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Proinflammatory Effects of Hypoglycemia in Humans With or Without Diabetes

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Severe hypoglycemic events have been associated with increased cardiovascular mortality in patients with diabetes, which may be explained by hypoglycemia-induced inflammation. We used ex vivo stimulations of peripheral blood mononuclear cells (PBMCs) and monocytes obtained during hyperinsulinemic-euglycemic (5.0 mmol/L)-hypoglycemic (2.6 mmol/L) clamps in 11 healthy participants, 10 patients with type 1 diabetes and normal awareness of hypoglycemia (NAH), and 10 patients with type 1 diabetes and impaired awareness (IAH) to test whether the composition and inflammatory function of immune cells adapt to a more proinflammatory state after hypoglycemia. Hypoglycemia increased leukocyte numbers in healthy control participants and patients with NAH but not in patients with IAH. Leukocytosis strongly correlated with the adrenaline response to hypoglycemia. Ex vivo, PBMCs and monocytes displayed a more robust cytokine response to microbial stimulation after hypoglycemia compared with euglycemia, although it was less pronounced in patients with IAH. Of note, hypoglycemia increased the expression of markers of demargination and inflammation in PBMCs. We conclude that hypoglycemia promotes mobilization of specific leukocyte subsets from the marginal pool and induces proinflammatory functional changes in immune cells. Inflammatory responses were less pronounced in IAH, indicating that counterregulatory hormone responses are key modulators of hypoglycemia-induced proinflammatory effects. Hypoglycemia-induced proinflammatory changes may promote a sustained inflammatory state.

Hypoglycemia is the most common complication of insulin therapy in people with type 1 diabetes (1). Patients with type 1 diabetes experience, on average, two hypoglycemic events per week and one severe event per year (2). The extent to which hypoglycemia contributes to cardiovascular disease risks in diabetes is debated: An association between severe hypoglycemia and increased mortality from cardiovascular events has been established in patients with type 2 diabetes (3–6) but is less consistent in type 1 diabetes (6–10), even though hypoglycemia occurs much more frequently in patients with type 1 than in those with type 2 diabetes (11).

An increase in circulating proatherothrombotic factors in response to acute insulin-induced hypoglycemia can link hypoglycemia to cardiovascular complications (12–14). In addition, hypoglycemia has been reported to increase leukocyte counts and circulating proinflammatory cytokines in both healthy individuals (15–18) and patients with type 1 diabetes (14,19), supporting the concept that hypoglycemia-induced systemic inflammation contributes to cardiovascular complications (12,13,15).

Adrenaline, the main counterregulatory hormone response to hypoglycemia in patients with type 1 diabetes, may play a role in the hypoglycemia-induced proinflammatory response. When adrenaline is administered to healthy individuals (normoglycemic conditions), it specifically mobilizes leukocytes equipped with cytotoxic effector potential from the marginal pool (vascular epithelium) (20). However,

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knowledge about the role of adrenaline in hypoglycemia-induced changes related to inflammation is lacking.

Patients with impaired awareness of hypoglycemia (IAH) are at particularly high risk of hypoglycemia (21) because they lack hypoglycemia warning symptoms and have attenuated adrenaline responses (1,22). If adrenaline contributes to hypoglycemia-induced proinflammatory responses, such effects may be altered in patients with type 1 diabetes and IAH. Under euglycemic conditions, patients with IAH were found to have higher leukocyte counts and a higher rate of endothelial dysfunction and preclinical atherosclerosis than sex- and age-matched patients without IAH (23). In accordance, Joy et al. (16) reported that antecedent hypoglycemia, which underlies the emergence of IAH, results in greater endothelial dysfunction, but inflammatory responses to hypoglycemia are not enhanced after prior hypoglycemia.

Thus, hypoglycemia has been shown to increase circulating proinflammatory cytokines, but the underlying mechanisms, the role of repeated hypoglycemia, and the relationship with counterregulatory hormone responses, particularly adrenaline, are incompletely understood. Because an enhanced proinflammatory state is not exclusively reflected in the levels of circulating cytokines, we studied the effects of acute hypoglycemia on the composition and inflammatory output of immune cells. We investigated these aspects by using ex vivo stimulations of peripheral blood mononuclear cells (PBMCs) obtained at various time points during hyperinsulinemic-euglycemic-hypoglycemic clamps in healthy participants and patients with type 1 diabetes. To assess the role of sympathoadrenal responses to hypoglycemia in inducing potential proinflammatory effects, we also included patients with type 1 diabetes and IAH characterized by impaired counterregulatory hormone responses to hypoglycemia.

RESEARCH DESIGN AND METHODS

Participants

We recruited 11 participants without diabetes, 10 patients with type 1 diabetes and normal awareness of hypoglycemia (NAH), and 10 patients with type 1 diabetes and IAH. Patients were otherwise healthy and did not use drugs that interfered with glucose metabolism other than insulin. Hypoglycemia awareness state, initially assessed by a Dutch version of the Cox questionnaire in which a score of 0–1 of 5 indicates normal awareness and a score ≥ 3 indicates impaired awareness (24,25), was determined on the basis of adrenaline and symptomatic responses to hypoglycemic clamp. Eighteen of the 20 patients were correctly characterized as having either IAH or NAH through the Cox questionnaire. The institutional review board of the Radboud University Medical Center (Nijmegen, the Netherlands) approved the study, and all participants gave written informed consent before participation.

