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Ethyl pyruvate and ethyl lactate down-regulate the production of pro-inflammatory cytokines and modulate expression of immune receptors

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ABSTRACT

Esters of α-oxo-carbonic acids such as ethyl pyruvate (EP) have been demonstrated to exert inhibitory effects on the production of anti-inflammatory cytokines. So far, there is no information about effects, if any, of ethyl lactate (EL), an obviously inactive analogue of EP, on inflammatory immune responses. In the present study, we provide evidence that the anti-inflammatory action of α-oxo-carbonic acid esters is mediated by inhibition of glyoxalases (Glo), cytosolic enzymes that catalyse the conversion of α-oxo-aldehydes such as methylglyoxal (MGO) into the corresponding α-hydroxy acids using glutathione as a cofactor. *In vitro* enzyme activity measurements revealed the inhibition of human Glo1 by α-oxo-carbonic acid esters, whilst α-hydroxy-carbonic acid esters such as EL were not inhibitory. In contrast, both EP and EL were shown to suppress the Lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1β, IL-6 and IL-8 from human immunocompetent cells, and modulated the expression of the immune receptors HLA-DR, CD14 and CD91 on human monocytes. Here, we show a crossing link between glyoxalases and the immune system. The results described herein introduce glyoxalases as a possible target for therapeutic approaches of immune suppression.

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Abbreviations: BGCD, *p*-bromobenzylglutathione cyclopentyl diester; EL, ethyl lactate; EP, ethyl pyruvate; HAGH, hydroxyacyl glutathione hydrolase (glyoxalase 2); IFN, interferon; Glo1, glyoxalase 1; GSH, L-glutathione; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MGO, methylglyoxal; NAC, *N*-acetyl cysteine; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline.

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1. Introduction

Recently, esters of pyruvate such as EP have been demonstrated to provide protection from ischemia/reperfusion-induced tissue injury and to rescue mice from endotoxin-induced lethality and from polymicrobially induced severe peritonitis [1,2]. The rationale behind pointed toward attenuation of release of early and late pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and high-mobility group box 1 (HMGB1). The molecular basis of EP action was suggested to be its interference with NF-κB signal transduction pathway, however, the detailed mechanism is not fully elucidated yet [3]. In the present study, we provide evidence that the anti-inflammatory action of EP is mediated by inhibition of glyoxalases. Glyoxalase 1 (Glo1, E.C. 4.4.1.5) and Glo 2 (E.C. 3.1.2.6) are cytosolic enzymes that catalyse the conversion of α-oxo-aldehydes into the corresponding α-hydroxy acids using L-glutathione (GSH) as a cofactor [4]. MGO is a reactive α-oxo-aldehyde that is mainly formed from non-enzymatic fragmentation of triose phosphates along the glycolytic pathway. MGO, when present in high concentrations, reacts rapidly with both, proteins and nucleic acids, and this is the presumed basis of its cytotoxicity [5].

To protect cells against damage caused by MGO, the glyoxalase system provides an efficient ubiquitous detoxification pathway. In many prokaryotic and eukaryotic cells, an increased MGO burden is prevented by up-regulation of glyoxalases. This enables tumor cells to escape intoxification by MGO formed paracatalytically via increased aerobic glycolysis [6].

Here, we show a link between glyoxalases and the immune system. Our studies reveal a remarkable and unexpected regulation of different immune functions by Glo1. Balanced inhibition of Glo1 in immune cells were found to modulate LPS-mediated release of pro-inflammatory cytokines, surface expression of receptors involved in antigen uptake and processing as well as Th1/Th2 cell responses upon antigenic and mitogenic stimulation. The results reveal glyoxalases as new targets for immune suppression.

2. Materials and methods

2.1. Reagents and cells

Ficoll-Paque Plus® was purchased from GE Healthcare Life Science (Freiburg, Germany). RPMI-1640 medium and heat-inactivated fetal calf serum (FCS) were purchased from Invitrogen (Karlruhe, Germany). Magnetic cell separation system (MACS®) was supplied by Miltenyi Biotec (Bergish Gladbach, Germany). GSH was purchased from Roth (Karlsruhe, Germany). Cell proliferation reagent WST-1 was obtained from Roche (Mannheim, Germany). BD™ Cytometric Bead Array (CBA Human Inflammation kit), BD FACS™ Lysing Solution and isotype control antibodies were obtained from BD Biosciences (Heidelberg, Germany). EP, EL, MGO, N-acetyl cysteine (NAC), Phytohemagglutinin (PHA), BCIP/NBT-Blue Liquid substrate, HEPES, $MgCl_2$, KCl, EDTA, glycerol, Triton X-100, phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), diamino benzidine (DAB), H_2O_2, protease inhibitors and LPS (*E. coli* Serotype O111:B4) were purchased from Sigma (Steinheim, Germany). Sodium dodecyl sulfate (SDS) was purchased from Serva (Heidelberg, Germany). Cellulose nitrate membranes were obtained from Whatman Schleicher & Schuell (Dassel, Germany). Trypan blue, phosphate-buffered saline (PBS) and Tris-buffered saline (TBS) were purchased from Seromed (Berlin, Germany). RNeasy Mini Kit was purchased from Qiagen (Hilden, Germany), AMV Reverse Transcriptase und Oligo (dT) Primer were from Promega (Heidelberg, Germany) and Ready-To-Go™ PCR beads from GE Healthcare (Munich, Germany). Goat serum was purchased from Dako (Glostrup, Denmark). APC-labeled anti-HLA-DR and PE-labeled anti-CD14 mAbs were obtained from Immunotech (Beckman Coulter, Krefeld, Germany), FITC-labeled anti-human CD91, anti-human Glo1 (clone 4 C10) and anti-mouse Glo1 (clone 4C7) mAbs from BioMac (Leipzig, Germany), biotin-labeled anti-human IFN-γ mAb (1-D1K) and streptavidin-labeled-horse radish peroxidase (HRP) from Mabtech (Hamburg, Germany). Rabbit anti-human β-actin Ab was purchased from Abcam (Cambridge, UK), goat anti-mouse HRP-Ab from Dako (Glostrup, Denmark), and Alexa 488-labeled goat anti-mouse Ig from Invitrogen (Karlsruhe, Germany). ELISPOT-plates (ELISPOT Multiscreen-IP, 45 μm) were delivered by Millipore (Beford, U.S.A). Cell culture plates were obtained from Greiner Bio-one (Frickenhausen, Germany).

Blood samples used in this study were drawn from healthy donors at the Blood Bank of Leipzig University Clinic, Germany. The experiments were approved by the local authorities and the informed consent of all participating subjects was obtained. Mouse macrophages (RAW 264.7, ATCC, No. TIB-71) were supplied by LGC Promochem (Wesel, Germany).

2.2. Glyoxalase assay

The determination of Glo1 activity has been adapted according to Mannervik et al. [7]. To evaluate the effect of EP and EL on Glo1 enzymatic reaction, variable concentrations of these compounds were pre-incubated with 2 mM GSH for 4 min and the reaction was started by addition of 2 mM MGO and the enzyme. IC_{50} values were calculated from the inhibition curves. In another set of experiments, Glo1 activity was evaluated at fixed concentrations of EP/MGO and variable concentrations of GSH. Human Glo1 was purified as formerly described [8].

2.3. Whole blood assay

LPS (10 ng/ml), test substances and 200 μl heparinized human blood were co-incubated with RPMI-1640 medium (1 ml final volume) in 24-well culture plates for 6 h at 37 °C with 5% CO_2. Following incubation, plates were centrifuged at 2000 × *g* for 10 min and supernatants were stored at −20 °C for later evaluation for pro-inflammatory cytokines.

2.4. Isolation of human blood monocytes

Peripheral blood mononuclear cells (PBMC) were separated from heparinized human blood by Ficoll-Paque Plus® separation and CD14+ monocytes were purified further by magnetic

cell sorting using human CD14-microbeads on an LS column as previously described [9]. Purity of separated CD14+ monocytes was >98%.

2.5. Synthesis of glyoxalase inhibitor

Preparation of the glyoxalase inhibitor BGCD was done as recently described [10].

2.6. Cytokine analysis using cytometric bead array

Assessment of cytokine levels in supernatants of human blood cell cultures was accomplished using CBA. The procedure was carried out according to the manufacturer's instruction. Briefly, 10 μl of each mouse inflammation capture bead suspension were mixed for each sample, and 50 μl of mixed beads were transferred to each assay tube. Standard dilutions or test samples (50 μl) were added to the appropriate sample tubes, PE detection reagent (20 μl) was added and the tubes were incubated in dark for 2 h at RT. Samples were washed (1 ml wash buffer/sample) and centrifuged at 200 × *g* for 5 min. Finally, wash buffer (250 ml) was added and samples were analyzed using a FACSCalibur and the CellQuest™ Software (BD Biosciences).

