM MG. The samples were incubated (37°C, 5% CO_2, humidified) for 2.5, 4 or 24 hrs. For assessment of intracellular cytokine, the samples were incubated in the absence or presence of monensin (3 μM, last 4 hrs of a 24 hrs culture). Stimulated cultures also contained lipopolysaccharide (LPS) (1 μg/ml, *Escherichia coli* 026:B6; Sigma, Pool, UK) or phorbol ester (phorbol 12-myristate 13-acetate [PMA] 10 ng/ml) plus ionomycin (2 μM) for the entire culture.

Antibodies

Antibodies (Abs) to CD14 (FITC, MΦP9), CD4 (PE, SK3), CD4 (FITC, SK3), CD3 (APC, UCHT1), HLA-DR (PE, L243), HLA-DR (APC, G46–6), CD69 (PE, L78), CD8 (APC, SK1) and CD83 (PE, HB15e) (BD Biosciences, San Jose, CA, USA); tumour necrosis factor (TNF)-α (FITC, B-D9), interferon (IFN)-γ (FITC, D9D10) and interleukin (IL)-10 (PE, JES3–9D7) (Serotec, Oxford, UK); CD3 (ECD, UCHT 1), CD8 (PC5, B9.11), CD19 (ECD, J4.119), CD14 (PC5, RM052) and HLA-DR (PC5, Immu-357) (Coulter Immunotech, High Wycombe, UK); HLA-ABC (FITC, W6/32) (eBioscience, San Diego, CA, USA) and IL-4 (PE, 3007) (R&D systems, Minneapolis, MN, USA) were used. Isotype-matched controls were obtained from the same manufacturers.

Flow cytometry

Surface Ab labelling was performed in FACs buffer as before [9]. For intracellular staining, before fixing, the cells were resuspended in 50 μl Leucoperm A (Serotec) diluted in 100 μl FACs buffer (15 min.), washed and resuspended in 100 μl Leucoperm B (Serotec) plus 5 μl anti-cytokine antibody (30 min.). Data were acquired using a BD FACS Calibur flow cytometer (BD Biosciences) (75,000–100,000 live cell events) and analysed using WinList 5.0 software (Verity, ME, USA). Single-colour samples stained with CD8 or CD3 were acquired in each experiment for objective compensation in WinList. Relative cell counts were obtained by simultaneous acquisition of Flow-count fluorospheres (Coulter Immunotech). Super-enhanced D_{max} normalized subtraction (SED) was performed using WinList software, giving percentage positive events and staining intensity ratios for the positive events compared with isotpype control staining. Mean fluorescence of staining for test histograms was also separately recorded. Populations were discriminated as follows: myeloid cells were defined as $(CD3 \text{ and } CD19)^-$ and $HLA\text{-}DR^+$. Within myeloid cells, putative DC were also $CD14^-$, monocyte-like cells $CD14^+$ and classic monocytes $CD14^{++\,(high)}$. T cells were taken as $CD3^+$ PBMC and also $CD8^{high}$ ($CD8^+$ T cells) or $CD8^-CD4^+$ ($CD4^+$ T cells).

RT-PCR

PBMC were resuspended in lysis buffer and mRNA was extracted using a μMACS isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Reverse transcription was undertaken using cloned AMV first-strand cDNA synthesis kit (Invitrogen Life Technologies, Paisley, UK). Primers were designed to span exons using Roche LightCycler primer design software (Roche Diagnostics Ltd., Lewes, East Sussex, UK). The primers used had the following sequences (5′ to 3′): IFN-γ forward CAGATGTAGCGGATAATGG, reverse GTATTGCTTTGCGTTGG (amplicon 273 bp); TNF-α forward TCGAACCCCGAGTGAC, reverse GGAGCACATGGGTGGA (amplicon 212 bp); IL-10 forward CTCCGAGATGCCTTCA, reverse GTTCACATGCGCCTTG (amplicon 201 bp) and UbcH5b forward AGTGTTCAGCAGGTCC, reverse

TCCGAGCAATCTCAGG (amplicon 318 bp). Roche LightCycler FastStart DNA Master SYBR Green I reagents and LightCycler instrument were used (45 amplification cycles of 95°C/10 sec., 60°C/5 sec. and 72°C/8 sec.).

ELISA

TNF-α ELISA was performed with supernatants from PBMC cultures, Endogen Human TNF-α ELISA kit reagents (Pierce Biotechnology, Inc., Rockford, IL, USA) and Dynatech Laboratories MRX plate reader (Dynatech Laboratories, Inc., Chantilly, VA, USA).

Bead separation of cells

PBMC were washed and resuspended in buffer as per manufacturer's instructions. Microbeads (Miltenyi Biotec) were added and the cells were incubated at 4–6°C for 15 min. The cells were washed and run on depletion (LD) or positive selection (MS) columns.

CFSE (carboxyfluorescein diacetate succinimidyl ester) staining of cells

Cells were washed twice in sterile PBS and resuspended at 2×10^7/ml had equal volume of CFSE added to a final concentration of 5 μM and were incubated in the dark for 3 min. One volume of FCS was added to stop reaction and the cells were washed twice and resuspended for counting and further culture.

Apoptosis assay

Cultured cells were washed and resuspended in 100 μl annexin V binding buffer plus 5 μl annexin V antibody (FITC; BD Bioscience, Oxford, UK) and 10 μl propidium iodide. The cells were acquired by flow cytometry within 3 hrs.

