

Acute and chronic neuroinflammation is triggered by diabetic ketoacidosis in a rat model

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To cite: Glaser N, Chu S, Hung B, *et al*. Acute and chronic neuroinflammation is triggered by diabetic ketoacidosis in a rat model. *BMJ Open Diab Res Care* 2020;**8**:e001793. doi:10.1136/bmjdr-2020-001793

► Supplemental material is published online only. To view, please visit the journal online (<http://dx.doi.org/10.1136/bmjdr-2020-001793>).

Received 22 July 2020
Revised 21 October 2020
Accepted 11 November 2020



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ABSTRACT

Introduction Cognitive decline is common in patients with type 1 diabetes and has been attributed to the effects of chronic hyperglycemia and severe hypoglycemia. Diabetic ketoacidosis (DKA) has only recently been suspected to be involved in causing cognitive decline. We hypothesized that DKA triggers both acute and chronic neuroinflammation, contributing to brain injury.

Research methods and design We measured concentrations of cytokines, chemokines and matrix metalloproteinases (MMP) in serum and brain tissue lysates in juvenile rats during and after DKA (during acute DKA, 24 hours and 7 days after DKA), and compared these to healthy controls and hyperglycemic controls. We also measured cytokine, chemokine and MMP concentrations in serum and brain tissue of adult rats (70 days) that had experienced DKA as juveniles and compared these measurements to those of adult diabetic rats without exposure to DKA.

Results During acute DKA in the juvenile rats, serum concentrations of CCL3, tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and MMP-9 were significantly increased. Serum concentrations of IL-2 and IL-17A increased 7 days after DKA recovery. In brain tissue lysates, concentrations of CCL3, CCL5, interferon (IFN)- γ and MMP-9 were significantly elevated during acute DKA. In adult rats that had DKA as juveniles (28 days previously), serum concentrations of IL-1 β and brain concentrations of IL-10 and IL-12p70 were elevated in comparison to diabetic rats without prior DKA. Composite scores for highly correlated cytokines and chemokines (mean z-scores for IL-10, IL-1 β , TNF- α , IL-17A, IFN- γ , CXCL-1 and CCL5) were also significantly elevated in adult rats with prior DKA.

Conclusions These data confirm that DKA causes acute systemic inflammation and neuroinflammation in a rat model. Importantly, the neuroinflammatory response triggered by DKA is long-lasting, suggesting the possibility that DKA-induced chronic neuroinflammation could contribute to long-term cognitive decline in individuals with diabetes.

INTRODUCTION

Decline in cognitive function is common in adults with type 1 diabetes (T1D), including greater risk of dementia.^{1 2} Cognitive impairments and structural alterations in the brain have also been documented in children with T1D.^{3–5} Chronic hyperglycemia and episodes

Significance of this study

What is already known about this subject?

- Decline in cognitive function is common in adults with type 1 diabetes (T1D), including greater risk of dementia. Cognitive alterations have also been described in children with T1D.
- Diabetic ketoacidosis (DKA) has recently been suspected to play a role in causing brain injury in patients with T1D; however, the mechanisms involved are unknown.

What are the new findings?

- We found that concentrations of inflammatory mediators (cytokines, chemokines and matrix metalloproteinases) were elevated in both blood and brain during DKA in a juvenile rat model. Patterns of inflammatory mediator elevation in brain differed from those in blood, suggesting a specific neuroinflammatory response.
- Importantly, adult rats that were exposed to DKA as juveniles had persistent evidence of neuroinflammation when compared with adult diabetic rats without past exposure to DKA.

How might these results change the focus of research or clinical practice?

- Our findings confirm that DKA causes acute neuroinflammation and suggest that chronic neuroinflammation may also be triggered by exposure to DKA. Both acute DKA-related brain injury and chronic neuroinflammation might play roles in causing cognitive decline in patients with T1D.
- Future research is needed to determine whether anti-inflammatory interventions could diminish brain injury during acute DKA and/or reduce chronic neuroinflammation to preserve cognition in patients with T1D.

of severe hypoglycemia have been hypothesized to be involved in causing cognitive decline, but recent data suggest that diabetic ketoacidosis (DKA) may also play a role.^{6 7} Life-threatening brain injury has long been recognized as an infrequent consequence of DKA in children; however, recent data suggest that more subtle brain injuries may

occur commonly during DKA and may contribute to cognitive decline.^{6–9} Investigating the role of DKA in contributing to cognitive decline is important because DKA occurs frequently in children with T1D. Approximately 30%–60% of children with new onset of T1D present with DKA, and subsequent episodes occur at a rate of 7%–10% per year.^{10–13}

Acute and chronic inflammation play important roles in many disease states. Exaggerated acute inflammatory responses may cause severe organ injuries in patients with sepsis or trauma, resulting in multiple organ dysfunction syndrome.^{14,15} Chronic low-level inflammation plays a role in causing atherosclerosis, Parkinson's disease and Alzheimer's disease, among others.^{16–18} Studies in animals and humans also demonstrate that DKA triggers a systemic inflammatory response.^{19–23} Previous work in our laboratory using a rat DKA model found that DKA causes a neuroinflammatory response involving reactive astrogliosis and activation of microglia^{24–26}; however, the mediators of this neuroinflammatory response have not been investigated, and the time course of neuroinflammation caused by DKA is unknown. We hypothesized that DKA would increase concentrations of proinflammatory cytokines and chemokines in brain tissue, particularly those involved in activating microglia or produced by activated microglia. Furthermore, we hypothesized that alterations in inflammatory mediators would persist for prolonged periods after resolution of DKA, contributing to chronic low-level neuroinflammation.

RESEARCH DESIGN AND METHODS

Overview

In these studies, we measured levels of inflammatory mediators (cytokines, chemokines and matrix metalloproteinases (MMPs) in blood samples and brain tissue lysates in juvenile and adult rats. We measured concentrations of inflammatory mediators at several time points during and after DKA and compared these measurements to those of rats with diabetes without DKA and healthy controls.

