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Hypoglycaemia induces a sustained pro-inflammatory response in people with type 1 diabetes and healthy controls

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Abstract

Aim: To determine the duration and the extension of the pro-inflammatory response to hypoglycaemia both in people with type 1 diabetes and healthy controls.

Materials and Methods: Adults with type 1 diabetes ($n = 47$) and matched controls ($n = 16$) underwent a hyperinsulinaemic-euglycaemic hypoglycaemic (2.8 ± 0.1 mmoL/L [49.9 ± 2.3 mg/dL]) glucose clamp. During euglycaemia, hypoglycaemia, and 1, 3 and 7 days later, blood was drawn to determine immune cell phenotype, monocyte function and circulating inflammatory markers.

Results: Hypoglycaemia increased lymphocyte and monocyte counts, which remained elevated for 1 week. The proportion of $CD16^+$ monocytes increased and the proportion of $CD14^+$ monocytes decreased. During hypoglycaemia, monocytes released more tumour necrosis factor-α and interleukin-1β, and less interleukin-10, after ex vivo stimulation. Hypoglycaemia increased the levels of 19 circulating inflammatory proteins, including high sensitive C-reactive protein, most of which remained elevated for 1 week. The epinephrine peak in response to hypoglycaemia was positively correlated with immune cell number and phenotype, but not with the proteomic response.

Conclusions: Overall, despite differences in prior exposure to hypoglycaemia, the pattern of the inflammatory responses to hypoglycaemia did not differ between people with type 1 diabetes and healthy controls. In conclusion, hypoglycaemia induces a range of pro-inflammatory responses that are sustained for at least 1 week in people with type 1 diabetes and healthy controls.

KEYWORDS
clamp, counterregulatory hormones, C-reactive protein, diabetes, hypoglycaemia, inflammation, white blood cells

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1 | INTRODUCTION

People with type 1 diabetes require insulin treatment to normalize their blood glucose levels and reduce the risk of developing microvascular and macrovascular complications.[1] Hypoglycaemia is the most common complication of insulin treatment and people with type 1 diabetes experience, on average, one to three hypoglycaemic events per week and one severe hypoglycaemic event per year.[2,3] Recurrent hypoglycaemic episodes can attenuate the counterregulatory response, and result in impaired awareness of hypoglycaemia (IAH).[4] People with IAH have a 6-fold increased risk of severe hypoglycaemia, defined as an event requiring external assistance for recovery.[5]

Since the early 1980s, studies have shown that hypoglycaemia induces a pro-inflammatory immune response, characterized by the upregulation and release of immune cells in the circulation.[6,7] Specifically, an increase in pro-inflammatory non-classical ($CD14^{-}CD16^{+}$) monocytes has been reported, which, in response to ex vivo stimulation, produce more pro-inflammatory cytokines, including tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-8.[8–11] Furthermore, hypoglycaemia has been found to increase several circulating inflammatory mediators, including high sensitive C-reactive protein (hs-CRP) and atherogenic markers (ICAM-1, VCAM-1 and E-selectin).[10,12,13]

A few studies have looked beyond the acute inflammatory response to hypoglycaemia, yet limited studies have been conducted in type 1 diabetes.[14–17] It has been reported that vasoactive substances and oxidative stress are upregulated until 24 hours after hypoglycaemia in type 1 diabetes and type 2 diabetes,[10,14] while circulating leukocyte levels were not different 48 hours after hypoglycaemia in healthy participants.[18] Two studies found inflammatory and prothrombotic markers to be elevated 7 days after hypoglycaemia in type 2 diabetes.[15,17] Together, these data show that hypoglycaemia can acutely mobilize and activate immune cells, at least in people with type 2 diabetes. It is unclear whether similar effects occur in people with type 1 diabetes (in whom hypoglycaemia occurs much more frequently), for how long those effects persist, and whether the inflammatory response is modified by prior exposure to hypoglycaemia in those individuals with IAH.

In this paper, we set out to investigate the acute and prolonged effects of a single hypoglycaemic event on immune cell composition, monocyte phenotype and function, and circulating inflammatory markers in people with type 1 diabetes and healthy controls. Furthermore, we assessed whether awareness state affects the inflammatory response to hypoglycaemia.

2 | METHODS

2.1 | Study design

This two-centre experimental study was performed at the Radboud University Medical Centre in Nijmegen, The Netherlands, and at the Nordsjællands University Hospital in Hillerød, Denmark. The study was approved by local institutional review boards and was performed according to the principles of the Declaration of Helsinki. All participants gave written informed consent.

2.2 | Participants

From August 2019 to March 2021, we recruited groups of people with type 1 diabetes from the outpatient diabetes clinics of each participating centre and people without diabetes matched for age and sex. Participants with type 1 diabetes included people with a normal awareness of hypoglycaemia (NAH), people with IAH and people with markedly high HbA1c (HH) (Figure S1). We observed no differences between the outcome of the subgroups and therefore decided to combine all groups to increase power. For the sensitivity analysis on awareness effect, we assigned the people with HH to either NAH or IAH based on awareness status. All participants were potentially eligible when they were younger than 80 years, had a body mass index (BMI) of 19-40 kg/m^2 and a blood pressure less than 140/90 mmHg. Key exclusion criteria were an HbA1c higher than 11.3% (> 100 mmol/mol) for people with type 1 diabetes or higher than 6.0% (> 42 mmol/mol) for healthy controls, pregnancy or breastfeeding or unwillingness to undertake measures for birth control. In addition, healthy controls were not allowed to use any medication, except for oral contraceptives. Exclusion criteria specific to the inflammatory analyses were use of immune-modifying drugs or antibiotics, use of statins that could not be interrupted, infection or previous vaccination in the previous 3 months and auto-inflammatory or auto-immune diseases other than type 1 diabetes. A complete overview of the inclusion and exclusion criteria can be found in (see the supporting information, Methods).