Experimental Design

All participants presented between 8:00 and 8:30 A.M. at the clinical research facility after an overnight fast and having abstained from caffeine, alcohol, and smoking for 24 h. Patients with diabetes received specific instructions to avoid (nocturnal) hypoglycemia the day before the clamp. Experiments were rescheduled in case of hypoglycemia in the 24 h before the clamp. Upon arrival, two intravenous cannulae were inserted, one into the antecubital vein of each forearm. One forearm was placed in a heated box (55°C) so that arterialized venous blood could be obtained for frequent blood sampling. The cannula in the contralateral arm was used for infusion of glucose 20% (Baxter, Deerfield, IL) and insulin (insulin aspart; Novo Nordisk, Bagsværd, Denmark). Baseline plasma glucose levels were determined (Biosen C-Line; EKF Diagnostics, Cardiff, U.K.), and a two-step hyperinsulinemic (60 mU/m^2/min)-euglycemic (5.0 $\pm$ 0.2 mmol/L)-hypoglycemic (2.6 $\pm$ 0.1 mmol/L) glucose clamp was initiated. Plasma glucose levels were determined every 5 min, and after a short euglycemic phase (~20 min), plasma glucose levels were gradually decreased to 2.6 mmol/L and maintained there for 60 min. Blood samples for measurement of adrenaline were taken at euglycemia and every 20 min during hypoglycemia. Insulin and glucagon were determined at euglycemia and at 60 min of hypoglycemia.

Analytical Methods

Plasma insulin was assessed by an in-house radioimmunoassay (26). After extraction (27), plasma glucagon was measured with a commercially available radioimmunoassay kit (Eurodiagnostica, Malmö, Sweden). Plasma growth hormone and cortisol were determined by routine analysis with an electrochemiluminescent immunoassay on a Modular Analytics E170 (Roche, Manheim, Germany). Plasma adrenaline and noradrenaline were analyzed by high-performance liquid chromatography combined with fluorometric detection (28). Peripheral total and differential white blood cell counts were determined by routine patient sample analysis (flow cytometric analysis on a Sysmex XE-5000).

Isolation of PBMCs and CD14$^+$ Monocytes

Blood samples were processed for isolation of cells immediately after being drawn to ensure equal quality of the samples because previous experiments showed that cytokine responses are altered when blood samples are processed for isolation at different time points after being drawn (data not shown). Isolation of PBMCs was performed by differential centrifugation over Ficoll-Paque PLUS (GE Healthcare). PBMCs were washed three times with PBS and counted with a Coulter counter (Coulter Electronics). CD14$^+$ monocytes were purified from freshly isolated PBMCs by using MACS MicroBeads (Miltenyi Biotec, Teterow, Germany) for positive selection according to the manufacturer's instructions.

Stimulation Experiments

For analysis of cytokine release, glucose consumption, and lactate production, 5×10^5 PBMCs or 1×10^5 monocytes were used per well in a 96-well plate. Cells were cultured in RPMI medium (no glucose; Gibco) supplemented with

10 μg/mL gentamicin (Gibco), 10 mmol/L pyruvate (Gibco), 10 mmol/L HEPES (Sigma-Aldrich), and 5.5 mmol/L glucose (Sigma-Aldrich) and stimulated with either RPMI medium, 10 ng/mL of the TLR4 agonist lipopolysaccharide (LPS) from *Escherichia coli* (Sigma-Aldrich), 10 μg/mL of the TLR2 agonist Pam_3CysSK_4 (Pam3Cys) (EMC Microcollections, Tübingen, Germany), 1 μg/mL *Mycobacterium tuberculosis* (H37Rv) lysate, or 1×10^6 heat-killed organisms/mL *Candida albicans* conidia for 24 h. Cell culture supernatants were collected and stored at −20°C.

Cytokine Measurements

The production of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) (R&D Systems), IL-10, IL-6 (Sanquin), and MCP-1 (eBioscience) was measured by ELISA. In the analysis of cytokine production by $CD14^+$ monocytes, participants were excluded when cytokine production upon stimulation of cells after both euglycemia and hypoglycemia was below detection limits. The number of nonresponders was comparable between groups.

Glucose Consumption and Lactate Measurements

Glucose and lactate concentrations were measured in cell culture supernatants. Measurements were based on an enzymatic reaction in which glucose or lactate is oxidized and the resulting H_2O_2 is coupled to the conversion of Amplex Red reagent to fluorescent resorufin by horseradish peroxidize. The fluorescence of resorufin (excitation/emission maxima 570/585 nm) was measured on a 96-well plate reader (BioTek). Glucose consumption was calculated by subtracting the glucose concentration measured in cell culture supernatants from that in culture medium incubated for 24 h without cells.

RNA Isolation and Quantitative Real-Time PCR

For mRNA expression analyses, PBMCs (1.5×10^6 PBMCs/condition) were lysed in TRIzol reagent (Invitrogen) directly after isolation and stored at −80°C until RNA isolation was performed according to the manufacturer's instructions. RNA was transcribed into cDNA by reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad). Primer sequences used for quantitative real-time PCR (qRT-PCR) are listed in Supplementary Table 2. Power SYBR Green PCR Master Mix (Applied Biosystems) was used for qRT-PCR in the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Expression data were normalized to the housekeeping gene human β_2M.