2.7. Flow cytometric analysis of cell surface receptors

Freshly drawn heparinized blood (200 μl) was diluted with RPMI-1640 medium up to a total volume of 1 ml in 24-well culture plates. LPS (10 ng/ml), test substances and blood were mixed. Following 6-h incubation (at 37 °C, 5% CO_2), sample were added to pre-cooled plastic tubes and washed three times with PBS-1% FCS. Cell suspensions (100 μl) were then incubated for 30 min at 4 °C with 10 μl of APC-labeled anti-HLA-DR mAb (1:10), PE-labeled anti-CD14 (1:10), FITC-labeled anti-CD91 (10 μg/ml) or respective isotype control mAb (10 μg/ml) for correcting unspecific binding. Red blood cells were then lysed by 2 ml lysing solution. Cells were centrifuged (200 × *g* for 10 min at 4 °C), washed twice with PBS-1% FCS and finally fixed with 250 μl PBS (2% para-formaldehyde). Fluorescence measurement and analysis were performed using a FACSCalibur and the CellQuest software (BD Biosciences).

2.8. ELISPOT experiments

ELISPOT-plates were coated with 100 μl anti-human IFN-γ mAb (1-D1K, 1 μg/ml) and incubated overnight. After washing the cavities with PBS, blocking was accomplished with PBS-10% FCS (150 μl/cavity) incubated for 1 h at 37 °C. Cell suspension (2×10^5 PBMC/ml) were transferred into each cavity and stimulated with 1.5 μl of a recombinant Rubella-like particles (6.4 mg/ml) [11] and with 0.2 μg/ml PHA, in the absence or presence of successive doses of EL in a final volume of 200 μl. After incubation at 37 °C for 18 h, the plates were washed with PBS (10% FCS) and then incubated for 2 h with 100 μl of the biotin-labeled anti-IFN-γ mAb (7-B6-1, 2 μg/ml). Detection was then accomplished by adding streptavidin-labeled-HRP (1:1000) and followed by incubation for 1 h. Color development was obtained by addition of 100 μl of BCIP/NBT-Blue Liquid substrate solution. After washing and air-drying, the spots were read with an ELISPOT-Reader (BIOREADER 3000, Bio-SYS, Karben, Germany).

2.9. Cell viability test

The viability of cells was measured by trypan blue exclusion. An aliquot of cell suspension (about 5×10^5 cells/ml) was centrifuged for 5 min at 100 × *g* and the supernatant was discarded. The cell pellet was re-suspended in 1 ml PBS or serum-free complete medium. 20 μl of cell suspension and 0.4% trypan blue were mixed, and the mixture was incubated at room temperature. Cells have been counted within 3–5 min by applying a drop of the trypan blue/cell mixture to a hemacytometer. The unstained (viable) and stained (nonviable) cells have been counted separately, and finally the percentage of viable cells (total number of viable cells per milliliter × 100/total number of cells per milliliter) was calculated.

2.10. Conventional RT-PCR

Total RNA was isolated from human monocytes and fibroblasts using the RNeasy Mini Kit and 1 μg was reverse transcribed using AMV Reverse Transcriptase und Oligo (dT) Primer in a volume of 10 μl. PCR was performed using 1 μl cDNA, ReadyToGo PCR-beads and 1 μl of each primer pairs. The forward (f) and reverse (r) primer sequences of GLO1 (GenBank accession no. NM_006708) are f: 5′-GGT CCC GTC GTC TGT GAT AC-3′; r: 5′-GAA TCG GGA CAG TGA TCC AT-3′; and of hydroxyacyl glutathione hydrolase (HAGH) (GenBank accession no. NM_005326) are f: 5′-TTT CTG CCA CAC AGA TTT GC-3; r: ′5′-CAG CAG GAA AGC CAG TTA CC-3′.

For all sets a 25 μl PCR reaction was subjected to 30 cycles, each of 30 s at 95 °C, 30 s at 58 °C, 2 min at 72 °C followed by a 10 min extension at 72 °C. PCR products (5 μl) were separated on 1% agarose gel and finally stained with ethidium bromide.

2.11. Immunoblot

Cellular extracts of human monocytes and mouse RAW 264.7 macrophages were obtained by cell lysis with buffer containing 10 mM Hepes, 2 mM $MgCl_2$, 15 mM KCl, 0.1 mM EDTA, pH 7.8, 1% glycerol, 0.1% Triton X-100, 1 mM PMSF, 1 mM DTT and 0.3% protease inhibitor cocktail. 20–40 μg protein of cell lysates were loaded to SDS-pore gradient gels (4–20%) and run under reducing conditions. Proteins were blotted to cellulose nitrate membranes and Glo1 was detected by monoclonal antibodies to human (clone 4 C10) or mouse Glo1 (clone 4C7) (1 μg/ml), in combination with goat anti-mouse Ig-HRP (1:1000). Beta-actin was analyzed for comparison using rabbit anti-β-actin Ig (1:2000). Color was developed using DAB/H_2O_2.

2.12. Confocal Laser Scan Microscopy (LSM)

Cellular localization of Glo1 was analyzed in mouse macrophages cultured in RPMI 1640 medium containing 1% penicillin–streptomycin supplemented with 10% FCS following incubation at 37 °C and 5% CO_2. Cells grown at chamber slides were fixed by 2% para-formaldehyde and blocked with 5% goat serum in TBS-0.3% Triton X-100 buffer (1.5 h). After

washing, the slides were incubated with 20 μg/ml anti-mouse Glo1 mAb (clone 4C7) in TBS buffer containing 0.3% Triton X-100 and 5% BSA, overnight. Finally, the slides were treated with 20 μg/ml Alexa 488-labeled goat anti-mouse Ig for 1 h. Immunostained cells were analyzed by LSM (Carl Zeiss, Jena, Germany) using a Plan Apochromat 62/1.4 Oil DIC; 2× zoom objective.

2.13. Statistical analysis

The data are presented as the mean ± S.D. of three independent experiments. For calculation of the $IC_{50,}$ values from dose-response curve data points were fitted with a logistic Hill equation:

$$Y = Y_0 + \frac{(Y_\infty - Y_0)}{1 + 10^{x-\log(IC50)}}$$

3. Results

3.1. Ethyl pyruvate inhibits glyoxalase 1 in vitro

We found that EP inhibited the enzymatic reaction of human Glo1. This enzyme catalyses the conversion of MGO and GSH to S-lactoylglutathione. The calculated IC_{50} values were 7.01 ± 1.9 mM measured at a substrate concentration of 2 mM (Fig. 1A). Surprisingly, the reduced form, EL, did not serve as an inhibitor for the isolated human enzyme in vitro indicating the necessity of a ''dicarbonyl'' structural element for inhibitory properties. The inhibitory effect of EP could not be abrogated by increasing GSH indicating that EP does not act by GSH displacement rather than by enzyme inhibition (Fig. 1B). To gain further insights into the effect of EP at cellular level, we examined the response of human immunocompetent cells to pyruvic and lactic esters.

3.2. Human and mouse monocytes/macrophages express glyoxalases

First, we studied the expression of Glo1 in monocytes and fibroblasts (for comparison) at mRNA and protein levels (Fig. 2). PCR revealed that human monocytes and fibroblasts express both GLO1 and HAGH indicated by the appearance of the 666 and 896 bp amplicons, respectively. Additionally, Sanger sequencing of the amplification products confirmed their identity. Western blot analysis using anti-Glo1 mAb revealed the presence of the enzyme (22 kDa) in the cytosolic extract of human monocytes as well as of mouse macrophages. As expected, the cytosolic localization of Glo1 immune reactivity could be verified by Confocal Laser Scanning Microscopy of mouse RAW 264.7 macrophages.