2.3 | Study procedure

All potentially eligible study participants were invited for a screening visit, which consisted of providing their medical history and undergoing a standard physical examination. Participants with diabetes were asked to complete several questionnaires to assess their awareness of hypoglycaemia (i.e. Clarke, Gold and Pedersen-Bjergaard[19–21]) with the use of standard cut-offs (Data S1). When at least two of these questionnaires fitted the diagnosis of IAH, a participant was classified as having IAH. HbA1c and kidney function (serum creatinine) were determined if this had not been carried out in the past 3 months before screening.

2.4 | Hyperinsulinaemic-euglycaemic glucose clamp

Participants with diabetes received an intermittently scanned continuous glucose monitoring (CGM) device (Freestyle Libre 1) for 14 days, which they starting 7 days before the experimental day. On the

experimental day, all participants underwent a hyperinsulinaemic euglycaemic-hypoglycaemic glucose clamp (Figure S2A), as described in detail previously.[17] Briefly, an intravenous catheter was inserted into an antecubital vein of one arm for continuous infusion of insulin Aspart (Novo Nordisk) at a rate of 1.5 mU/kg/min and a variable infusion of 20% glucose (Baxter B.V.). In a dorsal vein of the contralateral hand, a second catheter was inserted in retrograde fashion for frequent blood sampling, with the hand placed in a heated box (temperature ∼55°C) to arterialize venous blood. Plasma glucose levels were determined (Biosen C-Line; EKF Diagnostics) at baseline and every 5 minutes thereafter for the duration of the clamp. After a stable euglycaemic phase of 30 minutes, plasma glucose levels were gradually allowed to fall to 2.8 mmoL/L and were maintained at this level for another 60 minutes. At baseline and during euglycaemia and hypoglycaemia, symptom scores were determined using a slight modification of the Edinburgh Hypoglycaemia Score, as previously described.[22]

At baseline and twice during hypoglycaemia, blood was sampled for measurements of insulin and counterregulatory hormones (glucagon, epinephrine, norepinephrine, cortisol and growth hormone). At the end of euglycaemia and hypoglycaemia, then 1, 3 and 7 days after hypoglycaemia, extra blood was drawn under fasting conditions for additional analyses, as described below.

2.5 | Measurements

Serum creatinine was determined with an enzymatic assay on a Cobas 8000 c702 module (Roche Diagnostics). HbA1c was assessed by a TOSOH G8 high-performance liquid chromatography (HPLC) analyser (Sysmex) and a G11 HPLC-analyser (Sysmex). Plasma epinephrine and norepinephrine were measured by HPLC in combination with fluorometric detection. Plasma insulin was analysed with an in-house radioimmunoassay. Plasma hs-CRP concentrations were assessed by ELISA following the manufacturer's instructions (R&D Duoset ELISA Systems). Using the commercially available Olink Proteomics AB Inflammation Panel, 92 circulating plasma inflammatory proteins were measured (Uppsala, Sweden). Quality control was performed by Olink Proteomics; samples were excluded when their incubation control and detection control deviated +0.3 or −0.3 NPX from the plate median.[23] This resulted in the exclusion of six samples. Overall, 72 of the 92 (78%) proteins were detected in at least 75% of the plasma samples and were included in the analysis.

2.6 | Flow cytometry

Immune cell subset numbers were calculated based on cell numbers from whole blood differences measured on a Sysmex XN-450 (Sysmex) and Sysmex XN-9000 (Sysmex). Flow cytometry analysis was performed at one of the two participating study sites on 30 participants (controls: $n = 8$; participants with type 1 diabetes: $n = 25$). A total of 50 µL of whole undiluted blood was incubated for a duration of 15 minutes in the dark at room temperature with the following antibodies: CD16-FITC (dilution 1:20), CD14-PC7 (1:20), CCR2-BV421 (1:20) (BD Biosciences); CD41-PC5.5 (1:20), CD11b-BV785 (1:20) (ITK Diagnostics BV); HLA-DR-PE (1:10), CD56-APC (1:10), CD3-APC-750 (1:10), CD45-KO (1:10), CD36-APC-700 (1:10) (Beckman Coulter). Subsequently, 1 mL of lysis buffer (BD Pharm Lyse, BD Biosciences) was added, samples were mixed, incubated for another 10 minutes and then measured on a flow cytometer (Beckman Coulter FC500). To determine the position of analysis gates, single staining and fluorescence-minus-one control stains were used. Percentages were measured with flow cytometry. To analyse the flow cytometry data, Kaluza software (Beckman, Coulter) was used.

2.7 | Isolation of peripheral blood mononuclear cells and monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density centrifugation over Ficoll-Paque (GE Healthcare). Monocytes were isolated from PBMCs using magnetic activated cell sorting MicroBeads (Miltenyi Biotec) for CD14 negative selection according to the manufacturer's instructions. The purity of monocyte isolation was checked using a Sysmex XN-450 and Sysmex XN-9000.