Statistical Analysis

Data were tested for normality by using the Shapiro-Wilk test and Q-Q plots. Within-group differences were compared with paired Student *t* or Wilcoxon signed rank tests when data were not normally distributed. Between-group differences were analyzed by ANOVA followed by pairwise Bonferroni post hoc tests to delineate statistical significance and for nonparametric data, with the Kruskal-Wallis and post hoc Mann-Whitney *U* tests. For correlation analysis, Pearson correlation coefficient was used for normally distributed variables and Spearman rank sum test for nonnormally distributed data. All data are expressed as mean ± SEM unless otherwise specified. $P < 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS version 20 software (IBM Corporation).

RESULTS

Study participants were well matched for age, sex, and BMI (Table 1). Duration of diabetes and HbA_{1c} did not differ significantly between patient groups. Plasma glucose levels (Fig. 1*A*) and plasma insulin levels (data not shown) were similar in all groups during both the euglycemic and the hypoglycemic phase, whereas glucagon levels increased in response to hypoglycemia in healthy control participants but did not change in either patient group (Supplementary Table 1). Adrenaline levels during hypoglycemia were significantly lower in patients with IAH than in healthy control participants and patients with NAH (0.39 ± 0.07, 1.90 ± 0.46, and 1.94 ± 0.29 nmol/L, respectively).

Table 1—Participant characteristics

	Healthy control participants (*n* = 11)	Patients with type 1 diabetes and NAH (*n* = 10)	Patients with type 1 diabetes and IAH (*n* = 10)
Age (years)	24.5 ± 5.3	24.2 ± 5.1	25.3 ± 6.0
Sex			
Male	6	4	5
Female	5	6	5
BMI (kg/m^2)	22.9 ± 1.8	22.7 ± 2.2	23.4 ± 1.4
HbA_{1c}			
%	—	7.5 ± 0.6	6.9 ± 0.7
mmol/mol	—	58.5 ± 6.9	52.1 ± 7.8
Duration of diabetes (years)	—	10.9 ± 4.7	13.9 ± 8.1
Total daily insulin dose (IU)	—	48.2 ± 12.6	48.2 ± 13.2

Data are mean ± SD or *n*.