3.3. Ethyl pyruvate and ethyl lactate suppress the release of the pro-inflammatory cytokines

To prove our assumption that the proposed anti-inflammatory effect of EP is mediated via Glo1 inhibition, we performed whole blood assays in conjunction with endotoxin stimulation. Addition of LPS to heparinized human blood of healthy subjects induced the release of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, IL-8 but not IL-10. Co-incubation of cells with LPS and increasing concentrations of EP attenuated cytokine release in a concentration-dependent manner (Fig. 3A–D); exception was a slight increase of IL-1β at 1 mM EP. The IC_{50} values for cytokine release inhibition were 2.38 ± 1.1 mM, 5.537 ± 1.3 mM, 4.21 ± 0.9 mM, and 18.8 ± 5.7 mM for TNF-α, IL-1β, IL-6, and IL-8, respectively. Control experiments revealed the absence of any stimulatory effect of EP when used alone. Surprisingly, EL which was unable to inhibit Glo1 activity *in vitro*, exerted an inhibitory effect on pro-inflammatory cytokine production comparable to EP in the *ex vivo* whole blood assay

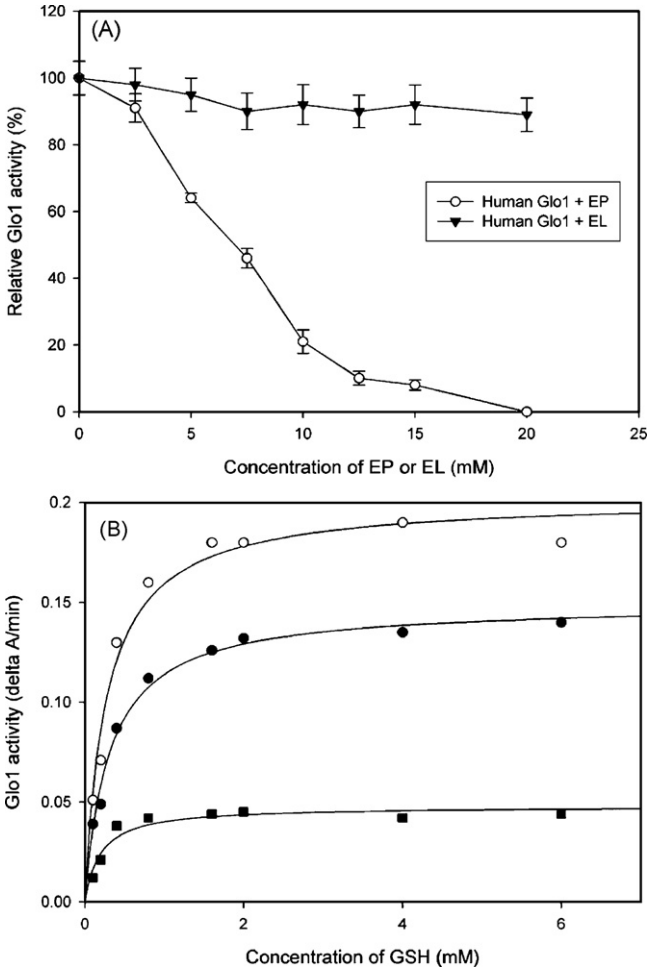

Fig. 1 – Effect of ethyl pyruvate (EP) and ethyl lactate (EL) on enzymatic activity of human glyoxalase 1. (A) Variable concentrations of EP or EL were incubated with 2 mM L-glutathione (GSH) and 2 mM methylglyoxal (MGO) for 4 min and the reaction was started by addition of 70 mU Glo1 in a 1-ml reaction volume. Enzymatic activity in the absence of EP or EL was set as 100% (ΔE/min = 0.13). (B) Variable concentrations of GSH were incubated with 0 (○), 5 mM (□) or 10 mM (■) EP, and the reaction was started by adding 2 mM MGO and 70 mU Glo1. The calculated values for V_{max} (ΔA/min)/K_m (mM) were 0.20 ± 0.01/0.27 ± 0.04 (○), 0.15 ± 0.01/0.31 ± 0.03 (□), and 0.048 ± 0.003/0.20 ± 0.05 (■).

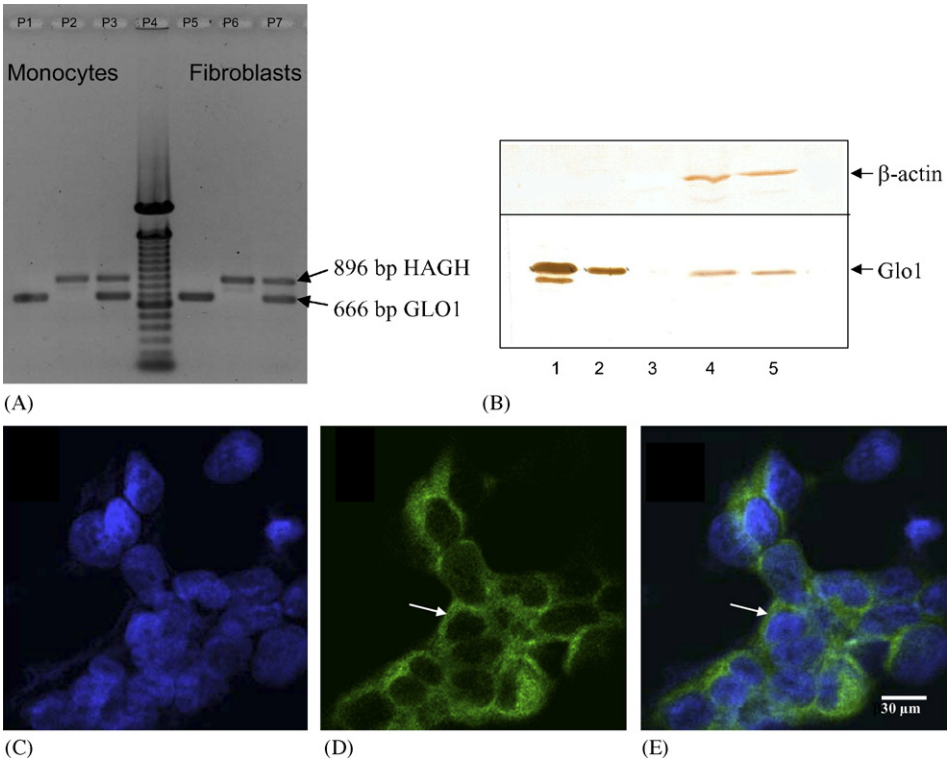

Fig. 2 – Detection of glyoxalase protein and mRNA in human and mouse cells. (A) RT-PCR for amplifying *GLO1*- and *HAGH*-cDNA (glyoxalase 2 gene) derived from human monocytes and fibroblasts. PCR products were generated and separated on 1% agarose gel and finally stained with ethidium bromide. P1 and P5: *GLO1*; P2 and P6: *HAGH*; P3 and P7: multiplex analysis of *GLO1* and *HAGH*. (B) Immunoblot of Glo1: Cellular extracts of human monocytes and mouse RAW 264.7 macrophages were immunoblotted for Glo1. 1: Purified human Glo1 (1 μg); 2: purified human Glo1 (0.1 μg); 3: free lane; 4: cell lysate form human monocytes; 5: cell lysate from mouse RAW 264.7 cells. (C–E) Immunofluorescence staining of Glo1 in mouse macrophages (C) RAW 264.7 macrophages were immunostained for Glo1 (green); (D) nuclei were stained with DAPI (blue), and (E) superimposed picture of C and D. Arrows show cytosolic localization of Glo1.

(Fig. 3E–H). The corresponding IC_{50} values were 5.67 ± 1.7, 10.43 ± 2.4, 8.88 ± 2.0, and 4.93 ± 1.2 mM for TNF-α, IL-1β, IL-6 and IL-8, respectively.

3.4. *Active site-directed inhibitor of glyoxalase 1 acts anti-inflammatory*

To verify that inhibition of the glyoxalase might be the cause for attenuation of the cytokine release, we tested the active site-directed inhibitor BGCD for comparison. BGCD does not inhibit Glo1 *in vitro*, however, following its de-esterification into *S*-*p*-bromobenzylglutathione in cells, a strong inhibition of Glo1 with a K_i value of 0.16 μM was described [4]. In the current study, BGCD, at remarkable low concentrations, reduced the LPS-induced cytokine release in the whole blood assay (Fig. 4). For example, the IC_{50} value for inhibition of TNF-α release amounts to 0.28 ± 0.1 μM, which is more than three orders of magnitude lower compared to that of EP (Fig. 3A) or EL (Fig. 3E).