2.8 | Monocyte stimulation

CD14 negative selected human monocytes (100,000 cells/well) were added to flat-bottom 96-well plates and stimulated with RPMI, 20 µg/mL of Pam3Cys (P3C) or 20 ng/mL of lipopolysaccharide (LPS) from *Escherichia coli* for 24 hours. The next day, the supernatants were collected and stored at −20°C until cytokine measurement. The production of TNF-α, IL-10, IL-1β and IL-6 in supernatants was determined by commercially available ELISA kits (R&D) for all participants in singular.

2.9 | Statistical analysis

Sample size calculation was based on ex vivo cytokine production of stimulated monocytes following hypoglycaemia, resulting in 16 participants per subgroup to find differences with an 80% power at the normal significance level of .05 (Data S1). Eventually, 16 healthy participants were included, along with 47 participants with type 1 diabetes (consisting of 15 participants with NAH, 16 participants with IAH and 16 participants with HH [Data S1]). First, the response to hypoglycaemia in people with diabetes was compared with the response in healthy controls. For additional sensitivity analyses, we compared different subgroups based on awareness state. All normally distributed data are shown as percentages or mean ± SD, unless otherwise indicated. All not normally distributed data were log-

transformed before analyses. Independent *t*-tests were used to compare baseline data, data obtained during euglycaemia and acute response of hypoglycaemia between participants with type 1 diabetes and healthy controls. The serial data were analysed with mixed model analysis. In this analysis, an unstructured covariance matrix for the correlation between and variance of the measurements was used. The dependent variable was the result of the measured variable of each time point and the independent variable was 'time'. Next to 'time', 'participant group' and their interaction terms were added as independent variables in the mixed model analysis to compare serial data between participants with type 1 diabetes and healthy controls. The *P* values reported are those of the model coefficients relevant to the question at hand. Pearson correlation was used to investigate the relationship between hypoglycaemia-induced increase in epinephrine and immune cell counts, phenotype and function, and Spearman correlation to relate circulating inflammatory markers to the increase in epinephrine.

Analysis and visualization of the Olink data were performed using the R programming language and R packages 'ggbiplot' and 'ggplot2'. A Wilcoxon matched-pairs test was performed to determine proteins that displayed significant changes from euglycaemia to each of the posteuglycaemia measurements. A Wilcoxon rank sum test was used to determine differences between subgroups for each time point. One subject was excluded from Olink analyses because of missing data. All proteomic analyses were false discovery rate (FDR) corrected for multiple testing. Independent *t*-tests were used in sensitivity analyses to test the impact of hypoglycaemia awareness status on the inflamma-

TABLE 1 Baseline characteristics

	Type 1 diabetes ($n = 47$)	Healthy controls ($n = 16$)
Male, *n* (%)	23 (48.9)	7 (43.8)
Age, y	47.0 [–]	47.5 [24.5-64.5]
Duration of diabetes, y	22.4 ± 13.7 (1.0-52.0)	–
HbA1c, mmol/mol	61.6 ± 9.9[a] (38.0-97.0)	33.6 ± 3.5 (28.0-39.0)
HbA1c, %	7.8 ± 0.9 (5.6-9.4)	5.2 ± 0.3 (4.7-5.7)
BMI, kg/m^2	26.4 ± 3.6[a] (20.0-33.5)	22.6 ± 2.8 (19.1-29.4)
Glucose-lowering medication		
Oral, *n* (%)	0 (0.0)	–
CSII, *n* (%)	21 (44.6)	–
MDI, *n* (%)	26 (55.3)	–
Insulin dose, IU/day	50.0 ± 23.2	–

Note: Data are presented as number (%), mean ± SD (range) or median [IQR].
Abbreviations: BMI, body mass index; CSII, continuous subcutaneous insulin infusion; MDI, multiple daily injections.
[a]$P < .05$ versus healthy controls.