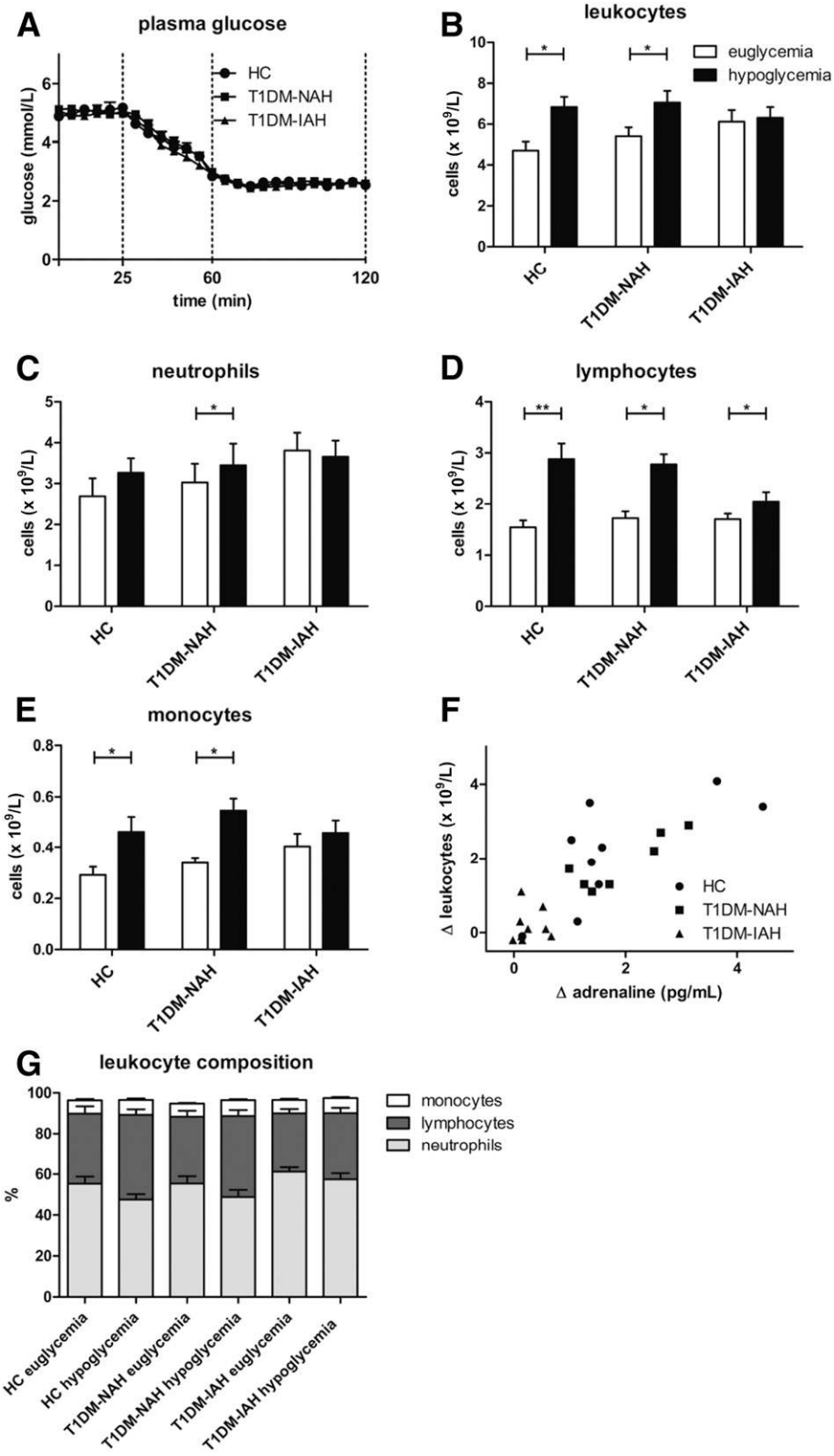

Figure 1—Hypoglycemia induces leukocytosis in healthy control (HC) participants and patients with type 1 diabetes mellitus (T1DM) and NAH but not in T1DM and IAH who have attenuated adrenaline responses to hypoglycemia. *A*: Time course of plasma glucose levels during the clamp. Dashed lines represent the end of the euglycemic phase and the beginning and end of the hypoglycemic phase. *B–E* and *G*: Number of circulating leukocytes (*B*), neutrophils (*C*), lymphocytes (*D*), and monocytes (*E*) and composition of leukocytes (*G*) measured with a routine patient sample analysis at euglycemia and after 1 h of hypoglycemia. *F*: Correlation between the difference in leukocyte numbers during hypoglycemia vs. euglycemia and the difference in adrenaline levels between hypoglycemia and euglycemia. $^{*}P < 0.05$; $^{**}P < 0.01$.

Hypoglycemia Increases Total Leukocyte Count

The total leukocyte count increased in response to hypoglycemia in healthy control participants and patients with NAH but not in patients with IAH (Fig. 1*B*). This increase was mainly due to an increase in the number of lymphocytes and, to a lesser extent, an increase in the number of monocytes (Fig. 1*C–E*). Consequently, neutrophil-to-lymphocyte ratios decreased in response to hypoglycemia (Fig. 1*G*). The change in total leukocyte count correlated positively with the adrenaline response to hypoglycemia (R^2 = 0.70; $P < 0.001$) (Fig. 1*F*). The positive correlation was strongest in lymphocytes (R^2 = 0.75; $P < 0.001$) but was also seen in monocytes (R^2 = 0.33; P = 0.003) and neutrophils (R^2 = 0.29; P = 0.007) (Supplementary Fig. 1). By looking at the separate groups, the positive correlation between the change in total leukocyte count and adrenaline response was significant in healthy control participants and patients with NAH, but not in patients with IAH.

Hypoglycemia Increases Ex Vivo Proinflammatory Cytokine Production

PBMCs from healthy control participants and patients with NAH isolated after 1 h of hypoglycemia and stimulated with the TLR4 agonist LPS produced more proinflammatory cytokines (IL-6, IL-1β, and TNF-α) than PBMCs isolated after euglycemia. Hypoglycemia had no effect on LPS-stimulated cytokine production of PBMCs isolated from patients with IAH (Fig. 2*A–C*). Hypoglycemia increased the production of the chemokine MCP-1 in healthy control participants (Fig. 2*D*) but did not affect levels of the anti-inflammatory cytokine IL-10 in any group (Fig. 2*E*).

Hypoglycemia enhanced the TNF-α response of PBMCs stimulated with Pam3Cys, *M. tuberculosis*, and *C. albicans* in all three groups. Hypoglycemia also increased the IL-6 response to *M. tuberculosis* in healthy control participants and patients with NAH but had virtually no effect on the IL-6 and IL-1β responses to Pam3Cys or *C. albicans* in any of the three groups (Supplementary Fig. 2). Altogether, hypoglycemia enhanced cytokine responses of PBMCs, with the most prominent increase in TNF-α responses (Fig. 2*F*). Of note, cytokine release of stimulated PBMCs from patients with IAH isolated during euglycemia tended to be higher than the cells from healthy control participants and patients with NAH, although the differences were not statistically significant.

Hypoglycemia Does Not Affect Glycolytic Metabolism of PBMCs

We then investigated whether hypoglycemia affected glycolytic metabolism of PBMCs. As expected, stimulation with LPS significantly increased glucose consumption and lactate production of PBMCs in all groups (Fig. 3*A* and *B*). However, no difference was found in either glucose consumption or lactate production between cells exposed to hypoglycemic versus euglycemic conditions, regardless of whether stimulated with LPS.

Hypoglycemia Generally Increases Expression of Markers for Demargination and Cells With Cytotoxic Effector Potential

Because adrenaline levels increase markedly in response to hypoglycemia and because adrenaline drives demargination of leukocytes (20), we investigated whether hypoglycemia altered gene expression levels of demargination markers in isolated PBMCs. Hypoglycemia increased the expression of the integrin CD11a in PBMCs of healthy control participants and patients with NAH but not in patients with IAH (Fig. 4*A*). Hypoglycemia also increased the expression of the chemokine receptor CX3CR1 in PBMCs of healthy control participants (Fig. 4*B*).

We next assessed the expression of marker genes of various immune cell types in PBMCs exposed to hypoglycemia or euglycemia (Fig. 4*C–G*). Hypoglycemia increased the expression of CD8 but not of CD4 or CD56 in PBMCs in all groups. Moreover, although hypoglycemia did not alter expression of CD14, it increased the expression of CD16, a marker for the nonclassic monocyte subset that produces more cytokines than the classic monocytes in response to certain stimulations (29).