Statistical analysis

Individual subtractions of isotypes from test histograms were assessed using Kolmogorov–Smirnov (K–S) statistics, with the calculation of critical D values (D_{crit}). Only a D_{crit} resulting in $P < 0.05$ was deemed significant; otherwise, the test results were taken as zero. A comparison of treatments was done using the paired t-test (SigmaStat software, SPSS, Inc., Chicago, IL, USA). *P*-values and standard deviations are shown.

Results

Previously described immunomodulation can be attributed to MG

An RM for the physiologically relevant AGE MG-derived hydroimidazolone was previously shown to block up regulation of CD83 on peripheral blood DC and monocytes and to impair DC stimulatory capacity [9]. Treatment of PBMC with MG alone exerted a comparable effect to RM on the percentage of monocytes/DC expressing CD83 (Fig. 1A) and the intensity of CD83 staining (data not shown). In addition, block of CD83 expression by RM could be prevented by pre-treatment with the MG scavenger N-acetyl cysteine (NAC) (Fig. 1B). Loss of DC stimulatory capacity was also seen after MG treatment of DC before addition to allogeneic T-cell responders in culture (Fig. 2). Responder cells treated with MG before being stimulated by allogeneic DC showed similar proliferation (CFSE assay) as buffer-treated controls (Fig. 2), suggesting that MG affects T-cell stimulation through damage to DC.

MG modulates cytokine production by myeloid and T cells

Before discovering that MG was the modulatory component of our RM, we had shown that RM could inhibit stimulated cytokine production from both myeloid and T cells.

Myeloid cell cytokine production

PBMC cultured with buffer or RM for 4 hrs showed low spontaneous production of the Th2 cytokine IL-4, the Th1 cytokine IL-12 and the immunoregulatory protein IL-10 by each of the $CD14^-$ (putative DC), $CD14^+$ (monocyte-like cell) and $CD14^{++}$ (classic monocyte) myeloid populations. In order to aid assessment of changes with treatment, myeloid cells were stimulated to produce cytokine by co-culture with LPS. After 4 hrs of stimulated culture, the RM reduced the number of IL-10-producing $CD14^+$ and $CD14^{++}$ cells compared with buffer (Fig. 3A), with no overall loss of myeloid cell number (data not shown). No effect of RM treatment on IL-4 and IL-12 production was detectable (data not shown). To confirm this loss of IL-10 protein, $CD14^+$ cells, purified by magnetic bead selection, were cultured with buffer or RM before mRNA was extracted for gene expression analysis by RT-PCR. Around four times less message for IL-10 was found in RM-treated cells compared with buffer controls, with little change seen in the housekeeping gene UbcH5B (data not shown). After 24 hrs of culture with buffer or RM, myeloid cells were producing little spontaneous IL-4, IL-12 or IL-10 and equivalent amounts of stimulated IL-10, suggesting that cells can 'recover' from the earlier RM-induced deficit.

T-cell cytokine production

Although myeloid cells were producing IL-10 in 4 hrs of stimulated culture, $CD8^+$ and $CD4^+$ T cells were not being induced to do the same and the RM had no detectable effect on induction of this cytokine (data not shown). T cells from LPS-stimulated PBMC cultures did however stain positive for IL-10 binding on their surface (data not shown), suggesting that loss of myeloid cell cytokine could have a later 'knock-on' effect.