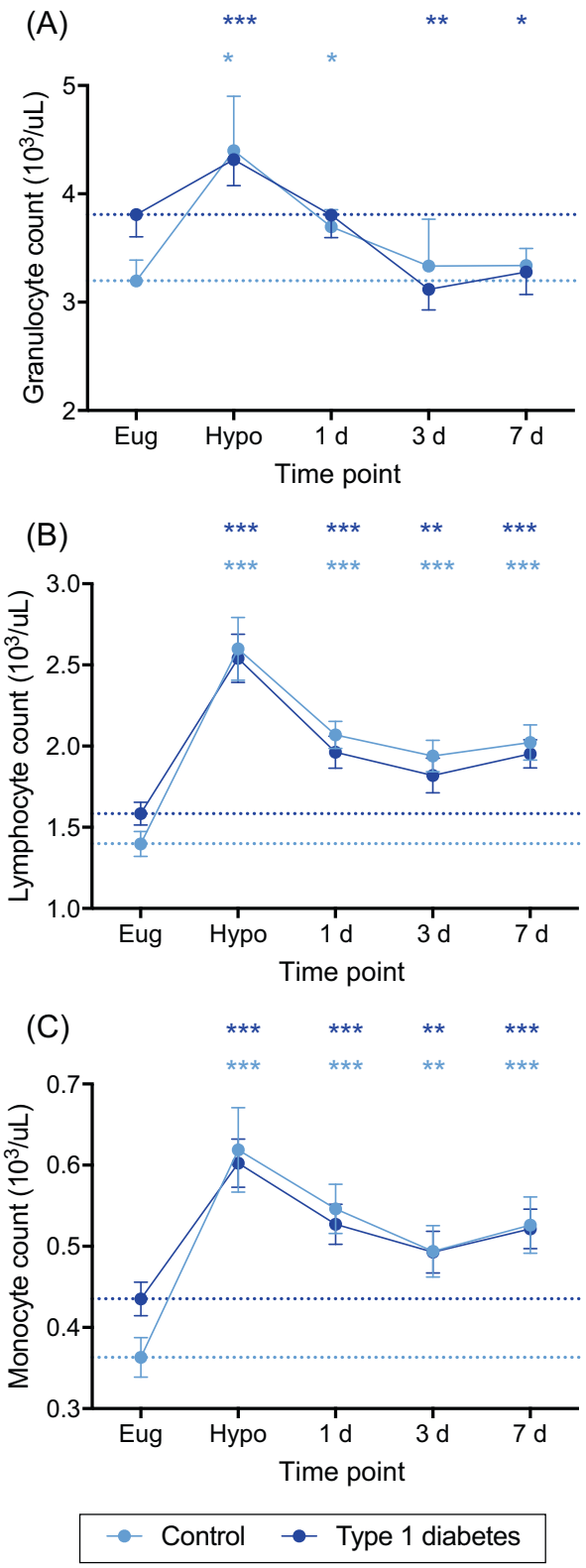


FIGURE 1 Counts (10^3/μL) of A, Granulocytes, B, Lymphocytes, and C, Monocytes for participants with type 1 diabetes (dark blue, $n = 47$) and healthy controls (light blue, $n = 16$). Data are presented as mean ± SEM; *$P < .05$, **$P < .01$ and ***$P < .001$ versus euglycaemia based on mixed model analysis. Eug, euglycaemia; Hypo, hypoglycaemia.

tory responses and CGM outcomes. CGM outcomes were calculated using the 'iglu' package[24] on participants with 6 days of CGM active time of 80% or higher after the clamp. Statistical analyses were performed using IBM SPSS Statistics 27, GraphPad Prism 5.03 or R Studio (version 1.4.1717). Alpha was set at .05 throughout, unless otherwise stated.

3 | RESULTS

In this study, a total of 47 participants with type 1 diabetes and 16 people without diabetes were included. Groups were well matched, except for higher BMI in the participants with type 1 diabetes (Table 1). Glucose levels for the euglycaemic phase during the clamp averaged 5.14 ± 0.44 mmoL/L (92.6 ± 7.9 mg/dL) and 5.20 ± 0.38 mmoL/L (93.8 ± 6.9 mg/dL) in participants with type 1 diabetes and healthy controls ($P = .589$), respectively, and 2.75 ± 0.09 mmoL/L (49.5 ± 1.7 mg/dL) versus 2.84 ± 0.19 mmoL/L (51.1 ± 3.4 mg/dL) ($P = .076$) for the hypoglycaemic phase (Figure S2A). All counterregulatory hormones, except for glucagon, increased significantly in response to hypoglycaemia in all participants (all $P < .001$; Figure S2B–D), but people with diabetes had a significantly lower increase in epinephrine and glucagon levels compared with healthy controls (both $P < .05$; Figure S2B,C).

At euglycaemia, granulocytes, lymphocytes and monocyte counts did not differ between participants with type 1 diabetes and healthy controls, but all cell counts increased in response to hypoglycaemia (Figure 1). Granulocyte counts fell below euglycaemic levels after 3 days and remained decreased in people with type 1 diabetes. In healthy controls, granulocytes increased numerically more than in people with type 1 diabetes, although this did not reach statistical significance, and remained elevated for 1 day before normalizing. In both groups, the levels of lymphocytes and monocytes remained elevated for the whole week (both $P < .001$).

Proportions of (pro-inflammatory) $CD16^+$ and (anti-inflammatory) $CD14^+$ monocytes did not differ between participants with type 1 diabetes and healthy controls at euglycaemia. Hypoglycaemia increased the proportion of $CD16^+$ monocytes in both groups (both $P < .001$), but significantly more in healthy controls ($P = .001$; Figure 2). Conversely, hypoglycaemia reduced the percentage of $CD14^+$ monocytes in participants with type 1 diabetes and healthy controls (both $P < .001$), again significantly more in healthy controls ($P = .002$). In both groups, this shift normalized within 24 hours after hypoglycaemia.

At euglycaemia, ex vivo LPS-stimulated levels of IL-1β, IL-6, TNF-α and IL-10, as well as P3C-stimulated IL-10 production, were significantly higher in people with type 1 diabetes compared with healthy controls (all $P < .05$; Figure 3). Hypoglycaemia resulted in significant increases in IL-1β and TNF-α production upon TLR-4 (LPS) and TLR-2 (P3C) stimulation in both type 1 diabetes and healthy controls (Figure 3A,B), but did not affect IL-6 production (Figure 3C). The anti-inflammatory cytokine IL-10 fell in response to hypoglycaemia in both groups following TLR-4 and TLR-2 stimulation (Figure 3D).