Hypoglycemia Increases Ex Vivo Cytokine Production of CD14$^+$ Cells

Because monocytes are the major producers of proinflammatory cytokines within the heterogeneous PBMC cell population, we specifically investigated the effect of hypoglycemia on the inflammatory function of CD14$^+$ monocytes. Hypoglycemia did not affect the percentage of isolated CD14$^+$ monocytes within the PBMC fraction in any of the three groups (Fig. 5*A*). When stimulated ex vivo, CD14$^+$ cells produced more proinflammatory cytokines, particularly TNF-α, if isolated after hypoglycemia compared with euglycemia (Fig. 5*B*). Nevertheless, CD14$^+$ cells did not have increased gene expression levels of surface markers characterizing proinflammatory monocytes (CD11a, CXRCR1, CCR5, CCR2) (Supplementary Fig. 3).

DISCUSSION

This study investigated the effect of acute hypoglycemia on the composition and inflammatory function of circulating immune cells. We demonstrate that exposure to hypoglycemia leads to demargination of specific immune cell subtypes and enhances the inflammatory response of PBMCs and CD14$^+$ monocytes. Of note, the hypoglycemic response of PBMCs was partly blunted in patients with type 1 diabetes and IAH, highlighting the role of adrenaline in immune cell recruitment and in the acute inflammatory response to hypoglycemia. The data support the concept that hypoglycemia shifts circulating immune cells toward a more proinflammatory state. When sustained, such an enhanced inflammatory state could contribute to atherogenesis in people with diabetes.

In line with previous findings (15), the results demonstrate that hypoglycemia induces leukocytosis. The strong correlation with adrenaline responses to hypoglycemia suggests a role for adrenaline, which is supported by our observations in patients with type 1 diabetes and