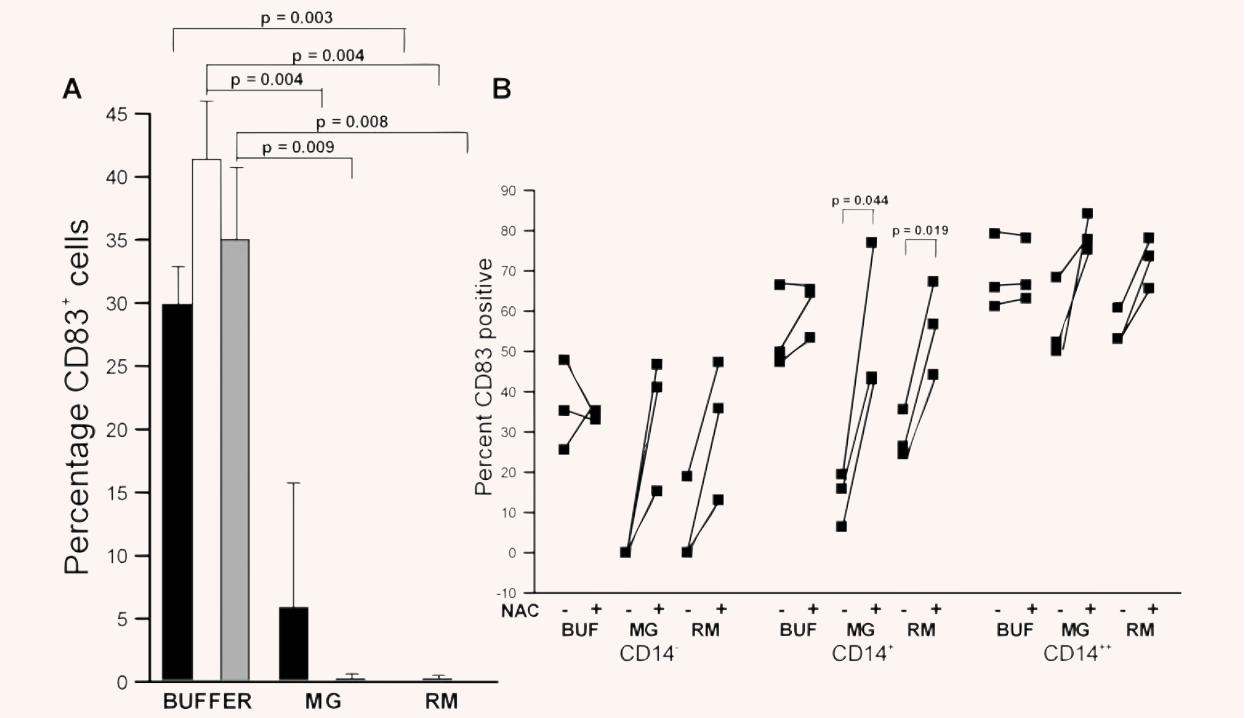

Fig. 1 Block of CD83 expression driven by MG. (**A**) Mean percent expression of CD83 on $CD14^-$ (black bars), $CD14^+$ (white bars) and $CD14^{++}$ (grey bars) myeloid cells from PBMC cultured for 2.5 hrs with sodium phosphate buffer (BUFFER), 500 μM methylglyoxal (MG) or reaction mixture (RM). Extension bars represent standard deviations of means from three experiments, and *P*-values are given for paired t-test comparisons between treatments. (**B**) Paired data sets (from separate experiments to those in (**A**)) for percent expression of CD83 on $CD14^-$, $CD14^+$ and $CD14^{++}$ myeloid cells from PBMC cultured for 2.5 hrs with sodium phosphate buffer (BUF), 500 μM methylglyoxal (MG) or reaction mixture (RM), with (+) and without (–) 600 μM N-acetyl cysteine (NAC). *P*-values from paired t-tests are shown for the effect of NAC.

To study T-cell production of pro-inflammatory cytokines, PBMC were cultured for 2.5 or 24 hrs with and without a mixture of ionomycin and phorbol ester (PMA); in conjunction, these molecules are good stimulators of IFN-γ and TNF-α, making assessment of change easier. In control 2.5 hrs cultures, spontaneous production of cytokine was low, but with stimulation, both CD4 and CD8 cells producing IFN-γ and TNF-α were detected as anticipated. RM treatment caused both $CD8^+$ and $CD4^+$ cells to produce less stimulated IFN-γ and TNF-α than controls (Fig. 3B). This loss was reflected in the proportions of cells producing cytokine (data not shown) and the intensity of anti-cytokine antibody staining (e.g. SED intensity ratio of IFN-γ staining: buffer 49.77, RM 1.48). Slight reduction in the numbers of $CD8^+$ cells was seen in the stimulated RM group compared with buffer (Fig. 4A), but this did not account for loss of $CD8^+$IFN-γ^+ or $CD8^+$TNF-α^+ cells. Supernatant from stimulated RM cultures contained less TNF-α than buffer cultures, confirming loss of intracellular protein production (Fig. 4B).

RT-PCR measurement of gene expression confirmed loss of IFN-γ, with mRNA levels in $CD3^+$ PBMC substantially reduced after 2.5 hrs of RM culture compared with buffer (Fig. 5A). Message for IL-10 in the same cells was also reduced, more so than for IFN-γ (Fig. 5A). In contrast to reduced TNF-α protein, the amount of TNF-α mRNA in RM cultures was raised in relation to buffer (Fig. 5A). The presence and purity of expected PCR products were confirmed by gel chromatography, reduction in IFN-γ and IL-10 products being obvious by eye (data not shown). Gene expression for the housekeeping gene UbcH5b did not vary significantly between treatments.

After 24 hrs of culture, no significant differences were detected between RM and buffer culture for stimulated production of IFN-γ and TNF-α, again suggesting cell 'recovery' from early insult. RT-PCR also revealed no variation in IFN-γ or TNF-α mRNA between culture conditions, but the level of IL-10 mRNA from RM-exposed, stimulated $CD3^+$ cells was approximately four times less than that from buffer-treated cells (Fig. 5B).

MG treatment of PBMC caused a similar loss in the numbers of stimulated IFN-γ^+ (Fig. 6) and TNF-α^+ $CD8/CD4^+$ T cells as RM treatment. MG action on T-cell cytokine production appears to be a direct effect (not reliant on APC orchestration), as depletion of $HLA\text{-}DR^+$, $CD19^+$ and $CD14^+$ cells from PBMC before culture did not prevent MG-induced loss of stimulated IFN-γ and TNF-α (Fig. 6).