At euglycaemia, seven proteins (hs-CRP, IL-18R1, CD244, LIF-R, CCL3, TNF and CCL4) were significantly higher in people with type 1 diabetes compared with healthy controls (Figure 4A,B). Hypoglycaemia resulted in increased levels of inflammatory proteins after 1 day and 7 days in both groups, although fewer proteins were significantly increased in the healthy controls (Figure 4C–F). Forty-one inflammatory proteins were significantly increased 1 day after hypoglycaemia (including hs-CRP, Fit3L, uPA, LIF-R, CD5, CD6, IL-10, TRANCE, IL-17C, IL-6 and FGF-21; FDR < 0.01; Figure S3A), all of which, except for FGF-19, IL-6, SCF and TWEAK, remained significantly elevated for the entire week. Furthermore, nine more proteins (CCL20, CCL3, CCL4, CXCL-9, EN-RAGE, IL12B, MCP2, TNF-β and TNFSF14) became significantly elevated after 7 days (Figure S3B).

In the whole group comprising all participants, the increase of granulocytes, lymphocytes and monocytes during hypoglycaemia was positively associated with the epinephrine response to hypoglycaemia (all $P < .05$; Figure S4A), whereas there were similar associations between increases of granulocytes and monocytes with the glucagon response, and between the increase of lymphocytes with the cortisol response (all $P < .01$). Also, the monocyte phenotype switch from classical to non-classical monocytes was associated with the epinephrine response ($P \leq .001$; Figure S4B). However, the increase in ex vivo cytokine production in stimulated monocytes was not correlated with the epinephrine response, nor were the increases in circulating inflammatory markers upon hypoglycaemia.

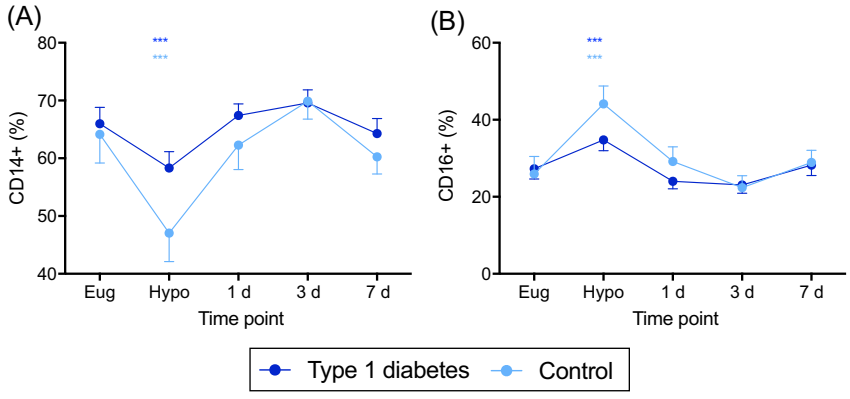
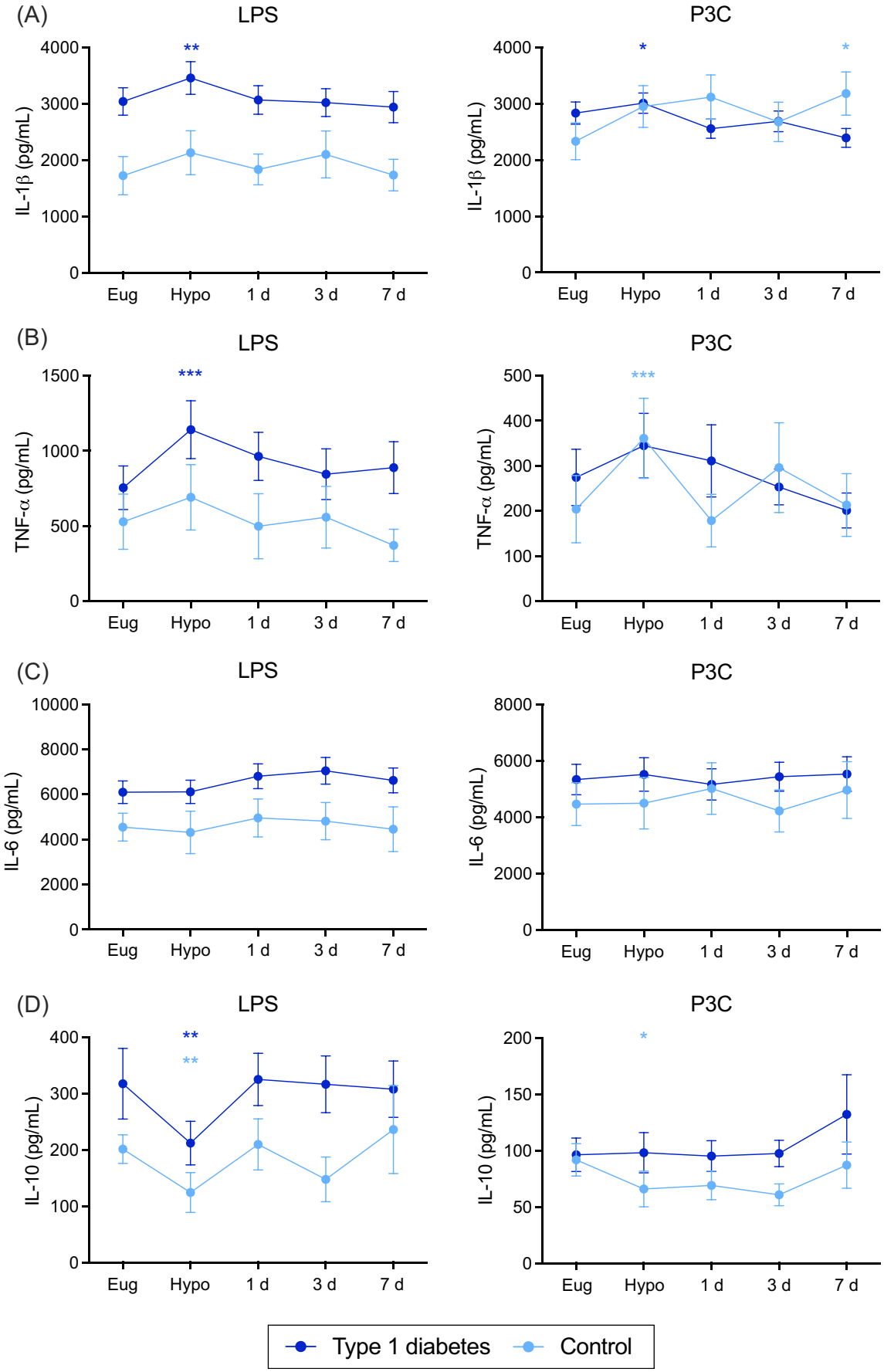