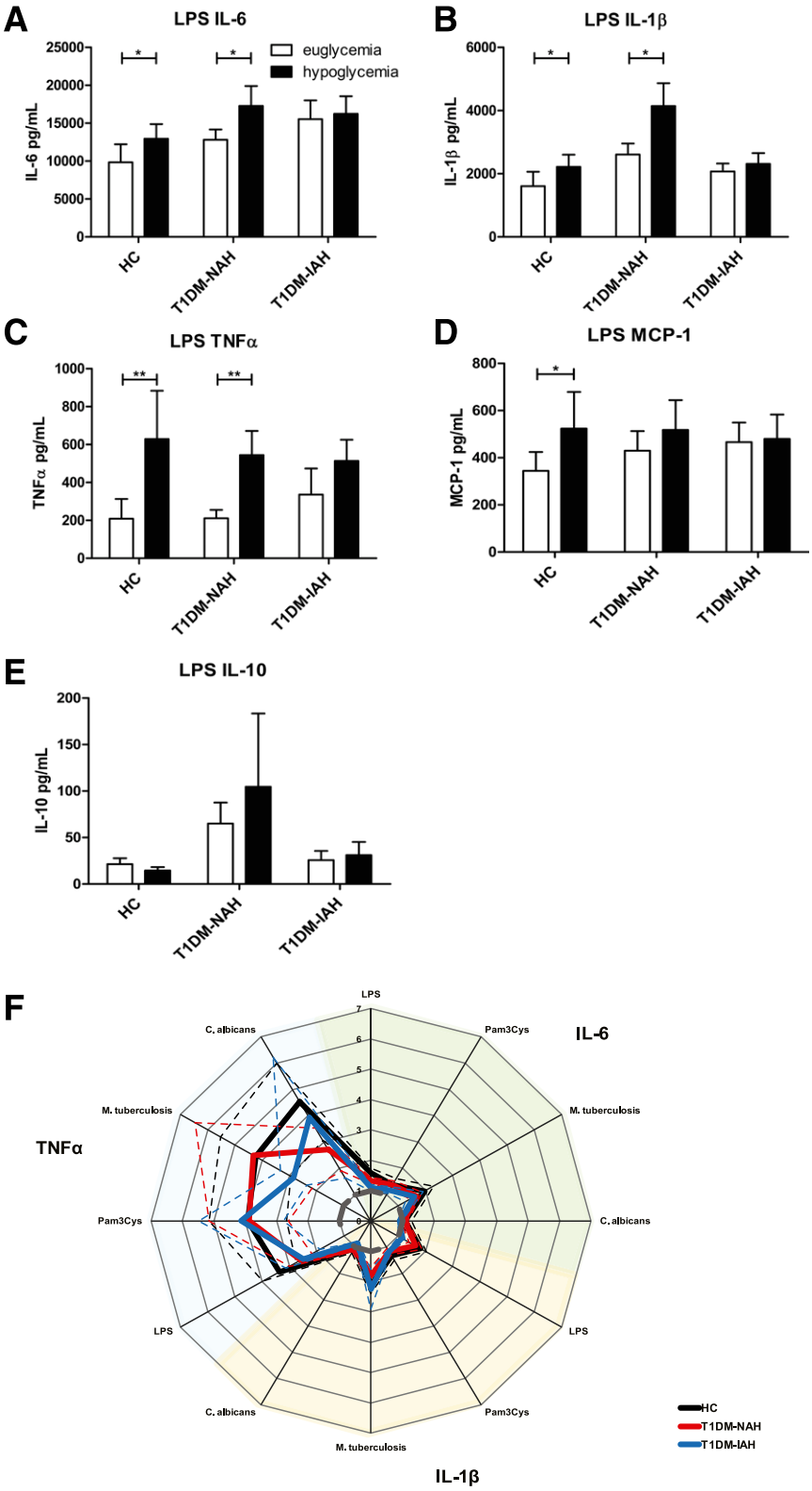

Figure 2—PBMCs isolated after hypoglycemia produce more proinflammatory cytokines than PBMCs isolated after euglycemia. *A–E*: IL-6 (*A*), IL-1β (*B*), TNF-α (*C*), MCP-1 (*D*), and IL-10 (*E*) production of PBMCs isolated from euglycemic or hypoglycemic conditions and stimulated for 24 h with LPS. *F*: Fold change in cytokine production (IL-6, IL-1β, TNF-α) by PBMCs upon hypoglycemia vs. euglycemia. The gray dashed line represents euglycemic values. PBMCs were stimulated with the TLR4 agonist LPS, the TLR2 agonist Pam3Cys, *C. albicans,* or lysate of *M. tuberculosis*. Data are mean (continuous lines) ± SEM (dotted lines). **P* < 0.05; ***P* < 0.01. HC, healthy control; T1DM, type 1 diabetes mellitus.

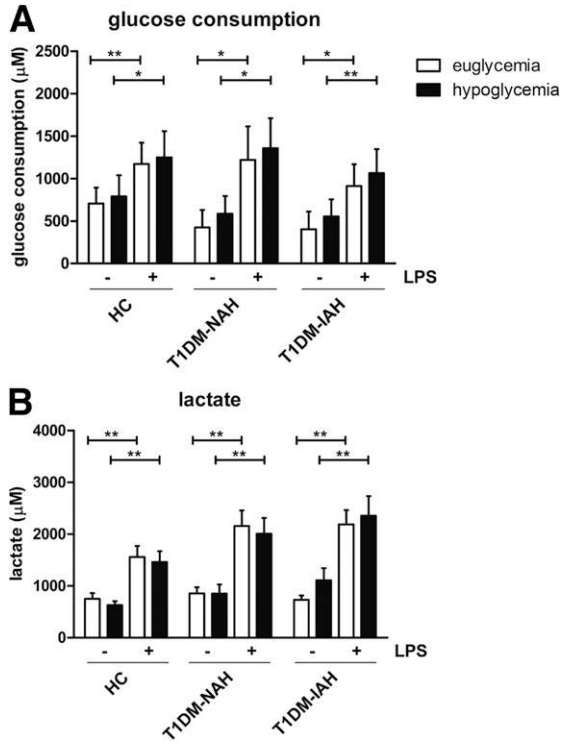

Figure 3—No differences in glycolytic metabolism of PBMCs isolated from hypoglycemia vs. euglycemia. Glucose consumption (*A*) and lactate secretion (*B*) measured in the supernatants of PBMCs isolated from euglycemic or hypoglycemic conditions and cultured for 24 h with or without stimulation with LPS. $^{*}P < 0.05$; $^{**}P < 0.01$. HC, healthy control; T1DM, type 1 diabetes mellitus.

IAH who have blunted counterregulatory hormone responses to hypoglycemia. These studies now extend previous findings by investigating the inflammatory function of isolated immune cells ex vivo. We observed that TNF-α production significantly increases in PBMCs exposed to hypoglycemia, independent of the pathogenic stimulus (LPS, Pam3Cys, *C. albicans*, or *M. tuberculosis)*, which strongly implies that the hypoglycemic event causes a universal potentiation of inflammatory function of the cells. Because equal numbers of PBMCs were used in stimulations to compare the two glycemic conditions, the increased levels of circulating proinflammatory cytokines found in previous studies (15,17–19,30) are not only due to the increase in the number of circulating immune cells in response to hypoglycemia but also likely reflect changes in the functional status of immune cells.

In contrast to TNF-α responses, the effect of hypoglycemia on IL-6 and IL-1β production was less pronounced and more variable between the various stimuli, suggesting that changes in pathogen-specific signaling pathways are involved. Such changes could affect either the expression of pattern recognition receptors on the cell surface or expression of their downstream effectors. If intracellular signaling pathways are indeed affected by hypoglycemia, this could also prime the immune cells to respond differently to other stimuli, such as proatherogenic factors.

PBMCs are a heterogeneous mix of cell populations, and changes in composition could explain the increased cytokine production observed in response to hypoglycemia. However, measurements of the cellular composition of several PBMC samples did not reveal major changes (percentage of lymphocytes, monocytes, and granulocytes) after hypoglycemia. Of note, CD11a and CX3CR1 gene expression levels were increased in PBMCs exposed to hypoglycemia, suggesting an increase in the number of demarginated cells (20). Recruitment of a distinct cell population with a different phenotype and function likely contributes to the observed change in inflammatory responses after exposure to hypoglycemia. Similar to leukocytosis experimentally induced by adrenaline (20), hypoglycemia increased the number of cells with cytotoxic effector potential, such as lymphocytes expressing CD8. Additionally, hypoglycemia increased gene expression levels of CD16, suggesting an increase in circulating $CD16^{+}$ monocytes and natural killer cells, also secondary to adrenaline-mediated leukocytosis (20). Future studies applying flow cytometric analysis of circulating immune cells to provide additional information on the specific surface expression of selected proteins on certain cell populations would be of particular interest.

Cytokine production was similarly altered in $CD14^{+}$ cells and PBMCs exposed to hypoglycemia. Although we cannot distinguish between the different monocyte subsets (classic, intermediate, nonclassic) within the population of isolated $CD14^{+}$ cells, the increased cytokine response of PBMCs is likely based on the enhanced cytokine production capacity of $CD14^{+}$ monocytes because the percentage of monocytes was similar at hypoglycemia and euglycemia.