3.5. *Methylglyoxal inhibits release of pro-inflammatory cytokines*

Inhibition of Glo1 is known to cause an increase of the intracellular concentrations of MGO, for which an anti-inflammatory effect has not been observed yet. Thus, if the intracellular elevation of MGO may account for the observed anti-inflammatory effects, supplementation of whole blood with the diffusible reactive MGO should mimic the effects of a glyoxalase inhibitor. Indeed, MGO decreased the release of pro-inflammatory cytokines upon LPS-stimulation in a concentration-dependent manner (Fig. 5). The inhibiting concentration ranged from 0.01 to 10 mM, which is almost comparable to the effective concentrations of EP and EL. However, differential effects of MGO with respect to the investigated cytokines were observed. Rather than being inhibited, the LPS-stimulated release of IL-8 is aggravated by MGO when compared to the other cytokines.

MGO, like other glyoxal compounds, is suggested to induce formation of reactive oxygen species (ROS) in different cells [12]. On the other hand, MGO is known to specifically modulate the function of proteins by reacting with arginine, lysine and cysteine residues of proteins [5]. Thus, quenching the action of MGO by co-incubation of blood with the NAC (antioxidant), quercetin (ROS scavanger) or arginine (MGO target) should annihilate the suppressive effect of MGO on cytokine release. To address this question, we stimulated whole blood with LPS in the presence of MGO and the above-mentioned different additives, separately. Neither arginine nor quercetin could

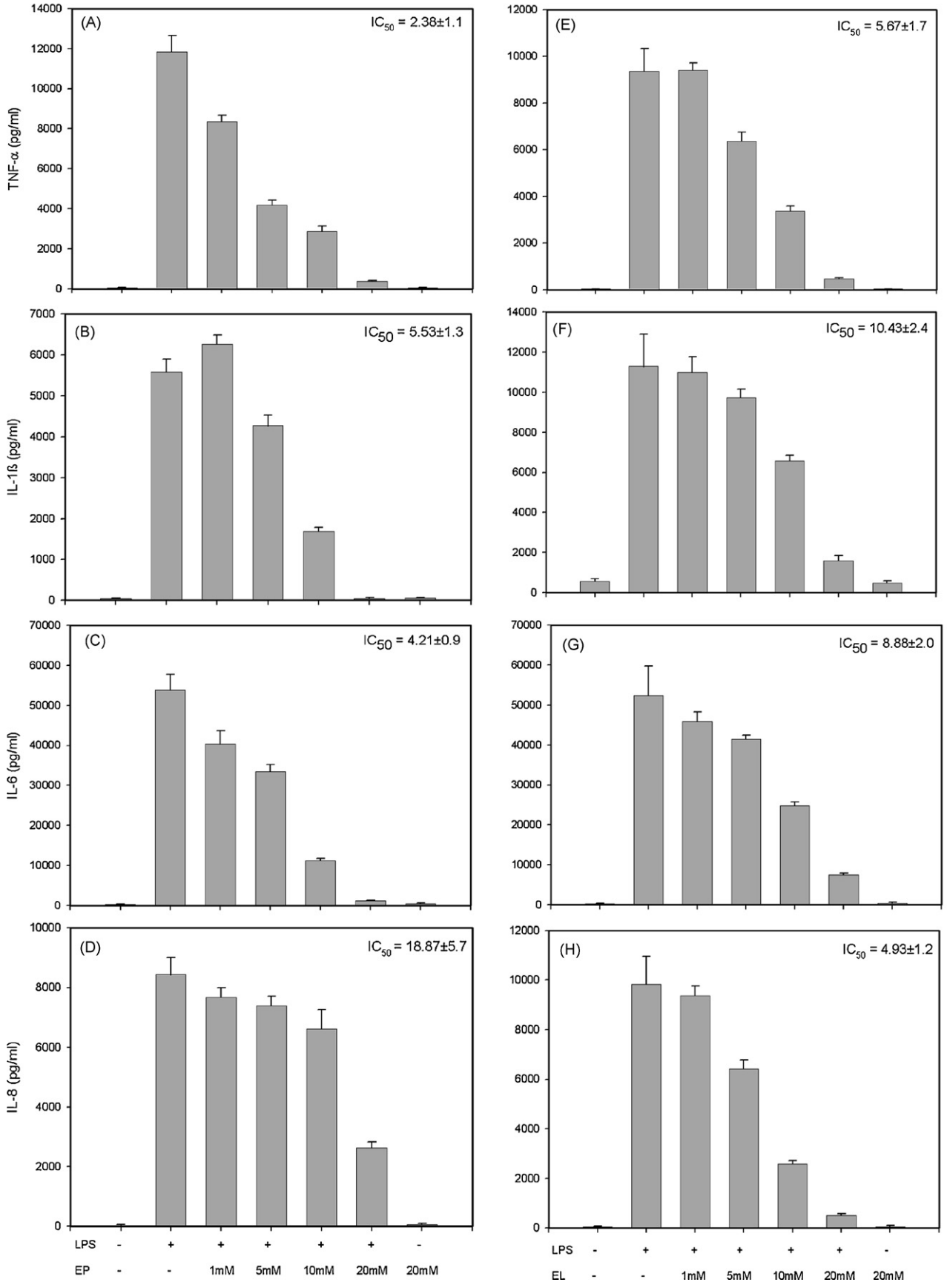

Fig. 3 – Ethyl pyruvate (EP) and ethyl lactate (EL) inhibit the release of inflammatory cytokines from LPS-stimulated human blood cells. Blood samples were incubated with LPS (10 ng/ml) and increasing concentration of EP (A–D) or EL (E–H) (1–20 mM), and analyzed for cytokines: TNF-α (A and E), IL-1β (B, F), IL-6 (C and G) and IL-8 (D and H).

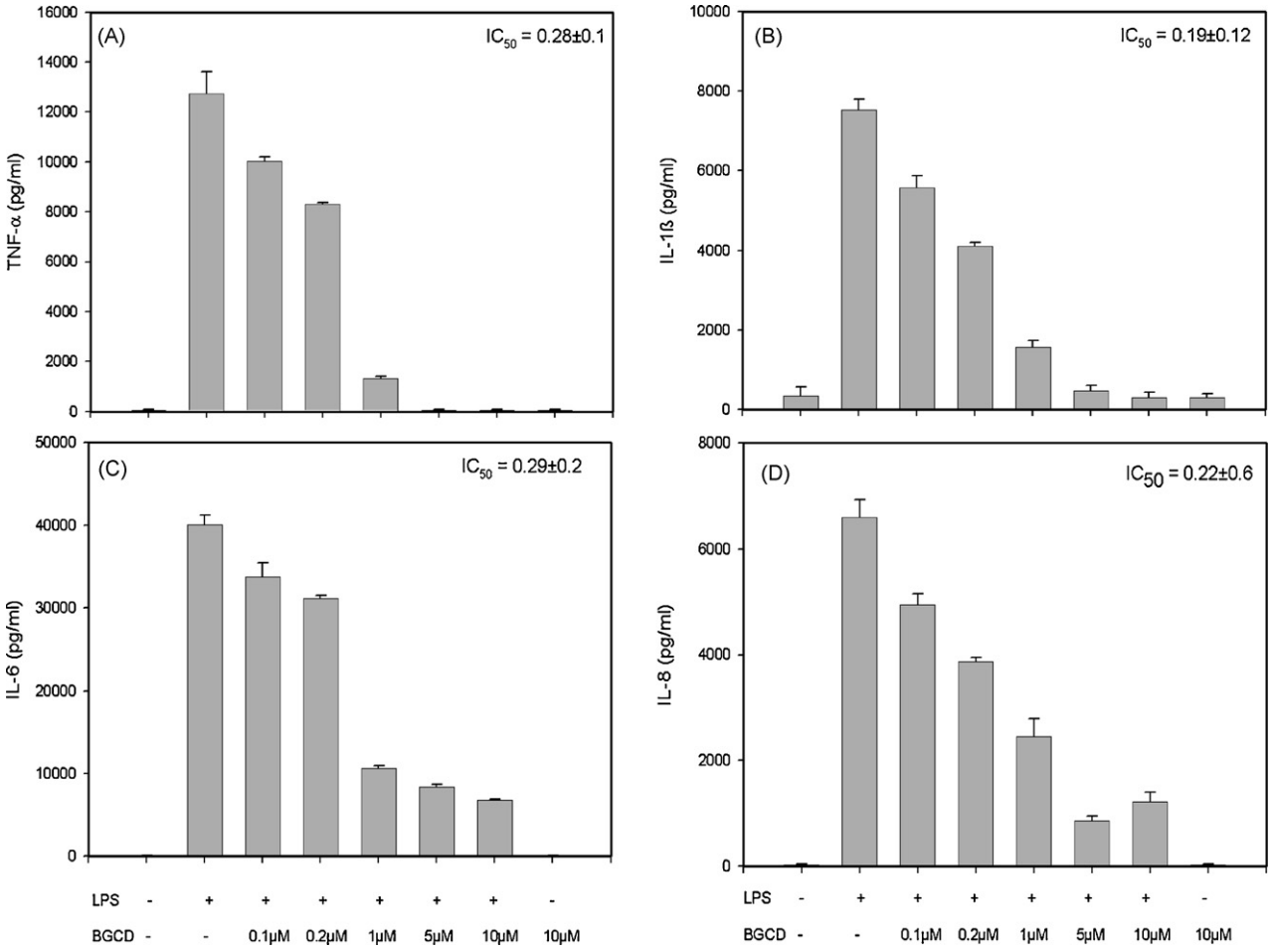

Fig. 4 – Inhibition of cytokine release from LPS-stimulated blood cells by the glyoxalase inhibitor BGCD. Blood samples were incubated with LPS (10 ng/ml) and increasing concentrations of BGCD (0.1–10 μM) and analyzed for the cytokines TNF-α, IL-1β, IL-6, and IL-8.