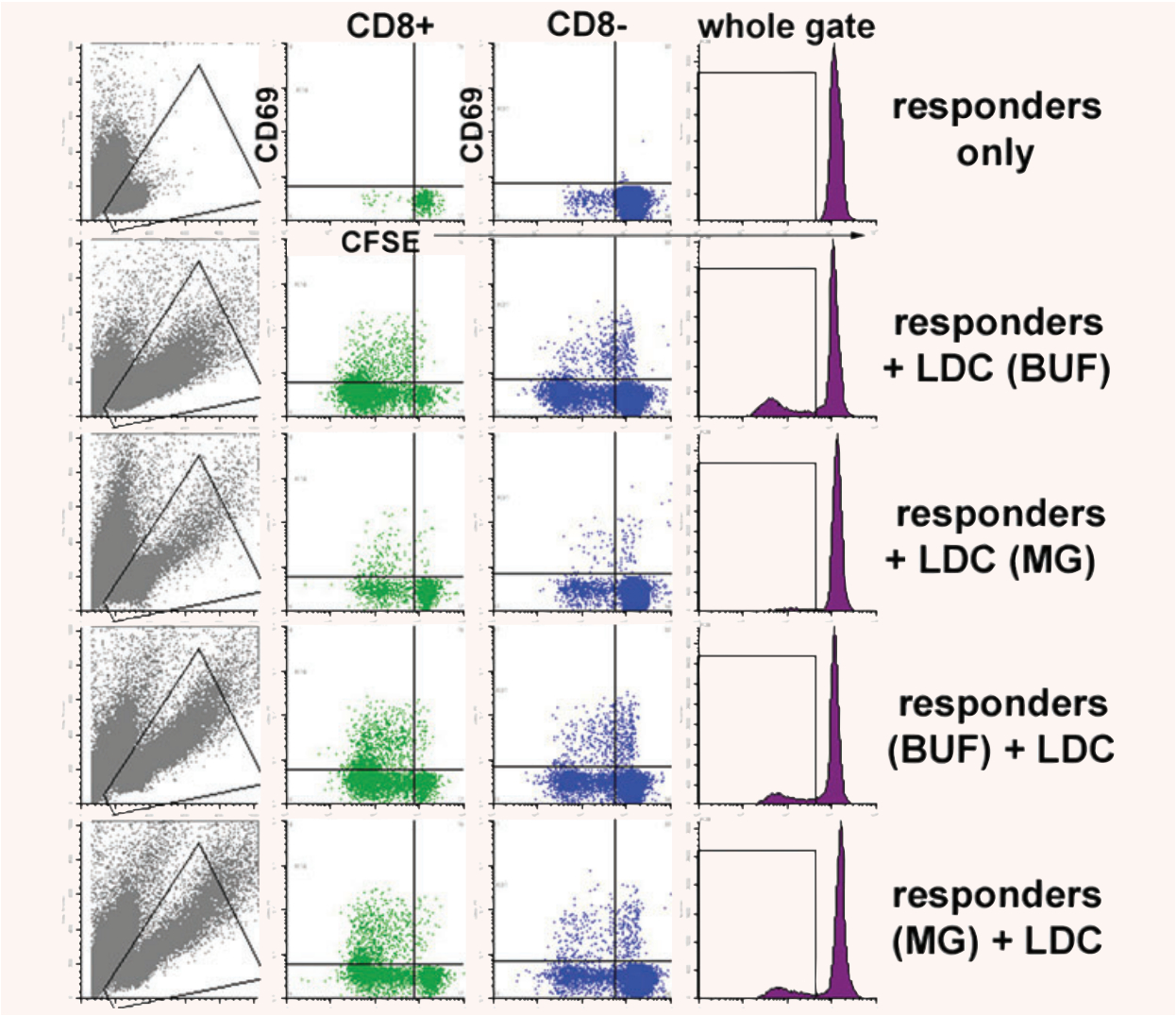

Fig. 2 MG inhibits proliferation in allogeneic culture. Figure shows example analysis histograms for division and activation of $CD3^+CD8^+$ (second column) and $CD3^+CD8^-$ (third column) responders (CD19/CD14/HLA-DR-depleted PBMC) after a 5-day culture with LDC stimulators (non-adherent myeloid cells enriched for DC). Previous to culture, responders and LDC were treated or not treated for 2.5 hrs with sodium phosphate buffer (BUF) or methylglyoxal (MG) and untreated responders were mixed with treated LDC and *vice versa*. First column shows forward and side scatter properties of 5-day cell cultures and columns 2 and 3 show the CFSE and CD69 expression of $CD8^+$ and $CD8^-$ responders, respectively. Final column shows CFSE fluorescence of the total gated population of cells (triangular gate on light scatter plot).

MG reduces expression of MHC class I on myeloid cells

Having shown the loss of DC stimulatory capacity after MG exposure, we were interested to see whether molecules involved in antigen presentation were reduced. We had observed no change in MHC II levels in myeloid cells after culturing with the RM (data not shown); thus, we tested expression of MHC I after MG exposure:

PBMC were cultured for 4 hrs with buffer or MG and expression of MHC I was measured on the $CD14^-$, $CD14^+$ and $CD14^{++}$ myeloid subsets. Regardless of treatment, staining for MHC I was seen on close to 100% of all myeloid cells (Fig. 7A). However, MG reduced the intensity of antibody staining on $CD14^-$ and $CD14^+$ cells compared with buffer, suggesting less MHC I protein per cell (Fig. 7B).