Another factor that may contribute to an altered inflammatory output of immune cells is a shift in cellular metabolism induced by changes in the metabolic environment. For instance, a highly active glycolytic metabolism has been shown to drive proinflammatory cytokine production in M1 macrophages and is important for activated effector T cells (31). However, similar glucose consumption and lactate production of cells isolated from hypoglycemic versus euglycemic conditions make it unlikely that changes in inflammatory responses are due to changes in glycolytic metabolism of immune cells. We cannot fully exclude an involvement of glycolytic metabolism because acute changes can occur in vivo but might be masked at the time point that we measured lactate levels in vitro.

The results revealed abrogation of hypoglycemia-induced leukocytosis and an attenuated inflammatory response to hypoglycemia in patients with IAH, potentially as a consequence of extensive prior exposure to hypoglycemia. The attenuated inflammatory response in patients with IAH underscores the contribution of counterregulatory hormones, especially adrenaline, in hypoglycemia-induced proinflammatory effects. Of note, hypoglycemia increased CD8 and CD16 expression in PBMCs of patients with IAH, indicating an increase in circulating cells with cytotoxic effector potential in these patients even while total leukocyte

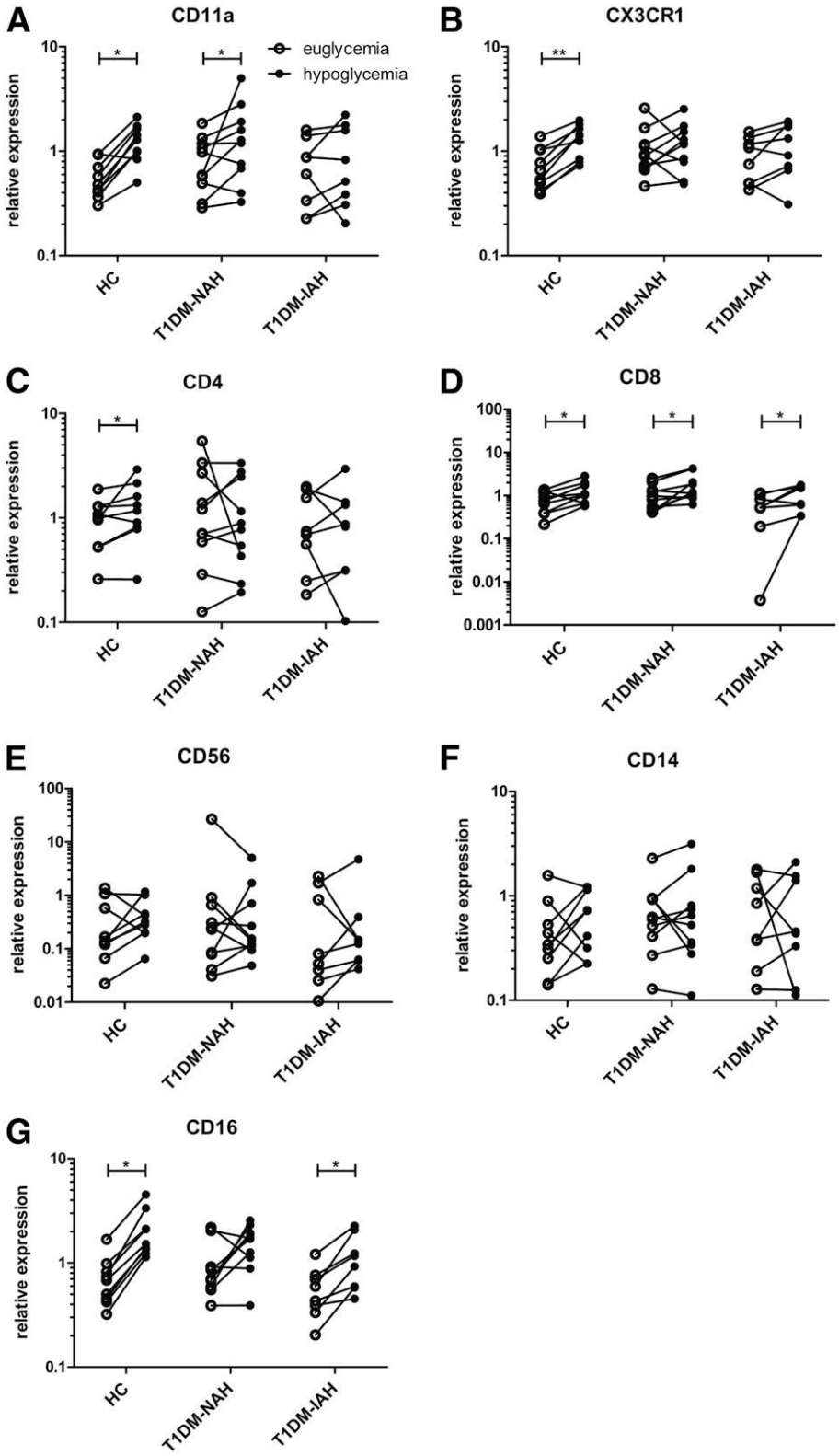

Figure 4—Increased expression of markers for demargination and cells with cytotoxic effector potential after hypoglycemia as assessed by qRT-PCR in PBMCs exposed to euglycemia or hypoglycemia. Relative expression of CD11a (*A*), CX3CR1 (*B*), CD4 (*C*), CD8 (*D*), CD56 (*E*), CD14 (*F*), and CD16 (*G*). *$P < 0.05$; **$P < 0.01$. HC, healthy control; T1DM, type 1 diabetes mellitus.