abolish the suppressive effect of MGO in contrast to NAC that partially reversed the MGO effect as exemplified by levels of IL-1β and IL-6, respectively (Table 1). This indicates that MGO may react with cysteine residues of target proteins or peptides involved in mediating cytokine suppression.

3.6. *Ethyl pyruvate modulates receptor expression in stimulated monocytes*

Next, we extended our studies and looked at surface expression of receptors involved in monocytic inflammatory reaction (CD14), antigen uptake (CD91) and presentation (HLA-DR), respectively. The expression of the aforementioned receptors increased after stimulation with LPS. Upon supplementation with EP, we observed a dramatic attenuation of LPS-induced stimulation of HLA-DR expression. The effect of EP on expression of CD14 was less compared to HLA-DR. On the contrary, EP, after an initial decline, stimulated the surface expression of CD91 on monocytes at higher concentrations (Fig. 6A–F). Obviously, the stimulatory effect of EP requires the presence of low concentrations of LPS because this effect was

Table 1 – Effect of methylglyoxal (MGO), *N*-acetylcysteine (NAC), arginine and quercetin on LPS-stimulated cytokine release of cultured human blood cells

Additives		IL-1β	IL-6
Unstimulated		2.1 ± 0.9	0.4 ± 0.1
LPS (10 ng/ml)		100	100
	+5 mM MGO	3.2 ± 1.0	1.4 ± 0.9
	+5 mM MGO + 10 mM NAC	13 ± 2.1	35 ± 3.9
	+5 mM MGO + 10 mM arginine	3.2 ± 1.9	1.2 ± 0.8
	+5 mM MGO + 1 mM quercetin	3.9 ± 1.5	0.4 ± 0.1

Diluted whole blood (1:5) was stimulated with 10 ng/ml LPS in the absence or presence of additives and incubated for 6 h at 37 °C and 5% CO_2. The percentage of cytokine release (IL-1β and IL-6) was compared to values obtained by LPS-stimulated control cells in the absence of any additives that were assigned to 100. The blank represents cytokine levels in the absence of LPS and additives. Values are presented as % mean ± S.D. (n = 3).

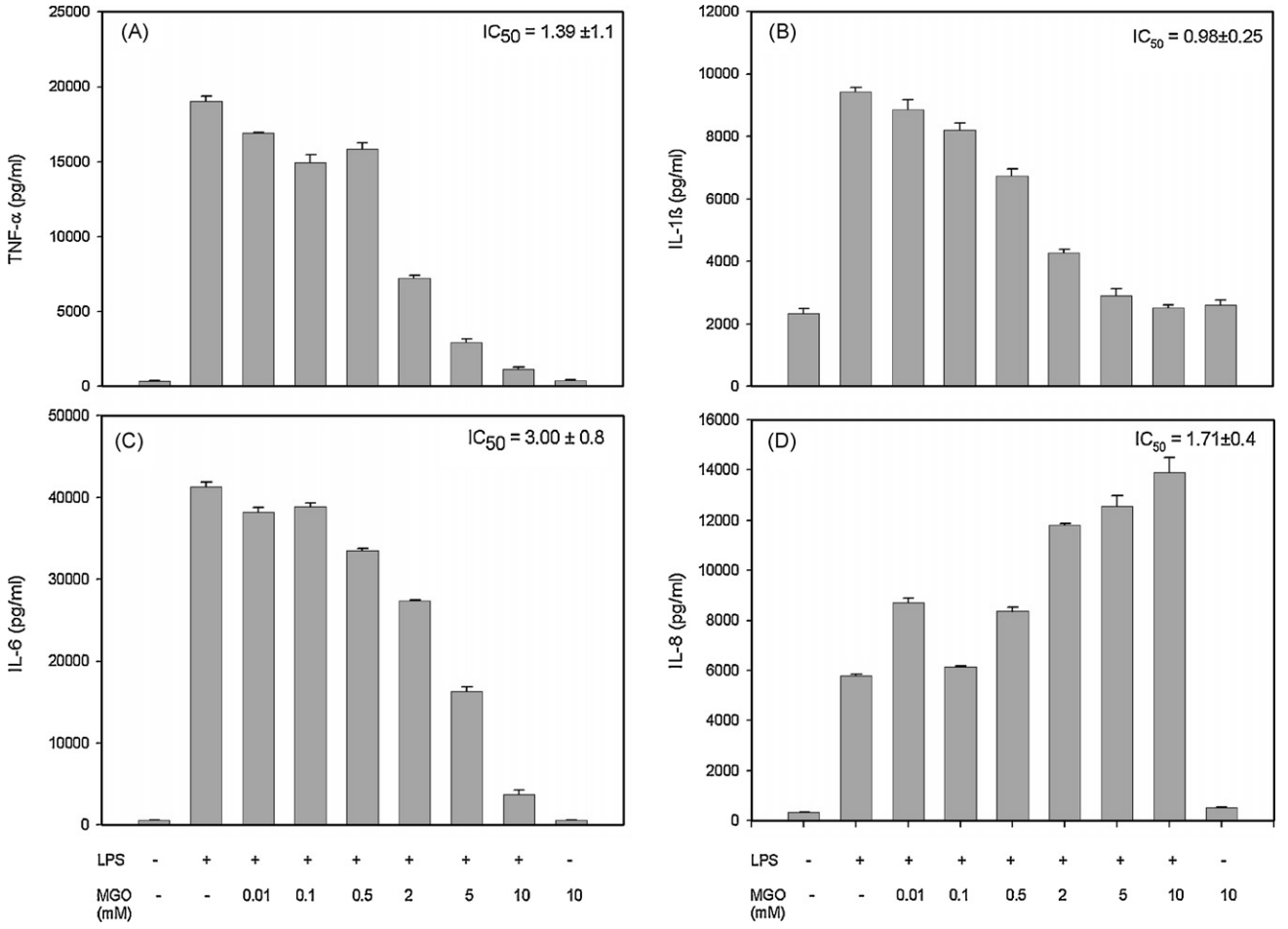

Fig. 5 – Inhibition of cytokine release from LPS-stimulated blood cells by methylglyoxal. Blood samples were incubated with LPS (10 ng/ml) and increasing concentration of MGO (0.01–10 mM) and analyzed for the cytokines TNF-α, IL-1β, IL-6, and IL-8.

not observed in samples without LPS. It is likely that LPS sensitizes monocytes for EP mediated expression of CD91. As expected, we found individual differences in response to LPS and EP in the blood from different blood donors. Likewise, MGO inhibited the expression of HLA-DR and CD14 but differed in case of CD91 expression (Fig. 7A–F).

3.7. *Ethyl lactate inhibits Th1/Th2 cell activation*

We could clearly show that glyoxalase inhibitors including their intracellular mediator MGO are effective modulators of the innate immune system. That raised the question whether the adaptive T helper (Th) immune responses can also be influenced by these compounds. To this end, we set up an experimental model of an antigen-specific stimulation of primed human blood cells. Blood cells of rubella-infected volunteers were re-stimulated with recombinant rubella-like particles (antigenic stimulation) together with PHA (mitogenic stimulation) in the absence or presence of EL. The Th1 cell response visualized by production of IFN-γ positive spots was analyzed by ELISPOT experiments (Fig. 8A). At increased concentration of EL in the 18-h PMBC assay, the number of IFN-γ positive spots was decreased. This effect applies for antigenic as well as mitogenic stimulation of PBMC. EL, at a concentration of 10 mM, suppressed the Th1 cell response by approximately 90% (Fig. 8B). To have an individual measure, the Th1 cell ratio, expressing the number of positive spots, in relation to the number of spots counted in PBMC samples without antigenic stimulus, was evaluated (Fig. 8D). To test the toxicity of EL on PMBC, we measured the viability of these cells by trypan blue exclusion and found that the overall viability of cells ranged between 87 and 95% in the presence of EL (Fig. 8C). It indicates that EL is non-toxic to human PBMC under the experimental conditions used. However, upon stimulation by antigens or PHA, a reduction of the cell number became evident.