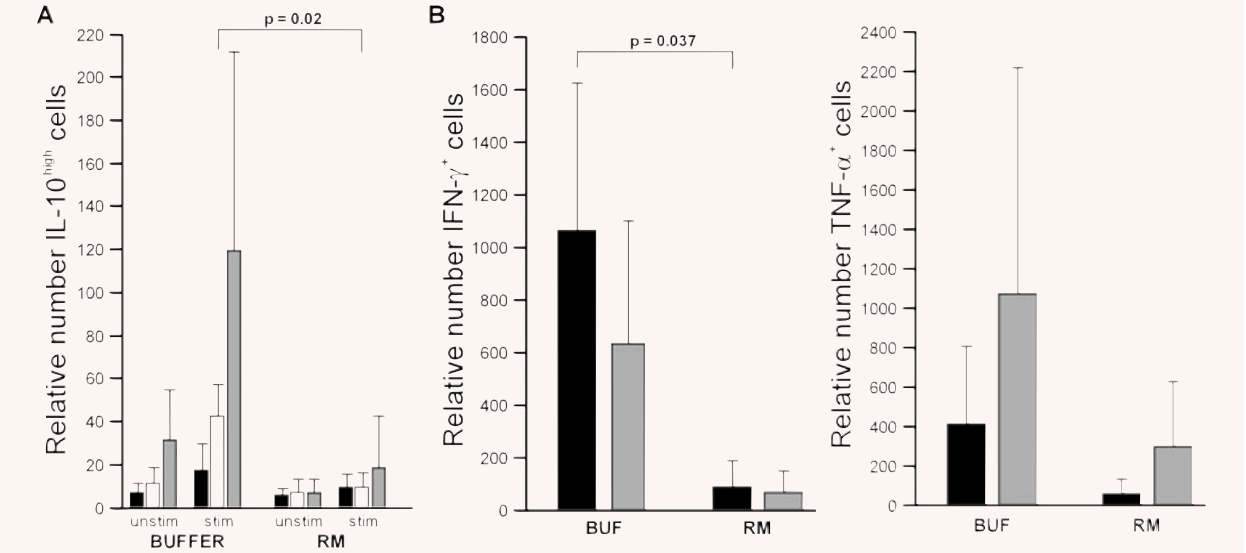

Fig. 3 MG within RM drives altered cytokine protein expression. (**A**) Mean relative numbers of IL-10^{high} CD14^{-} (black bars), CD14^{+} (white bars) and CD14^{++} (grey bars) myeloid cells from PBMC cultured for 4 hrs with sodium phosphate buffer (BUFFER) or reaction mixture (RM) plus monensin with (stim) and without (unstim) 1 μg/ml of LPS. (**B**) Mean relative numbers of IFN-γ^{+} and TNF-α^{+} CD8^{+} (black bars) and CD4^{+} (grey bars) T cells from PBMC cultured for 2.5 hrs with buffer (BUF) or RM plus a mixture of monensin, PMA and ionomycin. Extension bars represent standard deviations of means from three experiments, and *P*-values are given from paired t-test comparisons between treatments.

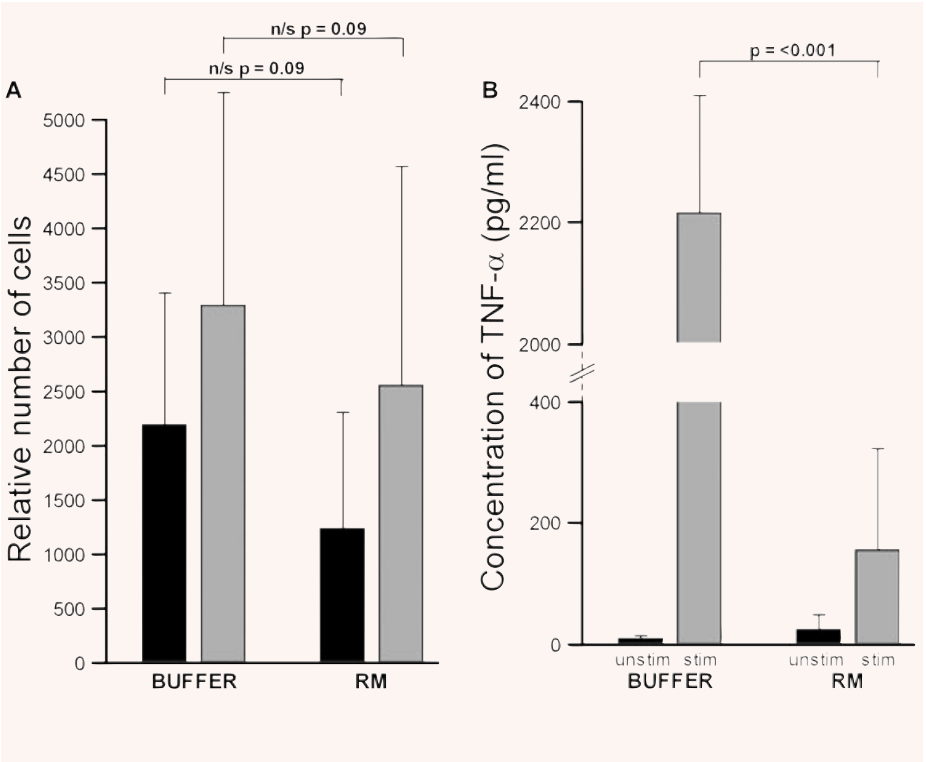

Fig. 4 Numbers of T cells and concentration of TNF-α in supernatants after stimulated culture. (**A**) Mean relative numbers of CD8^{+} (black bars) and CD4^{+} (grey bars) T cells from PBMC after 2.5 hrs of culture with either sodium phosphate buffer (BUFFER) or reaction mixture (RM) plus a mixture of monensin, phorbol ester and ionomycin in three similar experiments. Extension bars represent standard deviations of means. n/s = not significant. (**B**) Mean concentration of TNF-α from the supernatant of PBMC cultured for 2.5 hrs with (stim) or without (unstim) phorbol ester and ionomycin plus either sodium phosphate buffer (BUFFER) or reaction mixture (RM). Extension bars represent standard deviations of means from triplicate wells in three similar experiments, and *P*-value is from a paired t-test comparison between treatments.