Juvenile rat diabetes model

Sprague Dawley rats 4–5 weeks old ($n=6–7$ per group, 100 g, male and female, Charles River Laboratories, Wilmington, Massachusetts, USA) were used for these experiments. Rats were treated with streptozotocin (STZ; Sigma-Aldrich, St. Louis, Missouri, USA) 165 mg/kg via intraperitoneal injection to induce diabetes. Normoglycemic (NG) controls received an intraperitoneal injection of STZ vehicle (citric acid, Sigma-Aldrich). Rats were allowed unlimited access to water containing 10% dextrose (Fisher Scientific, Santa Clara, California, USA) in the first 24 hours after STZ injection to prevent hypoglycemia. Urine glucose and acetoacetate were measured daily using Multistix urinalysis strips (Bayer, Fisher Scientific). Rats treated with STZ were administered insulin 24 hours after STZ injection (1 unit of Novolin 70:30

insulin (Novo Nordisk, Princeton, New Jersey, USA) administered subcutaneously in the morning and two units in the evening). STZ-treated rats in the hyperglycemic (HG) control group continued to receive subcutaneous insulin injections for the duration of the experiments. Rats in the DKA groups received insulin injections for 6 days after STZ administration, at which time injections were held to induce DKA.

DKA groups

DKA was induced following 6 days of insulin maintenance by withholding insulin and giving a high fat diet (diet D12492, 60 kcal% fat; Research Diets, New Brunswick, New Jersey, USA) for 24 hours. Water was removed for 12 hours to promote ketosis and to increase the degree of dehydration, thereby increasing the similarity with human DKA in which prolonged vomiting and severe dehydration occur frequently. Rats were considered to have developed DKA when urine glucose and acetoacetate concentrations were ≥ 2000 and 160 mg/dL, respectively. DKA was confirmed by blood analyses (blood glucose ≥ 300 mg/dL and β -hydroxybutyrate > 3 mmol/L).

After establishment of DKA, rats were treated to resolve DKA by administering an intraperitoneal injection of 0.9% saline (8 mL/100 g body weight), followed by subcutaneous injection of regular human insulin (Novolin R, Novo Nordisk) 0.5 units/100 g body weight. After the first hour, 0.9% saline (4 mL/100 g body weight, intraperitoneal) and regular human insulin (0.5 units/100 g body weight, subcutaneous) were administered hourly until resolution of DKA (blood glucose concentrations below 200 mg/dL, venous pH above 7.30 and serum β -hydroxybutyrate concentrations below 0.5 mmol/L). Time until DKA resolution varied between 5 and 8 hours. Blood glucose and β -hydroxybutyrate were measured every 2 hours during treatment. Serum electrolytes and pH were measured at the beginning and end of treatment (I-STAT Portable Clinical Analyzer; Sensor Devices, Waukesha, Wisconsin, USA). Immediately after resolution of DKA, Novolin 70:30 (two units) was administered subcutaneously. Insulin treatment was then resumed as prior to DKA induction, and rats were allowed unlimited access to water and standard rat chow. NG and HG control groups underwent identical sham treatments (intraperitoneal puncture, subcutaneous puncture and blood sampling) without undergoing insulin and saline treatment.

Sampling time points

Blood and brain samples were obtained from rats with DKA. The following time points were evaluated: (1) acute DKA, 4 hours after beginning treatment with insulin and saline, before DKA resolution (corresponding to the peak time for brain injuries in children with DKA); (2) 24 hours after treatment for DKA; (3) 7 days after treatment for DKA; and (4) 28 days after treatment for DKA. Brain and blood samples were obtained from HG control rats of the same age and diabetes duration as the acute

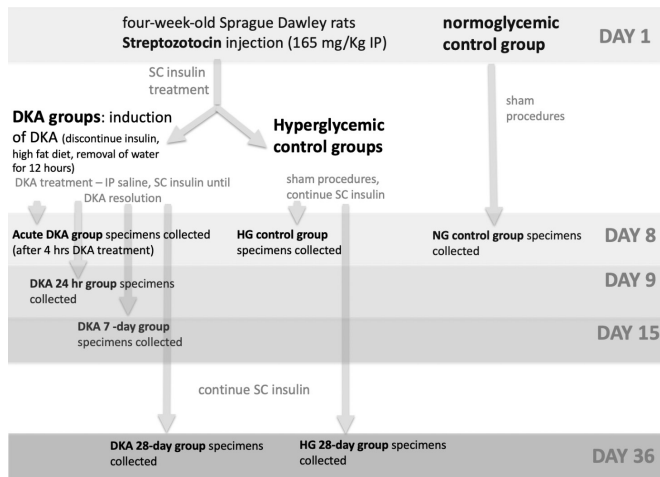


Figure 1 Overview of comparison groups and study procedures. DKA, diabetic ketoacidosis; HG, hyperglycemic; IP, intraperitoneal; NG, normoglycemic; SC, subcutaneous.

DKA group and the 28-day post-DKA group. Healthy (NG) control brain and blood samples were collected from rats identical in age to the acute DKA group. A summary of the treatment groups and sampling time points is presented in [figure 1](#).

Blood sample processing and brain lysate preparation

Rats were anesthetized using isoflurane and blood samples were collected from the left femoral artery. Blood samples were allowed to clot for 10 min, then centrifuged at 12 000 rpm for 5 min at room temperature. Serum (100–200 μ L) was collected and stored at -80°C . After blood samples were obtained, rats were decapitated using a guillotine. Brain samples were collected and dissected into regions according to previously published methods.²⁷ The left hemisphere was harvested intact. The right hemisphere was dissected into regions including cortex and hippocampus. Brain samples from all regions were typically collected within 10–12 min. Brain samples were initially frozen in microcentrifuge tubes placed in dry ice (-20°C) and then stored at -80°C .

Processing of brain tissue lysates followed previously published methods.²⁸ Brain samples were homogenized with Tissue Protein Extraction Reagent (T-PER; Thermo Fischer Scientific, Waltham, Massachusetts, USA) and Halt Protease Inhibitor Cocktails (Thermo Fischer Scientific). T-PER (2 mL) was used for every 100 mg of brain tissue with 1 μ L of protease inhibitor added for every 100 μ L of T-PER. Tissue was homogenized with a Dounce Homogenizer and then processed with an ultrasonicator (Branson Sonifier 4C15) for three pulses of 1 s each at 4°C . Brain tissue lysates were then centrifuged at 16 000 rpm for 10 min at 4°C and lysate supernatant was collected.