It is known that antigenic and mitogenic stimulation activate the Th1 and Th2 cell responses. A Th1 response is characterized by secretion of the cytokines IL-2, TNF-α and IFN-γ whereas cytokines characterizing the Th2 cell response are IL-4, IL-5, IL-6 and IL-10. We aimed at investigating whether EL can also modulate the function of different Th cells. Thus, we analyzed cytokine release in the supernatants of PBMC cultures after mitogenic stimulation (Table 2). We found that EL reduced Th cell activation and attenuated the release of the Th1 cytokines IFN-γ and IL-2, in addition to TNF-α as well as the Th2 responsive IL-10. No detectable amounts of IL-4 or IL-5 were found.

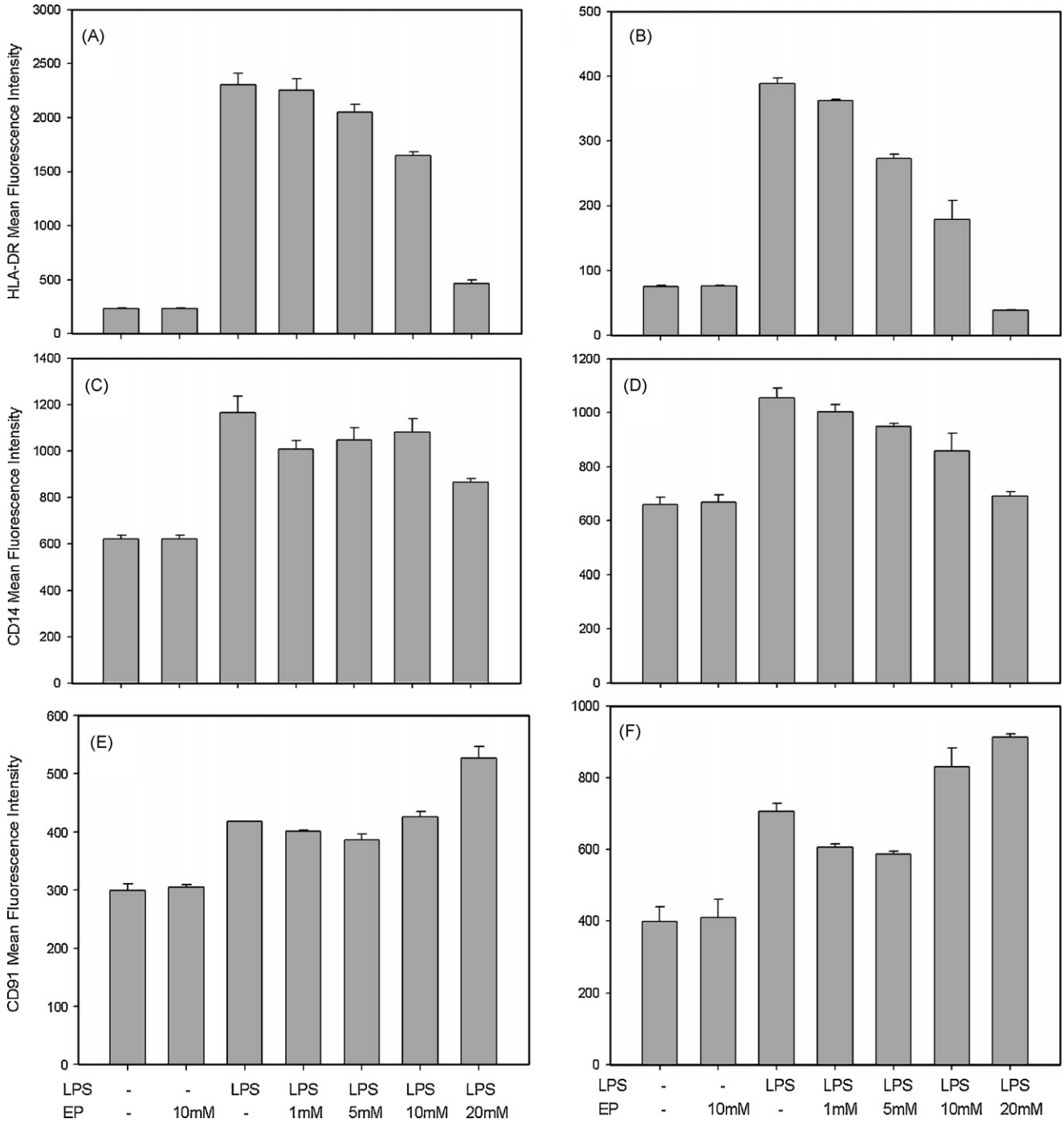

Fig. 6 – Effect of ethyl pyruvate on the expression of HLA-DR, CD14 and CD91 of LPS-stimulated human monocytes. Human blood cells from two volunteers were stimulated with LPS (10 ng/ml) in the presence of increasing concentrations of EP (1–20 mM). Cells were analyzed by flow cytometry for expression of HLA-DR, CD14, and CD91. Blood samples were obtained from G.B. (A, C, and E) and M.H. (B, D, and F). The mean fluorescence intensity (MFI) values were corrected for unspecific binding of control antibodies.

4. Discussion

The mechanism how EP acts is far from being solved. As a derivative of pyruvate, EP was proposed to mimic the well-known function of pyruvate as a ROS scavenger [13]. Pyruvate, like other α-ketoacids in general, reacts rapidly and non-enzymatically with H_2O_2 and thus decrease oxidative stress [14]. This characteristic, however, could not explain the various effects of EP in cells and tissues found so far. Also, the assumption that EP interferes with intracellular signal cascades such as NF-κB contradicts the fact that millimolar concentrations of EP are necessary to elicit cellular effects [3]. Recently, it was proposed that the effect of EP traces back to the depletion of cellular GSH pool.