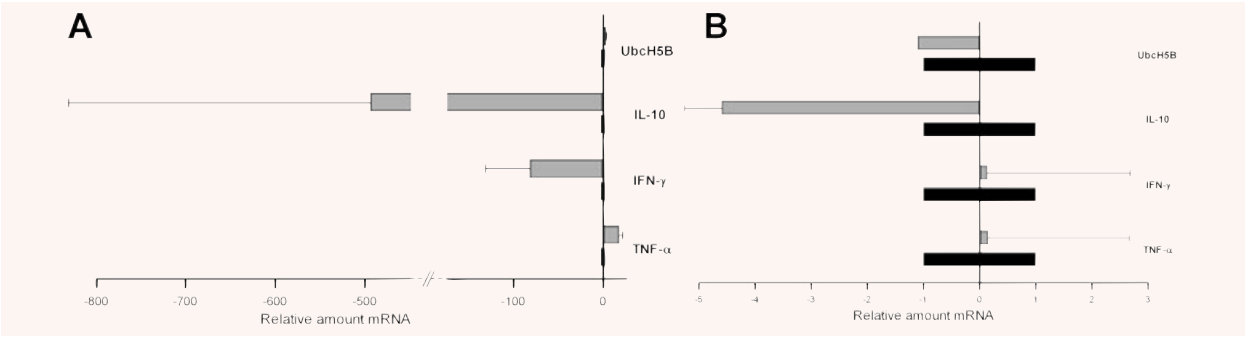

Fig. 5 MG within RM drives altered cytokine mRNA expression. (**A**) Amounts of mRNA for TNF-α, IFN-γ, IL-10 and housekeeping gene UbcH5B from $CD3^+$ (positively selected by magnetic bead separation) PBMC cultured for 2.5 hrs with PMA and ionomycin plus RM (grey bars) relative to buffer (black bars, given as ±1 in each case). Extension bars represent standard deviations of means from three experiments. (**B**) Mean relative amounts of mRNA for IL-10, IFN-γ and TNF-α as well as the housekeeping gene UbcH5B in $CD3^+$ cells from PBMC cultured for 24 hrs with either sodium phosphate buffer (black bars) or reaction mixture (grey bars) plus a mixture of monensin, phorbol ester and ionomycin (last 4 hrs). Amount of mRNA in reaction mixture culture is shown relative to buffer culture (in each instance given as ±1). Extension bars represent standard deviations of means from three similar experiments.

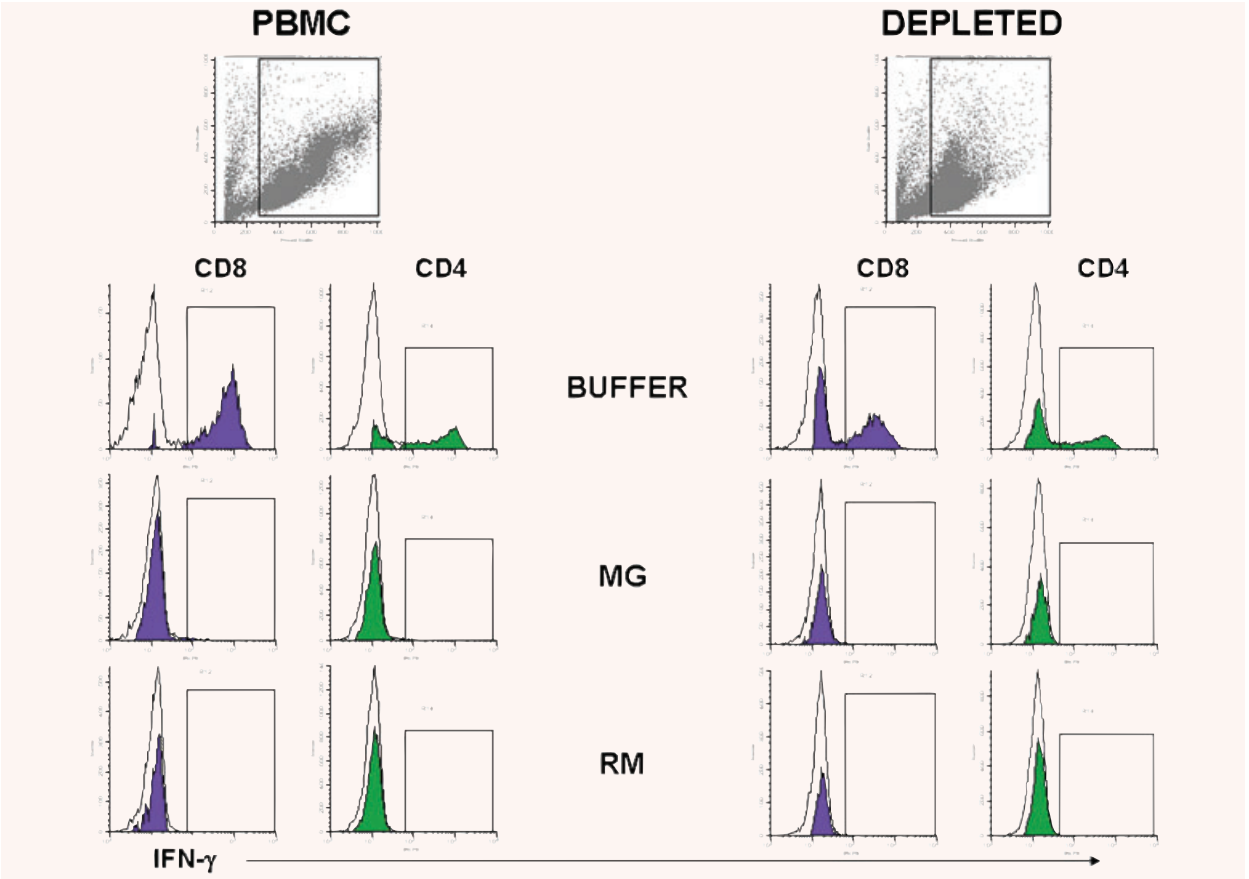

Fig. 6 Loss of IFN-γ production can be attributed to MG. Figure shows example test histograms for analysis of IFN-γ protein within $CD8^+$ and $CD4^+$ T cells from PBMC or PBMC depleted of $HLA\text{-}DR^+$, $CD19^+$ and $CD14^+$ cells cultured for 2.5 hrs with buffer, methylglyoxal (MG) or RM.