Cytokine and chemokine assays

To measure cytokine and chemokine concentrations in brain lysates and in serum, we used multiplex immunoassays (Invitrogen ProcartaPlex custom plates, Thermo

Fisher Scientific). Multiplex Immunoassays were used to measure CXCL1, CCL3, CCL5, interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , interferon (IFN)- γ , IL-2, IL-4, IL-6, IL-10, IL-12p70 and IL-17A. In these solid-phase sandwich immunoassays, microbeads of defined spectral properties coated with analyte-specific antibodies were incubated with brain tissue lysate or serum to capture the specific analytes. After washing, the beads were reacted with analyte-specific biotinylated detector antibodies which bound to the appropriate immobilized analytes. After additional washing, the beads were reacted with streptavidin-R-phycoerthrin (RPE), which bound to the biotinylated detector antibodies associated with the immune complexes on the beads, forming a four-member solid-phase sandwich. After a final washing to remove all unbound material, the beads were analyzed in a Luminex instrument. By monitoring the spectral properties of the beads and amount of associated RPE fluorescence for each brain lysate or serum sample and comparing to known standards, the concentration of each analyte was determined. MMP-9 and MMP-2 concentrations were also assessed in serum and tissue lysates, but multiplex assays were not available for these measures. Instead, these were measured using solid-phase sandwich ELISA (Rat Quantikine ELISA Kits, R&D Systems, Minneapolis, Minnesota, USA).

Statistical analyses

Statistical analyses were conducted in STATA/SE V.16. Pairwise contrasts among group means were estimated using the `xtmixed` command for general linear models with robust variance estimates to protect against unbalanced group sizes, heteroskedasticity and slight departures of measurement errors from normality assumptions. For comparisons of pH, glucose and electrolyte concentrations among treatment groups, separate models were fit for each biochemical measurement before DKA treatment and each measurement at the time of brain sample harvest. First, DKA groups were compared to confirm similar severity of acidosis and other biochemical derangements at the time of initiation of DKA treatment. The acute DKA group was used as the reference group for these comparisons. Next, DKA groups were compared with both NG control and HG control groups to document differences in biochemical measures at the time of removal of brain tissue.

To promote comparability across analytes and to support creation of composite variables, all cytokine and chemokine measurements were z-score transformed using the batch-specific NG mean for centering and with the standardization being with respect to the root mean square error across all groups for the same analyte and tissue type in a regression model with groups entered as fixed effects. All chemokine and cytokine levels in serum as well as the CCL3 values in the brain were natural log transformed prior to standardization, and the resulting standardized values were trimmed to lie between -4 and 4 to lessen the influence of skewed outliers. The

batch-specific standardization of brain chemokine and cytokine levels was performed separately by region, and then the batch-specific and region-specific z-scores were averaged together by animal, after verifying that group differences were not heterogeneous across brain regions. To improve the precision of estimates, we developed composite measurements based on theoretical and empirical considerations. For candidate items in a composite, internal consistency and item-rest correlations were conducted prior to forming the composite to verify the acceptability of combining the items together.

RESULTS

Acute DKA

To determine the effects of acute DKA on serum and brain concentrations of cytokines and chemokines, we induced DKA in juvenile rats and treated DKA with insulin and saline. We collected rat brain specimens during acute DKA (after 4 hours of treatment with insulin and saline, coinciding with the peak time of cerebral injury in children with DKA), 24 hours, and 7 days after insulin and

saline treatment. We compared levels of inflammatory mediators in these rats to those of NG and HG controls.

Glucose and electrolyte concentrations and pH values for rats in each of the experimental groups are presented in [table 1](#). There were no significant differences in pH, glucose or electrolyte concentrations among DKA groups before initiation of DKA treatment. Biochemical measurements in the acute DKA group at the time of brain sample harvesting (after 4 hours of insulin and saline treatment) documented expected improvements in acidosis and hyperglycemia. Comparisons between the HG control group and DKA groups before DKA treatment, as well as between the 28-day HG group and the 28-day DKA group confirmed similar levels of glycemia.

Serum

We found that acute DKA was characterized by elevations in serum concentrations of CCL3, TNF- α and IL-1 β ([figure 2A](#)). CCL3, TNF- α and IL-1 β concentrations were elevated only during acute DKA, and concentrations in the 24-hour and 7-day post-DKA groups were not

Table 1 Biochemical values in each experimental group

	NG control	HG control	Acute DKA	DKA 24 hours	DKA 7 days	DKA 28 days	HG 28 days
pH							
Before DKA treatment	N/A	N/A	6.81 (0.25)	6.77 (0.15)	6.73 (0.09)	6.64 (0.08)	N/A
At brain sample collection	7.40 (0.06)	7.33 (0.09)	6.99 (0.13)*†	7.40 (0.04)	7.34 (0.03)*	7.39 (0.04)	7.33 (0.04)*
Glucose (mg/dL/mmol/L)							
Before DKA treatment	N/A	N/A	440 (176)/ 24.4 (9.8)	386 (96)/ 21.4 (5.3)	364 (57)/ 20.2 (3.2)	459 (101)/ 25.5 (5.6)	N/A
At brain sample collection	148 (19)†/ 8.2 (1.1)	450 (263)*/ 25 (14.6)	97 (91)†/ 5.4 (5.1)	295 (270)/ 16.4 (15)	285 (359)/ 15.8 (19.9)	431 (102)*/ 23.9 (5.6)	600 (138)*/ 33.7 (7.7)
Sodium (mEq/L)							
Before DKA treatment	N/A	N/A	137 (4.0)	137 (1.6)	136 (3.3)	136 (4.4)	N/A
At brain sample collection	140 (0.8)†	135 (2.5)*	143 (14)	145 (19)	137 (3.5)	134 (0.5)*	136 (3.5)*
Chloride (mEq/L)							
Before DKA treatment	N/A	N/A	118 (7.5)	123 (6.7)	124 (3.1)	123 (3.1)	N/A
At brain sample collection	99 (2.3)	95 (4.5)	121 (16)*†	95 (6.1)	92 (1.0) *	91 (1.9)*†	95 (7.4)
Bicarbonate (mEq/L)							
Before DKA treatment	N/A	N/A	10 (4.3)	8 (1.1)	9 (1.6)	8 (1.0)	N/A
At brain sample collection	30 (1.5)†	28 (0.7)*	13 (5.5)*†	34 (4.2)*†	33 (3.6)†	33 (1.2)*†	27 (3.0)*
BUN (mg/dL/ mmol/L)							
Before DKA treatment	N/A	N/A	67 (53) 23.9 (18.9)	47 (18) 16.8 (6.4)	48 (14) 17.1 (4.9)	84 (22) 30.0 (7.8)	N/A
At brain sample collection	14 (2.4)/ 5.0 (0.9)	16 (2.3)/ 5.7 (0.8)	53 (54)*†/ 18.9 (19.3)	21 (8.4)/ 7.4 (3.0)	22 (7.1)*/ 7.7 (2.5)	22 (4.2)*†/ 7.8 (1.5)	21 (4.2)*†/ 7.5 (1.5)

Italicized values are mmol/L.