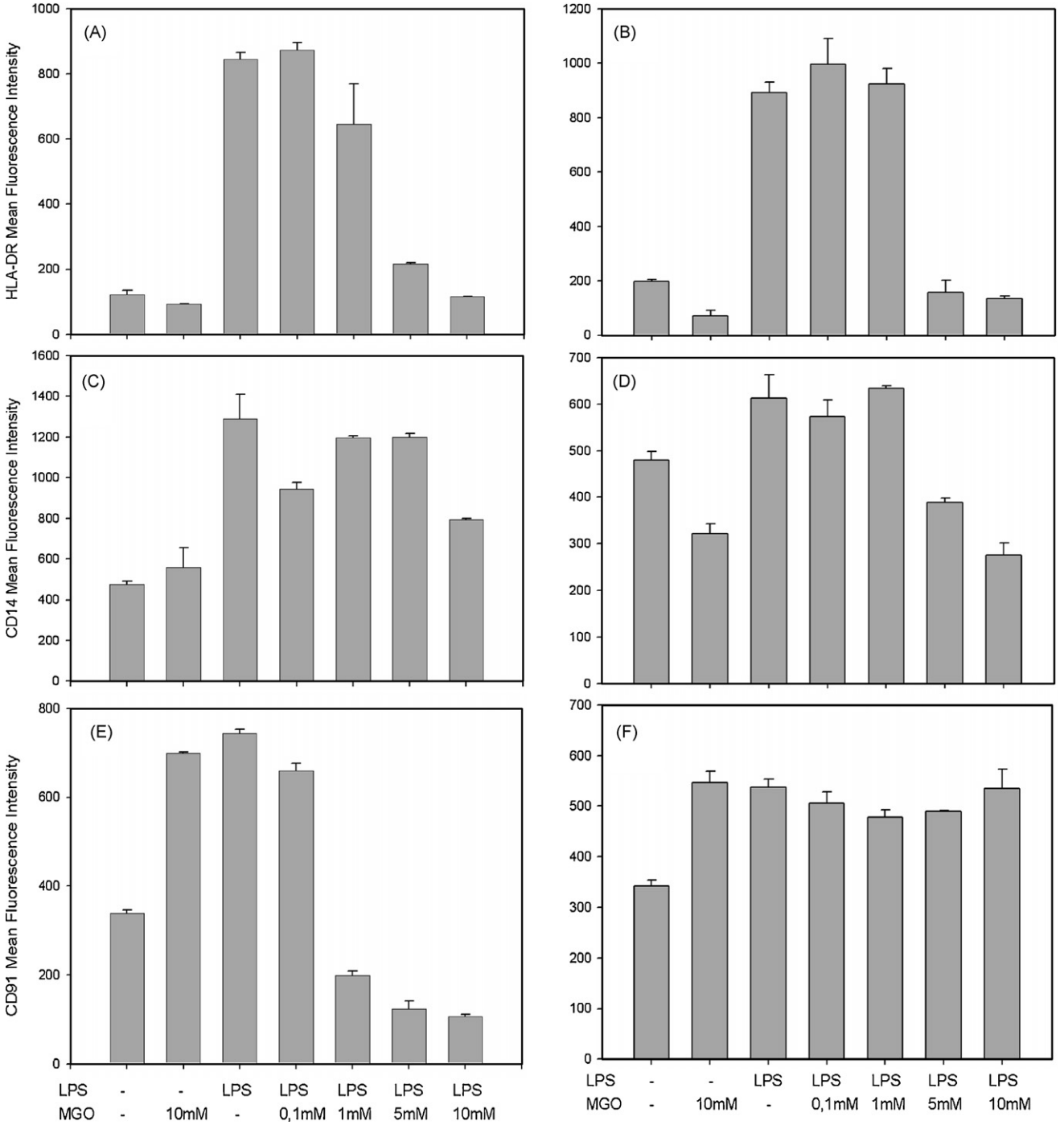

Fig. 7 – Effect of methylglyoxal on the expression of HLA-DR, CD14 and CD91 of LPS-stimulated human monocytes. Human blood cells from two blood donors were stimulated with LPS (10 ng/ml) in the presence of increasing concentrations of MGO (0.1–10 mM). Cells were analyzed by flow cytometry for expression of HLA-DR, CD14, and CD91. Blood samples were obtained from donor 1 (A, C, and E) and donor 2 (B, D, and F). The mean fluorescence intensity (MFI) values were corrected for unspecific binding of control antibodies.

Low intracellular GSH may promote an intracellular oxidized state accompanied by interference with NF-κB signaling cascades. Thus, inhibiting GSH depletion should counteract the anti-inflammatory effect of EP. However, using cell-penetrable GSH ethyl ester or NAC, which are suggested as precursors of GSH, exerted quite various effects [15]. Overall, there are many aspects concerning the mode of action of EP, which cannot be explained by the current results.

Surprisingly, we found that EP inhibited the enzymatic reaction of human Glo1. At present, the molecular basis of this phenomenon is not clear and needs further investigation. Noteworthy, the calculated IC_{50} values for EP reflect the effective EP concentrations at cellular level as shown by us and

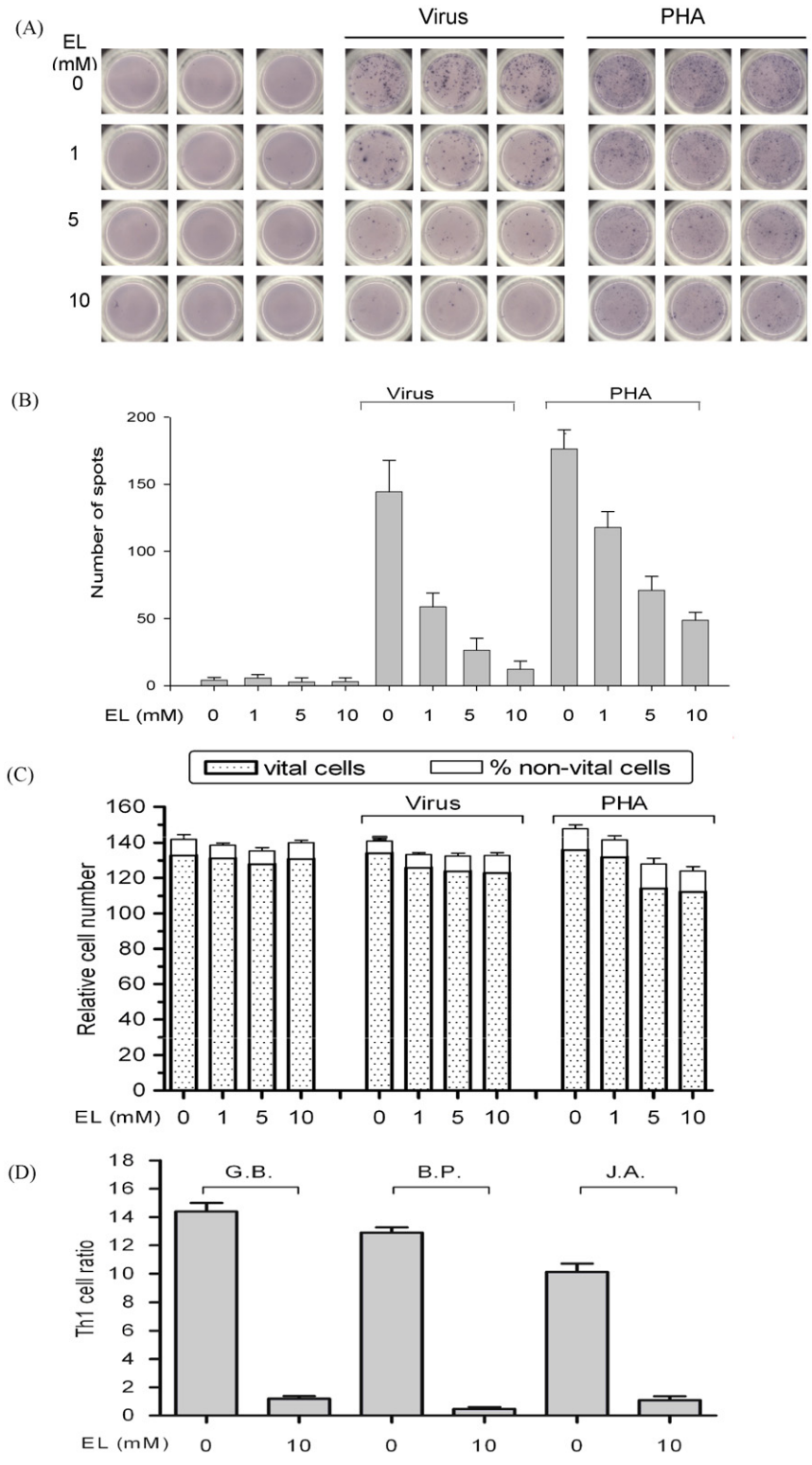

Fig. 8 – Ethyl lactate (EL) inhibits activation of T cells upon antigenic and mitogenic stimulation. (A) Ficoll®-isolated human PBMC were stimulated with Rubella-like particles or with phytohaemaglutinin (PHA) in the absence or presence of increasing doses of EL (1–10 mM). IFN-γ positive spots were evaluated using an ELISPOT-Reader. B) Statistical analysis of measured IFN-γ positive spots obtained from ELISPOT experiment shown in (A) ($n = 3$). (C) The effect of EL on vitality of cells. After incubation, as described in (A), cells were harvested and aliquots were counted using a haemocytometer ($n = 5$). Dotted columns (mean cell number ± S.D.); white columns (% non-vital cells ± S.D.). (D) Individual T cell response upon antigenic stimulation in the presence of EL. Blood from three volunteers, denoted G.B., B.P., and J.A., was analyzed as described in (A) and their Th1 cell ratio is depicted; expressed as number of positive clones in relation to the clones obtained in the absence of any stimulant.

others [15]. The finding that EL did not inhibit Glo1 *in vitro* let us assume that a ''dicarbonyl''-structural motif as formed by esters of oxo-carbonic, is a prerequisite for the observed inhibitory effects. This assumption was corroborated by accomplishing additional control experiments using chloropropionic acid ethyl ester (the keto-O moiety of EP is replaced by chlorine) that revealed no indication for enzyme inhibition (unpublished data).

To the best of our knowledge, the present study is the first to demonstrate that lactate esters are capable to abrogate the LPS-induced formation of inflammatory cytokines. The *in vivo* effects of EL can be best explained by the assumption that EL acts as a prodrug being converted intracellularly to EP by LDH [16]. Own *in vitro* measurements confirmed that assumption (unpublished data). We may then conclude that the effect of EL in different cells may depend on the expression level of LDH and the presence of distinct LDH iso-enzymes.