FIGURE 2 Proportion of monocytes for participants with type 1 diabetes (dark blue, $n = 25$) and healthy controls (light blue, $n = 8$) at all time points characterized by A, Classical monocytes $CD14^+$, and B, Non-classical monocytes $CD16^+$. Data are presented as mean ± SEM; ***$P < .001$ change versus euglycaemia based on mixed model analysis. Eug, euglycaemia; Hypo, hypoglycaemia.



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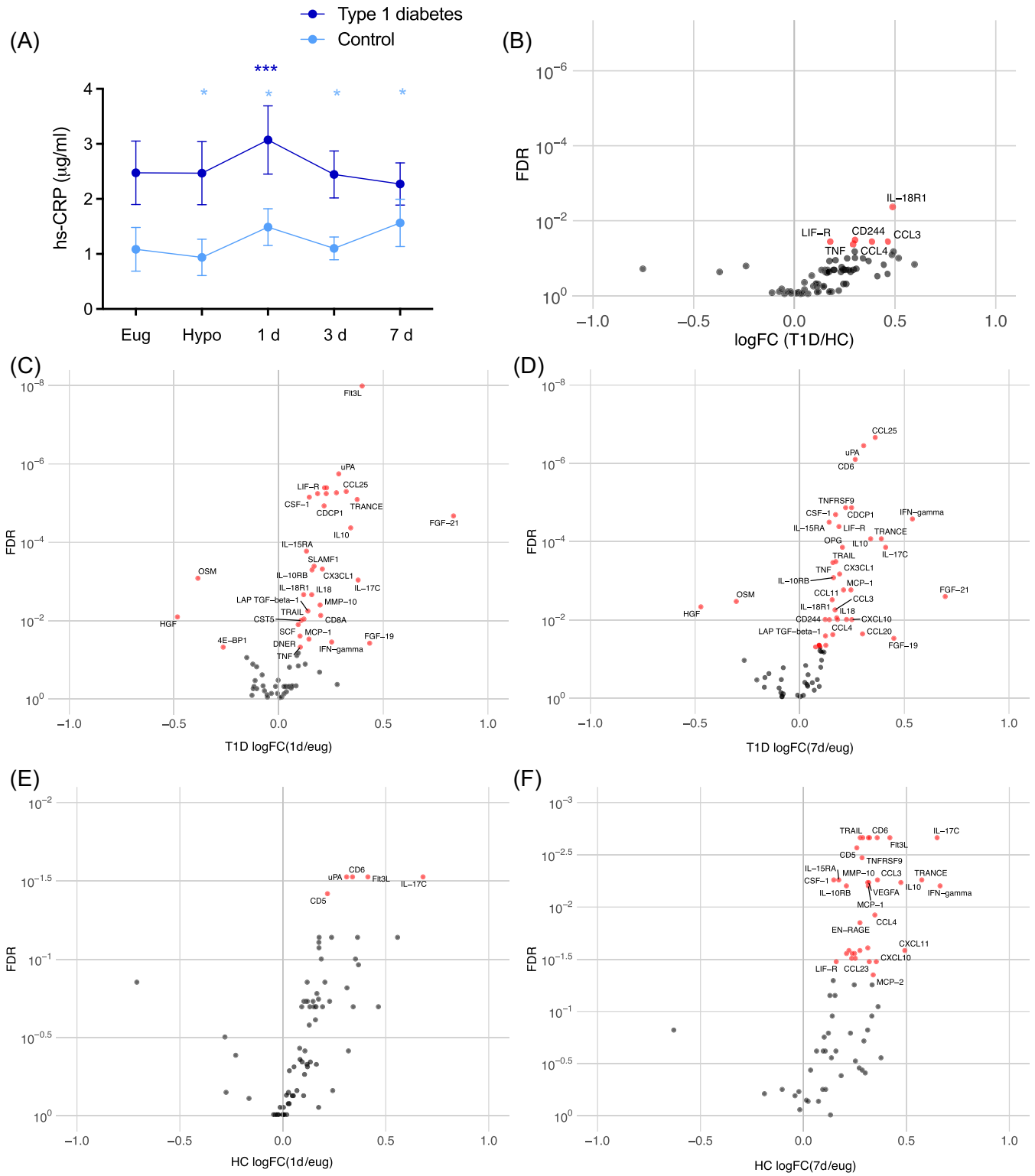