*Statistically significant pairwise difference in means compared with NG control ($p < 0.05$).

†Statistically significant pairwise difference in means compared with HG control ($p < 0.05$).

BUN, blood urea nitrogen; DKA, diabetic ketoacidosis; HG, hyperglycemic; N/A, not applicable; NG, normoglycemic.

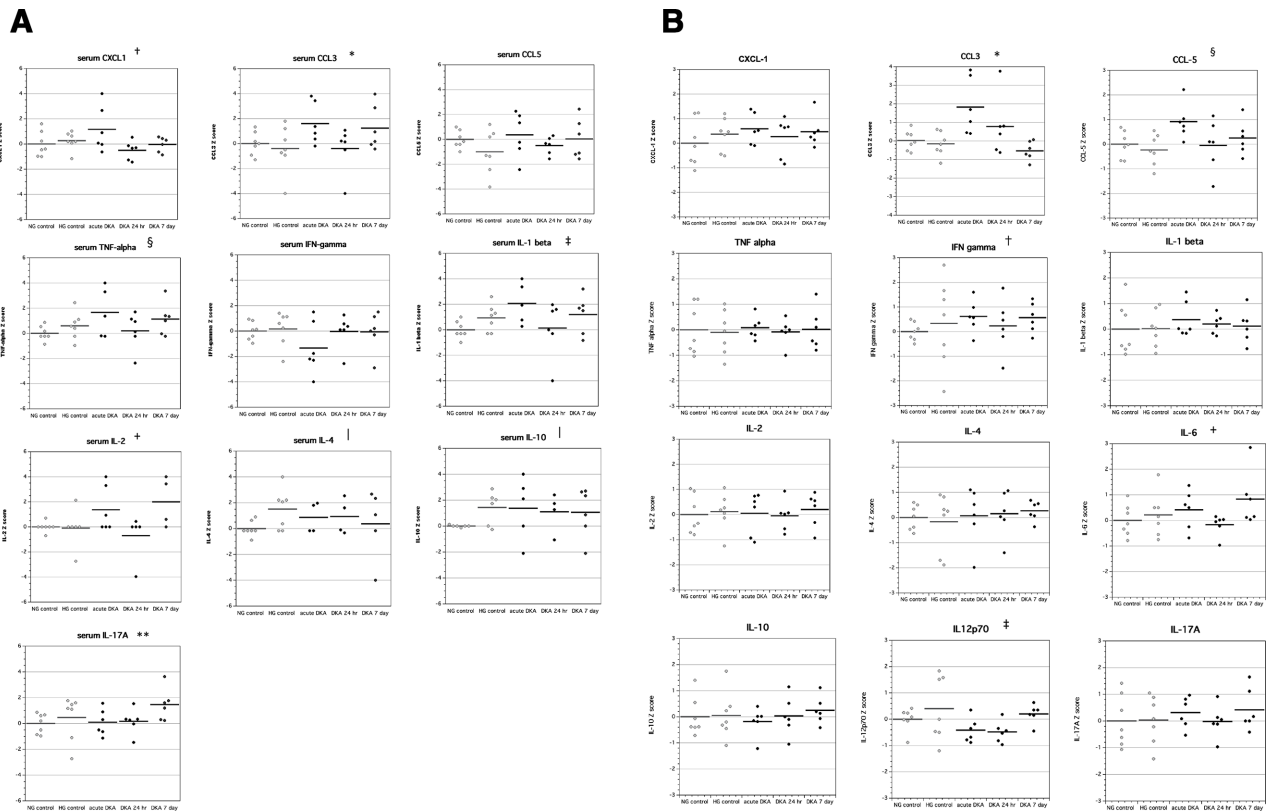


Figure 2 (A) Serum cytokine and chemokine levels during acute DKA and after recovery. Note: IL-6 and IL-12p70 concentrations were below the limits of detection in more than 70% of samples (data not shown). * $P < 0.05$ acute DKA with both NG control (mean difference 1.60, 95% CI 0.21 to 2.99) and HG control (mean difference 2.01, 95% CI 0.21 to 3.81). † $P < 0.05$ DKA 24 hours versus HG control (mean difference -0.74 , 95% CI -1.49 to -0.002). ‡ $P < 0.05$ HG control versus NG control (mean difference 0.94, 95% CI 0.01 to 1.81; $p < 0.01$), acute DKA versus NG control (mean difference 2.06, 95% CI 0.70 to 3.42; $p = 0.053$) for DKA 7 days versus NG control (mean 1.19, 95% CI -0.02 to 2.40). § $P < 0.05$ acute DKA versus NG control (mean difference 1.65, 95% CI 0.04 to 3.27) and DKA 7 days versus NG control (mean difference 1.14, 95% CI 0.09 to 2.18). + $P = 0.052$ acute DKA versus NG control (mean difference 1.37, 95% CI -0.01 to 2.75; $p < 0.05$) DKA 7 days versus HG control (mean difference 2.01, 95% CI 0.26 to 3.76) and DKA 7 days versus NG control (mean difference 2.11, 95% CI 0.04 to 4.18). | $P < 0.05$ HG control versus NG control (IL4: mean difference 1.50, 95% CI 0.35 to 2.66, and IL-10: mean difference 1.43, 95% CI 0.49 to 2.37). ** $P < 0.01$ DKA 7 days versus NG control (mean difference 1.46, 95% CI 0.39 to 2.53). (B) Brain cytokine and chemokine levels during acute DKA and after recovery. * $P < 0.01$ acute DKA group versus NG control and versus HG control (mean difference 1.81, 95% CI 0.62 to 2.99, and mean difference 2.00, 95% CI 0.77 to 3.20). § $P < 0.01$ acute DKA group versus NG control and versus HG control (mean difference 0.92, 95% CI 0.27 to 1.57, and mean difference 1.14, 95% CI 0.45 to 1.83). † $P < 0.05$ acute DKA group versus NG control (mean difference 0.62, 95% CI 0.07 to 1.17). + $P = 0.07$ DKA 7 day versus NG control (mean difference 0.83, 95% CI -0.07 to 1.74). ‡ $P < 0.05$ DKA 24-hour group versus NG control (mean difference -0.48 , 95% CI -0.90 to -0.06 ; $p = 0.07$), acute DKA versus NG control (mean difference -0.42 , 95% CI -0.88 to 0.03; $p = 0.08$), acute DKA versus HG control (mean difference -0.82 , 95% CI -1.74 to 0.10; $p = 0.06$). DKA 24 hours versus HG control (mean difference -0.88 , 95% CI -1.78 to 0.02]. z-Scores are adjusted for brain region and multiplex ELISA plate run. To mitigate the effects of outliers in the analysis of CCL3 data, these data were natural log transformed prior to calculation of z-scores, and z-scores were trimmed to the range of -4 to 4 SD. DKA, diabetic ketoacidosis; HG, hyperglycemic; IFN, interferon; IL, interleukin; NG, normoglycemic; TNF, tumor necrosis factor.