Inhibition of Glo1 may result in a marked increase of the intracellular and extracellular MGO concentration because the uncharged oxo-aldehyde leaks almost freely from the cell into the surrounding and vice versa [17]. Our finding that exogenously added MGO attenuated LPS-mediated cytokine release in the same manner as EP and EL indicates that MGO probably acts as an intracellular mediator of the action of Glo1 inhibitors, which concords previous reports [18]. The validity of the proposed mechanism is further demonstrated by the action of BGCD on cytokine release. Compared to BGCD, higher concentrations of MGO were required to exert detectable cellular effects. This might be due to partial use up of MGO by its reaction with abundant proteins present in the human plasma leading to lower levels of active and cell-diffusible MGO. MGO concentrations between 8 and 20 mM were found to be necessary to stimulate the Hog1 MAP kinase cascade in *S. cerevisiae* [19].

A number of different cellular effects of MGO have been described which are currently not fully understood. Some reports revealed that MGO is toxic to cells by depletion of GSH and ATP, modulation of mitochondrial membrane potential, induction of apoptosis and ROS production [12,20]. On the other hand, other reports were unable to assert any toxic effects of MGO [21,22]. It has been shown that MGO is capable to modify the function of very distinct proteins e.g. by activation of transcription factors in yeast [23], by distinct covalent modification of proteins like Hsp27 [24] or by modulation of enzyme activity of GAPDH [25]. It is still obscure whether the MGO-derived modifications of proteins are irreversible or transient. However, it is possible to conceive a system for the regulation of cellular metabolism and growth in which some MGO-derived protein modifications are transient, leading to temporary activation or inactivation of target proteins, whereas permanent MGO modifications of the same or different target molecules may result in cell death [26].

We clearly showed that EP, EL and MGO could abrogate many cellular effects elicited by LPS. Monocytes and macrophages respond to LPS by generation of ROS, secretion of cytokines and up-regulation of cell surface molecules. Many of these cellular responses are suggested to be mediated by activation of the NF-κB system. Taken the message that MGO and LPS are strong elicitors of early and extensive ROS, it is hard to grasp why they showed differential effects with respect to modulation of cytokines and surface receptors. It is our hypothesis that MGO targets specific regulatory proteins possibly via reacting with specific cysteine and/or arginine residues. Possible candidates of proteins might be the members of the NF-κB family because they share clusters of both amino acids in the DNA-binding region [27]. It has been suggested that the anti-inflammatory action of EP is due to the modification of cysteine residues of the p65 subunit of the NF-κB protein [15,28], however, modulation by MGO is more likely because of higher reactivity of MGO compared to EP. In line with our assumption, a recent report convincingly showed that MGO inhibited NF-κB activation by targeting Cys 38 of the p65 subunit [29]. This has been also highlighted by our finding of an anti-inflammatory effect of BGCD, which obviously may not react with amino acids of target proteins as it was proposed for EP [15]. Meanwhile a number of reports further deduced our assumption of a selective modulation by MGO of protein functions by blocking specific amino acids [30,31]. This could also explain the suppressive effect of EP, EL and MGO on LPS-induced expression of CD14 and HLA-DR because both immune receptors are regulated by Toll-like receptor in conjunction with NF-κB. Obviously, compared to CD14 and HLA-DR, the expression of CD91 might be differently regulated and warrants further studies.

We convincingly showed that specific functions of lymphocytes are modulated by inhibition of Glo1. To explain this effect, we hypothesize that an early step in activation of cells either by LPS, antigens or mitogens may be the activation of the glycolytic pathway. Earlier studies indicated that macrophage activation by LPS accounted for the potent stimulation of the macrophage glucose uptake and glycolytic flux by up-regulation of the GLUT1 [32]. Resting T cells consume glucose at low rate supplying energy to maintain normal housekeeping functions. Upon activation by antigen or mitogens, T cells respond by induction of cell growth and proliferation. The energy-demanding process of activation is accompanied by an increase in glucose utilization detectable within 1 h of stimulation. Interestingly, the increase in glycolysis is dramatically greater than the increase in oxygen consumption as recognized by increased lactate production [33]. This so called ''aerobic glycolysis'' resembles constitutive features of malignant cells [34]. Increased glycolytic throughput leads to enhanced accumulation of the highly reactive MGO. In resting cells, the expression of the enzymes Glo1 and Glo2 balances the production of MGO to prevent overproduction of MGO. In metabolic active cells, on the other hand, the produced MGO level may temporally exceed the degradation capacity of the two enzymes. For this reason, we hypothesize that the formed MGO acts as a ''glycolytic second messenger'' to regulate cellular processes like cytokine production or surface receptor expression. However, at sufficiently high and sustained levels of MGO, metabolically activated cells but only these are then guided into apoptosis while non-stimulated cell remain untouched. Thus, we assume that MGO may turn off temporary intracellular signals by modulating the expression level of regulatory proteins or by inducing apoptosis in a concentration-dependent manner. As the intracellular level of MGO is decreased with time, probably due to stimulation of metabolic enzymes, the signalling cascade may be restored.

Table 2 – Cytokine production of phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) in the absence or presence of ethyl lactate (EL)

	PHA-stimulated			
EL concentration (mM)	0	1	5	10
IFN-γ	13,641 ± 1,037	11,013 ± 997	4,436 ± 214	2,696 ± 269
IL-2	15,800 ± 192	1,303 ± 192	987 ± 134	824 ± 48
TNF-α	2,065 ± 44	822 ± 88	141 ± 82	10 ± 1.3
IL-10	4,906 ± 223	4,462 ± 244	3,800 ± 266	2,460 ± 97

Ficoll®-isolated human PBMC (5×10^5 cells/ml) were seeded in 24-well plates and treated with 1 μg/ml PHA for mitogenic stimulation and different concentrations of EL. After incubation at 37 °C the supernatant was analyzed for Th1/Th2 cytokines. Values are presented as mean ± S.D. ($n = 3$).

Recent studies obviously support our view. Aldose reductase (EC 1.1.1.21), another enzyme of glucose metabolism, has been linked to LPS-mediated cytokine release. It detoxifies a number of endogenous aldehydes including MGO and 4-hydroxynonenal as well as their glutathione conjugates in the presence of NADP [35]. It was found that the intracellular redox potential of GSH/GSSG decides whether MGO passes through the aldose reductase or glyoxalase pathway. Inhibition or genetic ablation of aldose reductase prevented LPS-induced activation of NF-κB and the concomitant cytokine production [36].

Our findings clue two additional aspects. First, tumors, exhibiting an increased glycolytic pathway, could disable invading immune cells by producing inhibitors of glyoxalases that may arrest macrophages or T cells in the vicinity of tumors. Such inhibitors could be MGO itself or other ''dicarbonyl'' compounds that easily diffuse the cell membrane and execute their effects on immune cells and thereby contribute to tumor escape mechanisms [37]. It can be anticipated that metabolically activated cells per se can produce endogenous glyoxalase inhibitors from the ''dicarbonyl''-type. For example, esters of pyruvate with branched or non-branched aliphatic alcohols might show even stronger inhibition of glyoxalase compared to EP. Second, the inhibition of glyoxalases may be a new approach to immunosuppression. The finding that only antigen-activated cells, which respond by increasing the glycolytic rate, are affected, offers the possibility to selectively target immune cells involved in antigen recognition and presentation. In addition, the stimulation of expression of CD91 in APCs by inhibition of glyoxalase fits well to the concept of immunosuppression because this receptor is known to clear body fluids from antigenic peptides bound to alpha2-macroglobulin [38] or intracellular chaperones [39] by receptor-mediated endocytosis. Because antigens when increasingly taken up by CD91 cannot be presented due to down-modulation of HLA-DR, that mechanism may act as a sink for antigenic molecules. The recent finding that indomethacin, an anti-inflammatory and anti-cancer agent, is a high-affinity inhibitor of Glo1 will draw a further attention to the remarkable functions of Glo1 in the metabolism of diverse cells [40].

Overall, we have collected data showing that EP is able to inhibit the activity of Glo1. Blood cells when activated, e.g. by LPS, antigens or mitogens, respond by enhancing glycolysis which consistently may generates MGO. We hypothesize that inhibition of Glo1 in metabolically activated cells blocks detoxification leading to a temporary increase of the MGO burden which then counteracts stimulant-driven immune reactions. Our data suggest that Glo1 might be a target for immune suppression, which might be of importance for application of glyoxalase inhibitors in tissue and organ transplantation.

Acknowledgments

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