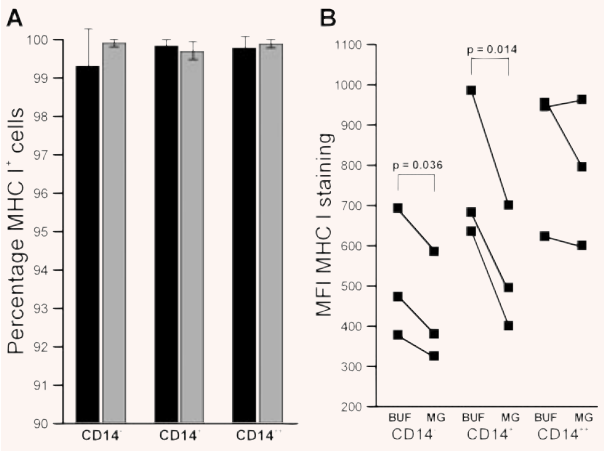

Fig. 7 MG reduces intensity of MHC I staining. (**A**) Mean percent expression of MHC class I on $CD14^-$, $CD14^+$ and $CD14^{++}$ myeloid cells from PBMC cultured for 4 hrs with buffer (black bars) or 500 μM MG (grey bars). Extension bars represent standard deviations of means from three experiments. (**B**) Paired data sets for mean fluorescence of MHC I staining on $CD14^-$, $CD14^+$ and $CD14^{++}$ myeloid cells from the same three experiments. *P*-values from paired t-test comparisons between treatments are given.

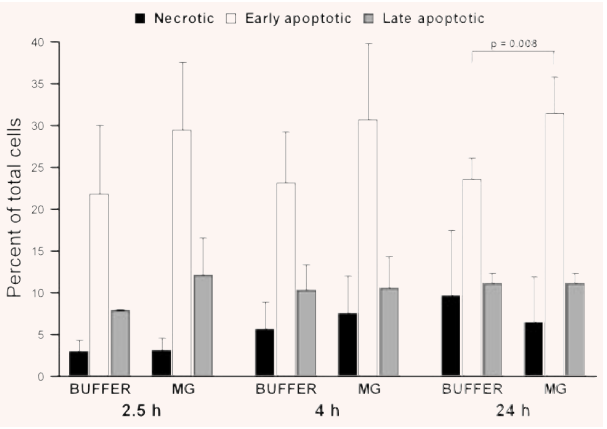

Fig. 8 MG induction of apoptosis. Figure shows mean percentage of total PBMC that stained as necrotic (propidium $iodide^+$), early apoptotic (annexin V^+) and late apoptotic (propidium $iodide^+$ and annexin V^+) after 2.5, 4 or 24 hrs of culture with sodium phosphate buffer or 500 μM MG. Extension bars represent standard deviations of means from three experiments, and *P*-value from paired t-test comparisons between treatments is given.

MG increases myeloid cell apoptosis

MG has been linked to cell death in other systems, largely through added oxidative stress [2]. We assessed whether MG was predisposing PBMC in our cultures to apoptosis or necrosis: cells were incubated with buffer or MG for 2.5, 4 and 24 hrs before staining with FITC-conjugated annexin V and the vital dye propidium iodide (PI). At 2.5 and 4 hrs, the proportions of necrotic (PI^+), early apoptotic (annexin V^+) and late apoptotic (annexin V^+/PI^+) cells were equivalent between treatments (Fig. 8). After 24 hrs of culture, MG increased the percentages of early apoptotic cells compared with buffer, but had not affected necrosis or late apoptosis (Fig. 8).

Discussion

MG is a potent precursor of glycation damage, which adds to tissue dysfunction in disease states of high MG concentration, such as diabetes mellitus. Here, we show that MG is also an effective modifier of immune function, responsible for blocking surface and cytokine protein expression and for reducing the stimulatory capacity of DC, after only short culture. MG also increases the propensity of cells to apoptose after longer culture periods. We suggest that this glycating agent may not only play a role in the increased rate of infection seen in diabetes patients but also has the potential for much farther-reaching consequences.

We previously showed that an RM for MG glycation of peptide could block up regulation of the DC surface protein CD83 and reduced DC potency for T-cell stimulation in allogeneic culture [9]. This RM could be seen by mass spectrometry to contain both MG-derived hydroimidazolone peptide and unreacted peptide, so we assumed that all of the highly reactive MG had been exhausted. However, MG binds initially reversibly to proteins, with flux in and out of solution [10]. We thus reconsidered that it may be the residual MG in solution that is driving immune modulation by the RM. We show here that culture of PBMC with MG indeed caused equivalent block of CD83 expression and loss of DC stimulatory capacity to RM and that CD83 expression could be recovered by pre-treatment with the MG scavenger NAC, in both MG and RM cultures. NAC is also a free radical scavenger by virtue of the same thiol group that is used to react with MG. It is thus possible that NAC confers some antioxidant benefit to cells, aside from removing MG from the system. Also, adding the equivalent RM without MG (buffered peptide under reaction conditions) has no effect on cellular protein expression [9].

We present data showing that RM blocks stimulated production of IL-10 from myeloid cells and of IFN-γ and TNF-α from T cells. Cytokines produced by myeloid cells can influence those in T cells that they stimulate [11, 12]. While myeloid cells in our cultures were producing IL-10, T cells were not being induced to do so. T cells in these cultures stained positive for IL-10 on their surfaces, suggesting that they were binding IL-10 made by the myeloid cells. This advocates that loss of IL-10 production by myeloid cells after exposure to MG may have an effect on the signals received by T cells and thus on their future cytokine production and function. While T cells in short cultures had not produced detectable IL-10 protein, mRNA for IL-10 was reduced after RM

culture, compared with buffer. This also suggests that the ability of T cells to produce IL-10 may be compromised by MG.