significantly different from control levels. However, we found that serum concentrations of IL-2 and IL-17A were increased in the 7 day post-DKA group. In the 24-hour post-DKA group, we found reduced serum concentrations of CXCL1 in comparison with concentrations measured in hyperglycemic controls. In hyperglycemic rats, we found that concentrations of IL-4 and IL-10 were elevated in comparison with NG control rats. Similar trends toward elevated levels of IL-4 and IL-10 were observed in the DKA groups, but these differences did not reach the level of statistical significance. Finally, we

found that serum concentrations of MMP-9 were significantly elevated during acute DKA in comparison with NG controls (figure 3). At 24 hours after DKA, serum MMP-9 concentrations declined substantially and were significantly lower than those observed in hyperglycemic controls. No significant alterations were observed in serum concentrations of MMP-2.

Brain tissue

In brain tissue lysates, we measured cytokine and chemokine concentrations in samples from the hippocampus,

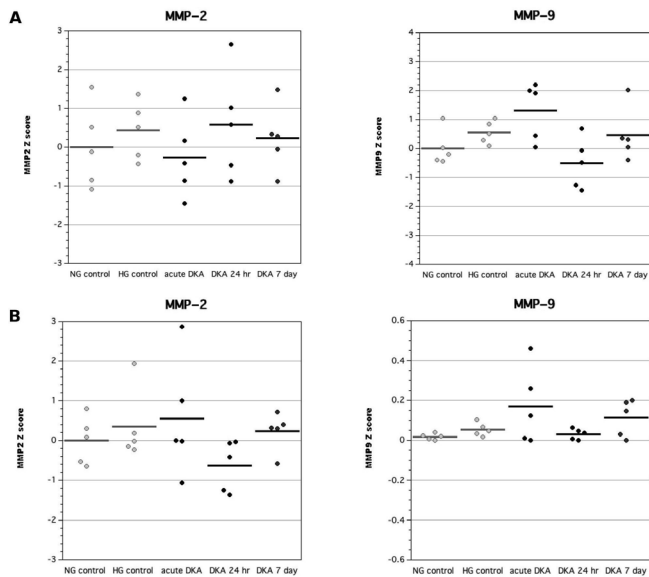


Figure 3 (A) Serum MMP concentrations during acute DKA and after recovery. (B) Brain MMP concentrations during acute DKA and after recovery. Serum MMP-2 concentrations were not significantly different among groups. Serum MMP-9 $p=0.06$ NG control versus HG control (mean difference 0.55, 95% CI -0.02 to 1.13 ; $p=0.006$); acute DKA versus NG control (mean difference 1.32, 95% CI 0.38 to 2.25 ; $p=0.006$); DKA 24 hours versus HG control (mean difference -1.07 , 95% CI -1.84 to -0.30 ; $p=0.08$); acute DKA versus HG control (mean difference 0.76, 95% CI -0.09 to 1.62). Brain MMP-2 $p=0.03$ DKA 24 hours versus NG control (mean difference -0.97 , 95% CI -1.86 to -0.09 ; $p=0.08$); DKA 24 hours versus HG control (mean difference -0.63 , 95% CI -1.33 to 0.08). Brain MMP-9 $p=0.02$ NG control versus HG control (mean difference 0.03, 95% CI -0.06 to -0.005 ; $p=0.05$); acute DKA versus NG control (mean difference 0.15; 95% CI -0.003 to 0.31 ; $p=0.01$); DKA 7 day versus NG control (mean difference 0.09, 95% CI 0.02 to 0.17). DKA, diabetic ketoacidosis; HG, hyperglycemic; MMP, matrix metalloproteinase; NG, normoglycemic.

cortex and whole hemisphere. Values from all three regions were similar in each rat; therefore, the three values were averaged to determine mean brain cytokine concentrations. These mean values were compared among groups. We found that concentrations of CCL3 and CCL5 in brain tissue lysates were significantly elevated during acute DKA in comparison to NG controls (figure 2B). Levels of these chemokines decreased by 24 hours and were not significantly different from control values at 24 hours and 7 days after DKA. We also found that brain tissue concentrations of IFN- γ were significantly elevated during acute DKA and that brain tissue concentrations of IL-12p70 were significantly reduced 24 hours after DKA. Finally, we found that brain concentrations of MMP-9 were significantly elevated in both hyperglycemic controls and during acute DKA compared with values observed in NG controls (figure 3). Brain tissue MMP-9 concentrations declined at 24 hours after DKA but were again significantly elevated 7 days after DKA recovery. Brain tissue concentrations of MMP-2

were significantly reduced 24 hours after DKA compared with levels observed in NG controls.

Long-term effects of DKA

To determine whether episodes of DKA alter chronic systemic inflammation and neuroinflammation related to diabetes, we measured serum and brain cytokine and chemokine levels in adult hyperglycemic rats (70 days old) who had experienced DKA as juveniles (28 days prior). These levels were compared with those of hyperglycemic control rats with similar duration and severity of hyperglycemia but without exposure to DKA.