FIGURE 4 Circulating inflammatory proteins: A, hs-CRP at all time points for participants with type 1 diabetes (T1D; dark blue, $n = 47$) and healthy controls (light blue, $n = 16$). Proteomics inflammation panel for B, participants with T1D ($n = 44$) compared with healthy controls ($n = 16$) at euglycaemia. Day 1, compared with euglycaemia for participants with C, T1D, and E, Healthy controls (HC). At day 7, compared with euglycaemia for D, T1D, and, F, HC. Red dots indicate proteins that are significantly different; Wilcoxon signed ranked or paired test ($P < .05$). Eug, euglycaemia; hs-CRP, high sensitive C-reactive protein; FDR, false discovery rate; Hypo, hypoglycaemia.

FIGURE 3 Ex vivo cytokine production of A, IL-1β, B, TNF-α, C, IL-6, and D, IL-10 upon LPS or P3C stimulation for participants with type 1 diabetes (dark blue, $n = 46$) and healthy controls (light blue, $n = 16$). Data are presented as mean ± SEM; *$P < .05$, **$P < .01$ and ***$P < .001$ versus euglycaemia based on mixed model analysis. Eug, euglycaemia; Hypo, hypoglycaemia; IL, interleukin; LPS, lipopolysaccharide; TNF, tumour necrosis factor.

In a sensitivity analysis, we examined the impact of awareness status in participants with type 1 diabetes. For this analysis, people with type 1 diabetes were categorized into subgroups determined by hypoglycaemia awareness status, that is, IAH ($n = 21$) or NAH ($n = 26$) (Table S1). The groups were well matched, except for a lower age in people with NAH compared with those with IAH. Baseline lymphocyte counts under euglycaemic conditions were higher in people with NAH compared with people with IAH ($P = .002$), whereas levels of pro-inflammatory proteins were significantly higher at all time points in IAH compared with NAH (Figure S5). Awareness status did not impact upon the effect of hypoglycaemia on circulating immune cell numbers and circulating inflammatory markers. Regarding the ex vivo monocyte function, only IL-6 production upon ex vivo stimulation with LPS was significantly lower in participants with IAH compared with those with NAH ($P = .02$; Table S2). To determine if these differences could be explained by additional hypoglycaemic episodes in the week after the clamp, we looked at the CGM recordings. There were no differences concerning the number and duration of hypoglycaemic episodes between people with IAH compared with those with NAH (Table S3).

4 | DISCUSSION

This study shows that hypoglycaemia induces a sustained pro-inflammatory response characterized by an increased number of white blood cells, change in monocyte phenotype towards a more pro-inflammatory type, enhanced ex vivo pro-inflammatory monocyte response and an increase of circulating pro-inflammatory markers. While most of the effects on monocyte phenotype and function did not last beyond 1 day, circulating levels of lymphocytes and monocytes and several circulating inflammatory markers remained elevated for up to 7 days. Increased levels of circulating immune cells and a switch to more pro-inflammatory monocytes following hypoglycaemia were associated with the epinephrine response, but the ex vivo stimulated production of cytokines and the production of pro-inflammatory proteins were not. These pro-inflammatory effects of hypoglycaemia were largely similar in people with type 1 diabetes and healthy controls and mostly irrespective of awareness status.

Hypoglycaemia has been shown previously to provoke the rapid mobilization of white blood cells in people with and without diabetes.[6,7,17,25,26] We extend these earlier published data by several new observations. First, we showed that, in particular, lymphocytes and monocytes remain elevated for up to 7 days after the hypoglycaemic event, suggesting that hypoglycaemia has persistent effects on the immune system. This is in line with previous work in people with type 2 diabetes.[17] Lymphocytes have a lifespan of approximately 1 week, while the lifespan of monocytes is only a few days, suggesting a second mechanism that sustains the increased number of monocytes. Epinephrine may potentially be involved in the underlying mechanism, as myeloid stimulating effects of epinephrine have been described,[27] which is supported by a positive correlation between total white blood cell count and the epinephrine response.

Besides the increased monocyte counts, we also found that hypoglycaemia induced a change of monocytes phenotype. The proportion of phagocytizing classical monocytes ($CD14^+CD16^-$) decreased and the proportion of more pro-inflammatory non-classical monocytes ($CD14^-CD16^+$) increased following hypoglycaemia, which is in line with previous findings.[17,25] A potential explanation for this acute shift could be that monocytes, which express β-2 adrenoreceptors, are able to shift from classical to non-classical forms by a catecholamine-dependent mechanism.[28] This is supported by the correlation between the epinephrine response and this phenotype switch.

The function of monocytes was altered towards a more pro-inflammatory profile, as defined by increased ex vivo TNF-α and IL-1β cytokine production, and decreased release of the anti-inflammatory cytokine IL-10. This observation, which is in line with previous findings in type 1 diabetes and type 2 diabetes,[9,17] suggests an imbalance between the production of pro-inflammatory and anti-inflammatory cytokines, further contributing to the creation of a more pro-inflammatory state.