Both RM and MG alone reduced stimulated production of pro-inflammatory cytokines from T cells, after only short culture. In the case of IFN-γ, this loss of protein could be attributed to reduced mRNA. Loss of TNF-α intracellular protein was confirmed within the supernatants by ELISA; however, the message for TNF-α was raised after RM exposure at this time. This discrepancy between message and protein suggests that the action of MG may be post-translational within the TNF-α pathway or that MG is altering the kinetics of TNF pathway activation/transcription.

MG also reduced the level of MHC I antibody staining on myeloid cells compared with buffer culture. Less amount of this antigen-presenting molecule on the cell surface may suggest internalization or shedding of protein, with consequences for efficient T-cell priming. The inhibition of NK cell effector function is related to MHC I expression on target cells [13]; thus, loss of MHC I after MG exposure could also induce cell killing.

MG has been linked to cell death in several cellular systems, largely through induction of ROS [2]. We found that MG increased the levels of early apoptosis only after 24 hrs of culture, with no obvious effects on necrosis. As this increased cell death was not obvious early, or to a large extent, it may suggest that death is triggered as a result of other MG-induced cellular changes and that the described loss of surface and intracellular proteins is independent of apoptosis induction.

The levels of MG are raised in diabetes patients, two- to six-fold compared with controls [14]. A recent estimate of MG in diabetes patients' blood was in the region of 0.2 μM [15]. However, the majority of MG is likely to be bound to protein [10] and the amount in free circulation would be much less than the actual level in the vasculature. Also, MG concentration will vary locally depending upon the supply of precursors, activity of detoxification systems and location of the cell/tissue. A concentration of 310 μM MG was reported in hamster cell cultures [16]. Thus, immune damage by 500 μM MG shown here may well be relevant *in vivo*, but more 'physiological' levels (50 μM) still inhibited CD83 up-regulation in myeloid cells (unpublished data).

We now start to understand how advanced glycation by MG may have roles in immune regulation in diabetes as well as in vascular dysfunction: We have seen that in DC, MG blocks expression of CD83, an important protein in DC:T-cell contact and stimulation [17]. We demonstrated a reduction in the capacity of these professional APCs to stimulate T cells in culture and disruption in production of pro-inflammatory and immunoregulatory cytokines at protein level, plus variable effects on gene expression. PBMC exposed to MG do not mount suitable defences against pro-inflammatory stimuli, which may include *in vivo* pathogens. This lack of reaction is unlikely to be an induction of tolerance, as DC were not up-regulating the inhibitory molecule immunoglobulin-like transcript-3 (ILT-3) (unpublished data) and lost the ability to produce IL-10. IL-10 plays a key role in limiting inflammatory reactions, including direct effects on the function of both myeloid and $CD4^+$ T cells [18].

This immune damage may have a role in diabetes, especially in the susceptibility to infection, with pathogens being able to thrive while immune defence is compromised. A number of studies have reported immune dysfunction in diabetes, with reduced Th1-associated chemokine receptors and cytokines [19], impaired T-cell proliferation in a rat model [20] and commonly, reduced IFN-γ production at the onset of disease [19, 21–23]. These changes are often not seen in long-standing disease, an observation easily explained by considering the self-promoting, escalating nature of tissue RAGE ligation. In established disease, immune dysfunction may be masked by a RAGE-associated pro-inflammatory and pro-adhesive milieu [24].

Although MG-induced damage in our system was short term, our model was a closed system, with contained populations of cells and limited dose of MG. *In vivo*, we would expect cells to encounter continually raised MG levels [14]. In this way, damage may be sustained, resulting in chronic immune dysfunction. The use of cellular cultures or animal models to assess damage by MG is necessary prior to investigating equivalent damage in patients. We used freshly isolated blood mononuclear cells, not matured or differentiated unnaturally by cytokines and growth factors, meaning that our results should, to a substantial extent, be applicable to *in vivo* reactions. The use of a single bolus of MG and a cellular *in vitro* system are limitations to this study. However, we are interested to further research by investigating the effect of repeated small doses of MG, the nature of the TNF-α protein/message dichotomy and the result of cytokine loss on the differentiation of T cells after stimulation by MG-exposed APC. It will also be important to identify key targets of MG damage, in cells of the immune system and elsewhere, in order to further knowledge of MG-associated disease and to provide direction for therapeutic advances: MG reacts with arginine, lysine and cysteine residues of proteins, as well as with guanine units in DNA [25]. Depending upon the configuration and environment of proteins, some residues will be more prone to attack from MG than others, creating 'hot spots' for glycation [26, 27]. Such damage is likely to affect the structure and function of many physiological proteins and enzymes, not just in an immunological setting. Bioinformatics analysis of receptor binding domains has shown that arginine and lysine are the most common amino acids at such sites of protein–protein interaction [28], and MG has already been implicated in the inactivation of cysteine active sites in cysteine proteases [29], which are involved in multiple physiological processes, including antigen presentation. The list of potential MG targets and the capacity for damage are thus huge, with disruption of protein binding and enzyme functions likely in numerous bodily settings. It may be interesting to perform proteomic analyses on immune cells exposed to MG to identify molecules containing AGEs. These altered proteins would provide clues to the key pathways of MG damage that lead to the observed immunomodulation.

The levels of related α-dicarbonyls glyoxal and 3-deoxyglucosone plus glycolytic by-product glucosamine are also raised in diabetes, accompanied by dysregulation of lipids and their metabolites. Any of these metabolic imbalances could also impact on immune function and are worthy of future investigation.

In conclusion, we have shown that methylglyoxal potently modifies immune function, causing multiple and varied deficiencies and reducing the ability of immune cells to respond appropriately to stimuli. We suggest that these findings may indicate a role for MG in the susceptibility of diabetes patients to infection, on top of the well-documented vascular dysfunction through AGE formation.

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