Serum

We found that serum concentrations of IL-1 β were elevated in adult diabetic rats that had DKA as juveniles compared with adult diabetic rats without prior exposure to DKA (online supplemental figure 1). Concentrations of TNF- α and CXCL1 also tended to be higher in rats with prior exposure to DKA but these differences did not reach the level of statistical significance. Serum levels of MMP-9 and MMP-2 were not significantly different in adult rats with and without prior DKA (online supplemental figure 2).

Brain tissue

In brain tissue lysates, we found that concentrations of IL-10 and IL-12p70 were significantly elevated in rats with prior exposure to DKA compared with diabetic rats without prior DKA (figure 4). Concentrations of CCL5 also tended to be increased in the DKA group, but this difference was just short of statistical significance ($p=0.057$). Finally, we observed that brain tissue concentrations of MMP-2 were significantly reduced in the DKA group (online supplemental figure 2).

Composite measures of inflammation

Within each brain region, we observed that concentrations of several cytokines and chemokines (IL-10, IL-1 β , TNF- α , IL-17A, IFN- γ , CXCL-1 and CCL5) were strongly correlated with each other (Cronbach alpha=0.84). We explored whether changes in mean z-scores for this cluster of brain inflammatory mediators were influenced by DKA. Although we did not observe significant changes in this inflammatory mediator grouping during acute DKA or during the immediate recovery period, we found that mean z-scores for this inflammatory mediator cluster were significantly elevated in adult rats that had experienced DKA as juveniles compared with adult diabetic rats without exposure to DKA (mean difference 0.51, 95% CI 0.05 to 0.97 ; $p=0.03$).

In serum samples, we also found that concentrations of several inflammatory mediators were strongly correlated, and we formed a composite score averaging standardized CXCL1, CCL3, IL-1 β and TNF- α values (Cronbach alpha=0.87). Mean z-scores for this serum inflammatory mediator group were significantly elevated during acute DKA as well as 7 days after DKA (acute DKA vs NG control mean difference 1.45, 95% CI 0.09 to 2.81 ; DKA

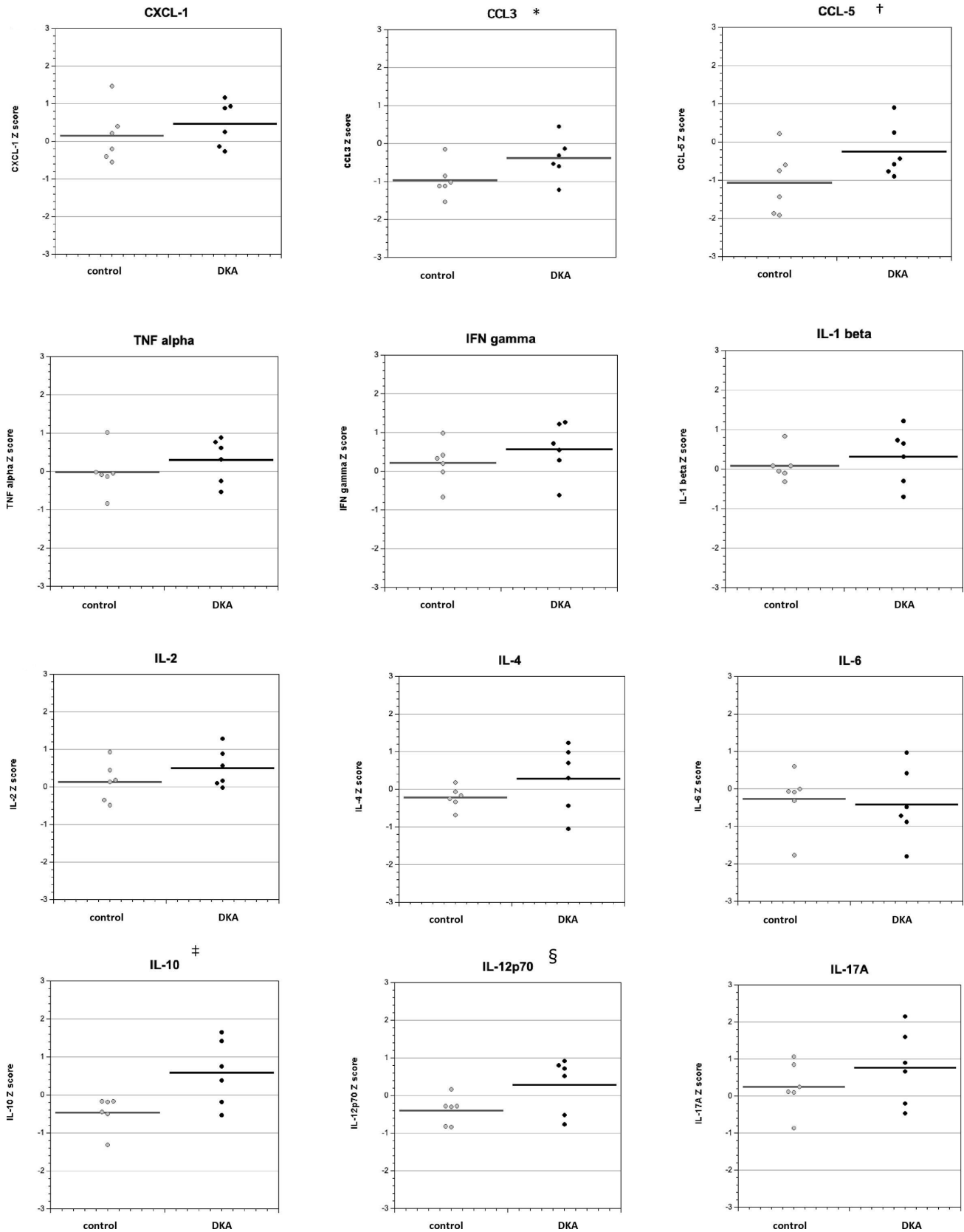


Figure 4 Cytokine and chemokine levels in brain tissue lysates of adult diabetic rats with and without DKA as juveniles. * $P=0.04$ (mean difference 0.57, 95% CI 0.02 to 1.12). † $P=0.057$ (mean difference 0.80, 95% CI -0.02 to 1.63). ‡ $P=0.006$ (mean difference 1.04, 95% CI 0.30 to 1.78). § $P=0.04$ (mean difference 0.67, 95% CI 0.05 to 1.30). z-Scores are adjusted for brain region and multiplex ELISA plate run. To mitigate the effects of outliers in the analysis of CCL3 data, these data were natural log transformed prior to calculation of z-scores, and z-scores were trimmed to the range of -4 to 4 SD. DKA, diabetic ketoacidosis; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

7 day vs NG control mean difference 0.88, 95% CI 0.13 to 1.63; $p < 0.05$ for both comparisons). In addition, levels of this serum inflammatory mediator group were significantly higher in adult rats that had experienced DKA as juveniles compared with adult diabetic rats without exposure to DKA (mean difference 1.36, 95% CI 0.07 to 2.65; $p = 0.04$).