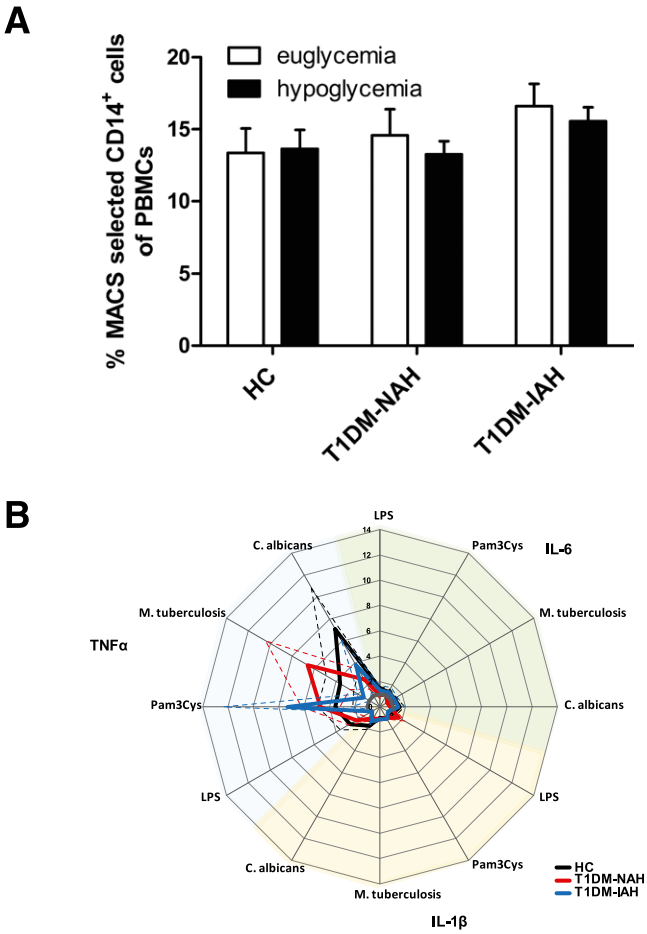

Figure 5—CD14$^+$ monocytes isolated after hypoglycemia produce more proinflammatory cytokines than CD14$^+$ cells isolated after euglycemia. *A*: Percentage of CD14$^+$ cells isolated by MACS. *B*: Fold change in cytokine production (IL-6, IL-1β, TNF-α) by CD14$^+$ cells isolated from hypoglycemic vs. euglycemic conditions. CD14$^+$ cells were stimulated with the TLR4 agonist LPS, the TLR2 agonist Pam3Cys, *C. albicans*, or lysate of *M. tuberculosis*. Data are mean (continuous lines) ± SEM (dotted lines). HC, healthy control; T1DM, type 1 diabetes mellitus.

count did not increase upon hypoglycemia. This finding might be explained by the minimal, albeit still significant, increase in adrenaline levels in response to hypoglycemia. Leukocyte numbers and cytokine responses during euglycemia appeared to be higher but were not significantly elevated in patients with IAH compared with healthy control participants or patients with NAH. Although attributing this trend to prior exposure to hypoglycemia is tempting, a larger sample size would be required to address this question.

One could speculate that an attenuated proinflammatory response to acute hypoglycemia as observed in the patients with IAH might provide some protection against harmful effects of subsequent hypoglycemia. Frequent hypoglycemic events, typical for patients with type 1 diabetes, have been reported to protect against hypoglycemia-induced mortality (32) or neuronal damage (33) in rats. These adaptive effects of recurrent hypoglycemia appear to be in line with the reported absence of increased cardiovascular mortality in patients with type 1 diabetes and IAH compared with those with NAH (10) but contrasts with studies focusing on vascular effects that reported higher rates of preclinical atherosclerosis in patients with repeated hypoglycemia (23) and greater proatherothrombotic responses and endothelial dysfunction after recurrent hypoglycemia (16). Studies are needed to determine the long-term consequences of repeated hypoglycemia and IAH on inflammation and immune cells, their inflammatory function, and their involvement in atherogenesis.

The strengths of this study include the use of glucose clamps in three matched groups of participants under similar glycemic conditions, which enabled us to differentiate between the impact of diabetes and IAH. A larger sample size would have allowed us to differentiate better between patients with IAH and NAH, especially with regard to baseline values. Although we analyzed gene expression of demargination markers and of specific cell types, flow cytometric analysis would have provided a more detailed characterization of changes in composition and inflammatory status of leukocytes. Future research should focus on mechanistic studies in lymphocytes and look into the role of neutrophils to extend the current gene expression data. Another limitation of this study is that participants with and without diabetes were healthy and relatively young. Inflammatory responses to hypoglycemia might differ in older patients, in those with a history of cardiovascular disease, or in those with poor glycemic control.

We conclude that hypoglycemia leads to demargination, an increase in circulating immune cells with cytotoxic effector potential, and an induction of proinflammatory functional changes in PBMCs and CD14$^+$ monocytes. Acute inflammatory responses to hypoglycemia were partly blunted in patients with type 1 diabetes and IAH, highlighting that counterregulatory hormone responses are key modulators of proinflammatory responses to hypoglycemia. These data indicate that hypoglycemia induces a shift in inflammatory function of immune cells, which could promote a sustained proinflammatory state in patients with diabetes.

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