The sustained inflammatory effects of hypoglycaemia are further supported by increased levels of pro-inflammatory proteins in the circulation, most of which remained elevated for up to 7 days. These finding are similar to previous reported long-term effects of hypoglycaemia in people with type 2 diabetes.[17] Apart from the previously reported increase in hs-CRP,[10] a well-known marker for the development of atherosclerosis,[29] we also found several other proteins that have not been reported before to increase in response to hypoglycaemia in type 1 diabetes. An example is MCP-1, which plays a role in monocyte migration from the bone marrow.[29] Another highly expressed marker was uPA, which is a multifunctional domain protein that is highly expressed by cells in the atherosclerotic plaque.[30] Overall, these findings confirm the presence of long-term inflammation following hypoglycaemia and provide us with new inflammatory proteins that play a role in the pathophysiology of the pro-inflammatory response to hypoglycaemia in type 1 diabetes.

In contrast to the correlation between epinephrine and circulating immune cell numbers and phenotype, epinephrine did not appear to be correlated with the ex vivo release of pro-inflammatory cytokines and proteins in the circulation. This suggests that epinephrine may be a driving force in the acute inflammatory response to hypoglycaemia (perhaps via binding to the β2 adrenoreceptors on monocytes and lymphocytes), but not, or much less so, in the longer term inflammatory response. Whether the changes in the circulating markers by hypoglycaemia are an indirect effect of epinephrine or are explained by another mechanism cannot be derived from our data. Besides epinephrine, we also found a positive correlation between cortisol and circulating immune cell numbers. Glucocorticoids can affect leukocyte migration from the bone marrow, which is in line with our findings.[31,32]

We found increased levels of inflammatory markers in participants with IAH at all time points. IAH is associated with an increased risk of developing severe hypoglycaemia,[5] which in turn is associated

with the development of cardiovascular complications.[33–36] Although previous research did not find increased levels of inflammation in people with IAH,[37] our data suggest people with IAH are at a high risk of recurrent hypoglycaemic events and that each event continues to provoke an inflammatory response, which could affect the vessel wall. In the brain, recurrent episodes of hypoglycaemia have been associated with increased levels of oxidative stress and inflammation,[38] which is in line with our findings on a systemic level. This may be an additional reason for people with diabetes to avoid IHSG level 2 and level 3 hypoglycaemic events. As stated before, chronic low-grade inflammation is an important risk factor for the development of vascular complications.[39–41] We found acutely and prolonged elevated monocyte and lymphocyte counts, impaired ex vivo cytokine production and increased circulating inflammatory markers following a hypoglycaemia event, which may contribute to a more chronic pro-inflammatory state[42] and consequently to the development of diabetes-related complications, including cardiovascular disease.[36] Whether the repeatedly reported association between history of (severe) hypoglycaemia and cardiovascular risk[33] is related to hypoglycaemia-induced inflammation cannot be answered by the present study.

Interestingly, we found a similar pro-inflammatory effect in people with type 1 diabetes compared with previous work in people with type 2 diabetes.[17] This suggests that the pro-inflammatory effect of hypoglycaemia is largely independent of the type of diabetes, and even the presence of diabetes altogether. Although the effect of hypoglycaemia appears to be similar in people with type 2 diabetes, the levels of inflammatory markers do appear to be higher at baseline, including the number of circulating immune cells. This is line with the presence of chronic low-grade inflammation in people with type 2 diabetes, which is also important in the development of insulin resistance.[43]

Our study has several limitations. First, the hypoglycaemia induced with the hyperinsulinaemic glucose clamp may differ from hypoglycaemia experienced in daily life, even although using this technique guaranteed equivalent glycaemic conditions in all participants. Second, phenotyping of monocytes was only performed at one of the two study centres, which limits the study's statistical power. Nevertheless, this method is highly sensitive to site-specific effects and was only used to examine underlying mechanisms. Third, the samples were collected at both study sites, which could have introduced bias. We corrected for this effect by performing paired analysis for each individual and by conducting the analysis of all the samples at one study site. Fourth, although we tried to match the participants in both participant groups, the BMI of those people with type 1 diabetes was significantly higher, which may have affected our results.[44,45] Our study also has several strengths. First, it included a large number of participants. Second, we provided an extensive, comprehensive assessment of the inflammatory profile, including inflammatory cell counts, composition of cells, function of cells and circulating inflammatory proteins representative for multiple levels of the immune system. Finally, unlike most previous studies on inflammatory profiles, our investigations continued for up to 1 week after the hypoglycaemic event.

In conclusion, this study shows that hypoglycaemia induces a pro-inflammatory response, both at the cellular and protein level, that is sustained for at least 1 week in people with type 1 diabetes and healthy controls. These effects of hypoglycaemia are largely irrespective of the presence of type 1 diabetes or the hypoglycaemia awareness status, with some of them appearing—at least partly—related to the hypoglycaemia-induced epinephrine response. Future studies need to reveal whether recurrent hypoglycaemic events contribute to a state of chronic low-grade inflammation and increased risks of vascular complications.

AUTHOR CONTRIBUTIONS

CEMV, TWF, CJT, RS, UB and BEdG designed the study. CEMV, TWF and JIPvH performed the experiments and collected the data. JIPvH and CEMV analysed the data and wrote the first version of the manuscript. All authors discussed the results and implications and provided feedback on the manuscript at all stages. UB and BEdG are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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CONFLICT OF INTEREST STATEMENT

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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