Finally, we assessed differences in average z-scores for CCL3 and CCL5 in brain tissue lysates. These chemokines serve similar functions as chemoattractants involved in recruitment and activation of microglia and monocytes.^{29–32} The mean brain CCL3/CCL5 z-score was significantly elevated during acute DKA (acute DKA vs NG control mean difference 1.38, 95% CI 0.61 to 2.16; acute DKA versus HG control mean difference 1.56, 95% CI 0.77 to 2.36; $p < 0.001$ for both comparisons) and remained elevated in adult rats that had experienced DKA as juveniles compared with diabetic rats without DKA exposure (mean difference 0.63, 95% CI 0.15 to 1.12; $p = 0.04$).

CONCLUSIONS

Acute, severe brain injuries in children with DKA are infrequent but can be life-threatening.³³ More subtle effects of DKA on the brain in children are common, resulting in subclinical cerebral edema, alterations in metabolic measures and alterations in cerebral microstructure.^{7 9 34} Recent data suggest that DKA may be associated with declines in cognition and that these deficits may worsen or become more apparent over time^{6 7}; however, the cause of DKA-related brain injuries remains poorly understood. In previous studies using the same rat DKA model as in the current report, we documented reactive astrogliosis and activation of microglia in the hippocampus during DKA and found persistent reactive astrogliosis 10 weeks after recovery, suggesting a prolonged neuroinflammatory response.^{24 26} We also found that DKA was associated with long-term cortical neuron loss and that rats exposed to DKA had deficits in memory and exhibited delayed learning of a water maze task.^{26 35} The current data further elucidate potential mechanisms responsible for DKA-related brain injuries by demonstrating that DKA triggers a neuroinflammatory response involving production of specific inflammatory mediators. Patterns of elevation in inflammatory mediators in the brain differed from those in serum, suggesting that the neuroinflammatory response does not simply reflect systemic inflammation. DKA causes acute dramatic increases in brain levels of CCL3 and CCL5, chemokines that stimulate microglial recruitment and activation and monocyte accumulation in injured brain.^{29 36–38} Importantly, concentrations of CCL3, IL-10 and IL-12p70, as well as composite measures involving multiple correlated inflammatory mediators were significantly increased in brain samples from adult rats that had experienced DKA as juveniles. To our knowledge, these data are the first to demonstrate persistent neuroinflammation after DKA

and suggest the possibility that DKA induces chronic inflammation that could contribute to cognitive decline in patients with T1D.

We found that brain concentrations of several cytokines and chemokines were altered by DKA; however, the most immediate and dramatic elevations were observed in levels of CCL3 (macrophage inflammatory protein (MIP)-1 α) and CCL5 (Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES)). We observed that levels of CCL3 were markedly increased during acute DKA, declined during the immediate recovery period and again were significantly increased 28 days after DKA. Similar patterns were observed with regard to CCL5, although differences in CCL5 levels 28 days after DKA were just short of statistical significance. Brain IL-10 and IL-12p70 levels were not elevated during acute DKA but were significantly elevated 28 days after DKA. An early increase in brain levels of CCL3 and CCL5 with a delayed rise in IL-10 and IL-12p70 levels has also been documented in brain microdialysis studies of adults with traumatic brain injury (TBI), another condition that can result in long-term cognitive decline.³⁹ IL-10 is a critical inhibitor of IL-12, and concurrent elevation in concentrations of these cytokines may reflect a counterbalancing response.⁴⁰

We also found that IL-17A levels in serum were significantly increased 7 days after DKA, suggesting peripheral T-cell activation. A delayed rise in IL-17A in both serum and brain has also been documented after TBI and has been proposed to be involved in causing secondary brain injury.⁴¹ IL-17A contributes to blood–brain barrier disruption and immune cell traffic into the central nervous system (CNS) and is thought to play an important role in sustaining a proinflammatory environment.⁴² Chronic elevations in serum concentrations of several cytokines, including IL-1 β and TNF- α , have also been demonstrated after TBI, suggesting additional similarity to DKA.⁴³

CCL3 and CCL5 have been shown to be important mediators of neuroinflammation following neonatal hypoxia, as well as ischemic stroke in adults.^{29 36–38} Both CCL3 and CCL5 are produced by brain microvascular endothelial cells, as well as by microglia and astrocytes.^{29–32} Elevated levels of CCL3 and CCL5 are associated with accumulation of microglia and monocytes in regions of brain injury and with increased production of proinflammatory cytokines.^{29–32} CCL3 also contributes to blood–brain barrier disruption following ischemic stroke.⁴⁴ Vasogenic cerebral edema occurs frequently in children with DKA,⁹ and elevated levels of CCL3, along with other inflammatory mediators, may help to explain this finding. In rodent models of ischemic brain injury, inhibition of both CCL3 and CCL5 has been shown to improve neurological outcomes.^{29 37 45} Increased expression of both CCL3 and CCL5 has also been found in chronic neurodegenerative conditions, including Alzheimer's disease.^{46–48} Our data suggest the possible involvement of these chemokines in both acute, severe DKA-related brain injuries, as well as in persistent

neuroinflammation that might contribute to cognitive decline in patients with diabetes.

Blood levels of IL-1 β and TNF- α were elevated during DKA, and IL-1 β elevations persisted in adult rats exposed to DKA as juveniles. IL-1 β and TNF- α were also highly correlated with levels of several other cytokines and chemokines in both blood and brain and were components of inflammatory mediator composite scores that were affected by DKA. These cytokines are important mediators of cerebral vasospasm during cerebral injury⁴⁹ and have been found to alter the function of various ion channels, contributing to edema formation, among other effects.⁵⁰ Cerebral hypoperfusion prior to DKA treatment and aberrations in cerebral blood flow during DKA treatment have been hypothesized to be involved in causing DKA-related cerebral injury.⁵¹ Furthermore, altered function of brain Na-K-Cl cotransporters has been shown to be involved in generating cerebral edema during DKA in the rat model.⁵² Involvement of IL-1 β , TNF- α and other cytokines in altering cerebral blood flow and causing cellular and interstitial edema during DKA should be further investigated.

Our data also document alterations in levels of MMPs during acute DKA and after recovery. MMPs are endopeptidases that degrade tight junction proteins and endothelial basement membranes, allowing blood-borne proinflammatory or neurotoxic proteins to invade the CNS.^{53,54} MMPs serve as mediators of blood-brain barrier dysfunction and as neuroinflammatory signaling molecules.⁵⁵ MMPs may also play beneficial roles in tissue repair and promotion of synaptic plasticity after brain injury.⁵⁶ In these experiments, we found elevated concentrations of MMP-9 in serum and in brain lysates during DKA, and reduced concentrations of MMP-2 in brain lysates. These findings are similar to our previous studies in children in which we found elevated MMP-9 and reduced MMP-2 concentrations in serum during acute DKA.⁵⁷ Chronic hyperglycemia has also been shown to be associated with elevated serum MMP-9 concentrations, consistent with our findings.⁵⁸ It is possible that elevated MMP-9 concentrations might contribute to vasogenic cerebral edema during DKA in children.⁹ Interestingly, MMP-2 concentrations in brain lysates were persistently reduced in adult rats that had experienced DKA as juveniles. The significance of this reduction is unclear but could possibly result in diminished capacity for repair and synaptic plasticity related to loss of beneficial MMP functions.⁵⁶

Similar to the findings in our rat model, several previous studies have documented increased serum levels of inflammatory mediators in adults and children with DKA.^{19–23} Elevated serum concentrations of IL-6, IL-8 (CXCL8), TNF- α , and IL-1 β have been found in adults with acute DKA.^{22,23} In children, acute DKA results in increased serum levels of CXCL1, IL-8, IL-6, IL-10, IFN- α 2, IFN- γ , TNF- α and IL-1 β and reduced levels of CXCL10.^{19–21} Plasma from children with DKA, as well as mixtures of cytokines simulating those in DKA plasma,

has been shown to increase adhesion of polymorphonuclear leukocytes to cultured microvascular endothelial cells.²⁰ In a mouse model, DKA was associated with increased circulating levels of IL-6, IL-8, MCP-1, IL-10, sE-selectin, sICAM-1, and sVCAM-1, and stimulation of cultured microvascular endothelial cells with mouse DKA plasma caused cellular activation (increased reactive oxygen species and activation of nuclear factor kappa B), upregulated E-selectin, ICAM-1, and VCAM-1, and increased leukocyte adhesion.⁵⁹

The current study has limitations that should be taken into account when interpreting the data. The number of rats in each group was relatively small ($n=6$ or 7), and statistical power to detect differences of smaller magnitude was therefore limited. Several of the observed differences between groups would likely have reached the level of statistical significance had the groups been larger. Our current results therefore highlight only the most robust DKA-related changes in inflammatory mediators. A study with larger groups will be needed to more clearly define changes in inflammatory mediators that are more subtle. Furthermore, we used both male and female rats for these experiments, and the groups were not of sufficient size to investigate possible differences between sexes. It should also be kept in mind that our findings describe neuroinflammatory responses to DKA in a rodent model and human neuroinflammatory responses may differ from those observed. Finally, in our experiments, rats were studied 28 days after DKA. Evidence of persistent neuroinflammation was present at 28 days in this study, but whether this represents chronic neuroinflammation versus a prolonged recovery phase will require further investigation.

In summary, our data demonstrate that DKA causes both a systemic inflammatory response and a neuroinflammatory response. Chemokines involved in recruitment and activation of monocytes/microglia (CCL3 and CCL5) appear to play a prominent role. Most importantly, our data demonstrate persistent neuroinflammation after recovery from DKA, suggesting that DKA might trigger a chronic neuroinflammatory response that could contribute to neurodegeneration and declines in cognition over time in patients with diabetes. Our data also suggest similarities between DKA and TBI, a condition in which repetitive episodes result in increased chronic neuroinflammation.⁴³ These findings raise concerns about detrimental effects of repeated episodes of DKA. Recent data suggest that anti-inflammatory interventions may be beneficial in preventing neurodegeneration after TBI, even when administered long after recovery,⁴³ underscoring the importance of investigating possible connections between DKA, chronic neuroinflammation and diabetes-related cognitive declines.

Acknowledgements We gratefully acknowledge the assistance of Boram Lee in helping us to optimize the procedures for brain tissue lysate processing. We are also very grateful to Dr Yi-Je Chen for his help in conducting the small animal procedures. We appreciate the helpful assistance of Dr JoAnn Yee in conducting the multiplex assays for these studies. We also appreciate the help of Neeharika

Muppa, BS, Kathryn Morris, BS, and Emily Moody in caring for the diabetic animals and conducting other laboratory procedures.

Contributors NG obtained funding, contributed to study design, supervised data collection and data analysis, drafted and revised the manuscript. SC contributed to study design, participated in data collection, assisted with data analysis, reviewed and revised the manuscript. BH contributed to study design, participated in data collection, reviewed and revised the manuscript. LF contributed to study design, participated in data collection, reviewed and revised the manuscript. HW contributed to study design, assisted in supervising data collection, reviewed and revised the manuscript. DT contributed to study design, analyzed the data in collaboration with NG, reviewed and revised the manuscript. MEO contributed to study design, assisted in supervising data collection, assisted with data analysis, reviewed and revised the manuscript.

Funding These studies were supported by American Diabetes Association basic science grant (grant number 1–17-IBS-186, awarded to NG).

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval This study was conducted in accordance with the Animal Use and Care Guidelines issued by the National Institutes of Health and was approved by the Animal Use and Care Committee at University of California Davis.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplemental information.

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