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ARTICLE

A proinflammatory CD4$^+$ T cell phenotype in gestational diabetes mellitus

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Abstract

Aims/hypothesis Numerous adaptations of the maternal immune system are necessary during pregnancy to maintain immunological tolerance to the semi-allogeneic fetus. Several complications of pregnancy have been associated with dysregulation of these adaptive mechanisms. While gestational diabetes mellitus (GDM) has been associated with upregulation of circulating inflammatory factors linked to innate immunity, polarisation of the adaptive immune system has not been extensively characterised in this condition. We aimed to characterise pro- and anti-inflammatory CD4$^+$ (T helper [Th]) T cell subsets in women with GDM vs women without GDM (of similar BMI), during and after pregnancy, and examine the relationship between CD4$^+$ subsets and severity of GDM.

Methods This is a prospective longitudinal case–control study of 55 women with GDM (cases) and 65 women without GDM (controls) at a tertiary maternity hospital. Quantification of proinflammatory (Th17, Th17.1, Th1) and anti-inflammatory (regulatory T cell [Treg]) CD4$^+$ T cell subsets was performed on peripheral blood at 37 weeks gestation and 7 weeks postpartum, and correlated with clinical characteristics and measures of blood glucose.

Results Women with GDM had a significantly greater percentage of Th17 (median 2.49% [interquartile range 1.62–4.60] vs 1.85% [1.13–2.98], $p = 0.012$) and Th17.1 (3.06% [1.30–4.33] vs 1.55% [0.65–3.13], $p = 0.006$) cells compared with the control group of women without GDM. Women with GDM also had higher proinflammatory cell ratios (Th17:Treg, Th17.1:Treg and Th1:Treg) in pregnancy compared with the control group of women without GDM. In the control group, there was a statistically significant independent association between 1 h glucose levels in the GTT and Th17 cell percentages, and also between 2 h glucose levels and percentage of Th17 cells. The percentage of Th17 cells and the Th17:Treg ratio declined significantly after delivery in women with GDM, whereas this was not the case with the control group of women. Nevertheless, a milder inflammatory phenotype persisted after delivery (higher Th17:Treg ratio) in women with GDM vs women without.

Conclusions/interpretation Dysregulation of adaptive immunity supports a novel paradigm of GDM that extends beyond hyperglycaemia and altered innate immunity.

Angela Sheu and Yixian Chan are joint first authors. Frederic Sierro and Sue Mei Lau are joint senior authors

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Research in context

What is already known about this subject?

- The innate immune system is activated in pregnancies complicated by gestational diabetes mellitus (GDM). However, less is known about the polarisation of cells in the adaptive immune system in this condition

What is the key question?

- Is the adaptive immune system dysregulated in pregnancies complicated by GDM?

What are the new findings?

- Women with GDM had a greater proportion of circulating proinflammatory $CD4^+$ T cells and increased proinflammatory-to-anti-inflammatory $CD4^+$ ratios, compared with control women of similar age and BMI
- A milder proinflammatory phenotype persisted in the postpartum period in women with GDM

How might this impact on clinical practice in the foreseeable future?

- Further studies should explore the effects of maternal inflammation on the short- and long-term outcomes of GDM and examine ways in which adaptive immunity can be modulated for the benefit of the mother and fetus

Keywords Adaptive immunity · Diabetes · Gestational diabetes mellitus · Inflammation · Pregnancy · T cells · Th17

Abbreviations

GCT	Glucose challenge test
GDM	Gestational diabetes mellitus
IA-2	Islet autoantibody-2
LGA	Large for gestational age
PBMC	Peripheral blood mononuclear cell
SGA	Small for gestational age
Th	T helper
Treg	Regulatory T cell

Introduction

Gestational diabetes mellitus (GDM) has adverse effects on the mother and fetus with long-term associated risks for both [1]. The paradigm of GDM has evolved from a disorder solely related to hyperglycaemia to one involving dysregulated placental function [2] and activation of the immune system [3]. The immune system is conceptually split into two broad subsystems: the 'innate' immune system can be triggered rapidly but does not usually confer long-lasting immunity; the 'adaptive' immune system is slower but confers long-lived immunological memory following initial activation, resulting in more efficient responses to subsequent encounters with the same antigen. Both systems synergise to maintain protection and homeostasis.

Now, common complications of pregnancy such as recurrent miscarriage and pre-eclampsia have been linked to a disruption in adaptive immunity [4–6]. We hypothesised that GDM, like pre-eclampsia, is also a disorder of adaptive immunity. If this is true, then management of GDM could also be targeted at modulating the immune system to achieve the most favourable environment for short- and long-term fetomaternal outcomes. As adaptive immunity has a memory, this could also have long-term implications on maternal metabolic disease and future risk of GDM.

$CD4^+$ T cells, a major component of the adaptive immune response, have been classified into T helper (Th) subsets. Each of these subsets has specific, largely cytokine-mediated functions: Th2 cells are essential to the induction of humoral immunity [7] and Th1 and Th17 cells are proinflammatory and enhance the cytotoxic and/or phagocytic activity of macrophages, neutrophils and natural killer cells by secreting IFN-γ or IL-17, respectively. Th17 cells also defend against extracellular pathogens, enhancing barrier function through induction of proinflammatory pathways in epithelial cells [7]. Conversely, several $CD4^+$ subsets have demonstrated suppressive roles, with regulatory T cells (Tregs) being prototypical [7]. After interaction with their cognate antigen–MHC complex, Th polarisation of $CD4^+$ T cells depends on the cytokine milieu. There is a level of plasticity and interplay between these subsets. For example, Th17.1 cells secrete cytokines typical of both Th17 and Th1 cells and are considered to have increased inflammatory potential [8]. An imbalance between proinflammatory and regulatory $CD4^+$ subsets is observed in certain disorders. Th17 cells also play an important role in allograft rejection [9] and they are increased in autoimmune conditions such as rheumatoid arthritis, psoriasis and multiple sclerosis [10].

Chronic, low-grade inflammation is observed in insulin resistance and plays a role in the pathogenesis of type 2 diabetes [11, 12]. Adipocytes release proinflammatory cytokines which increase secretion of proinflammmatory cytokines from localised immune cells, perpetuating local and systemic

inflammation [13]. Alterations in lymphocyte numbers, subset distribution and/or function have been observed in type 2 diabetes, including a higher percentage of circulating Th1 and Th17 cells [13, 14].

Pregnancy is an immune-tolerant state in which the semi-allogenic fetus is not rejected by the mother [15]. The Treg pool expands in, and is required for, normal pregnancy [16]. A disruption to the balance of pro- and anti-inflammatory T cells is associated with recurrent miscarriage [4, 17]. A decreased percentage of circulating Tregs and an increased percentage of Th17 cells have been reported in pre-eclampsia [5, 6], implying that immune tolerance is important for fetal wellbeing.

The adaptive immune system has not been extensively characterised in GDM. Increased total numbers of lymphocytes have been reported in peripheral blood from women with GDM vs women without GDM [18]. A small case–control study showed increased activation markers in $CD4^+$ and $CD8^+$ T cells in GDM, associated with an imbalance in co-stimulatory vs co-inhibitory molecules [19]. Another study found increased percentages of activated $CD4^+$ and $CD8^+$ T cells [20]. More recently, decreased Treg suppressive function has been reported in women with GDM [21]. In these last two studies, women with GDM were significantly heavier than those without GDM. This is an important point, given that obesity itself is associated with alterations in $CD4^+$ inflammatory phenotype in non-pregnant individuals [22]. $CD4^+$ Th subsets were not evaluated in any of these studies.

The aim of this prospective, longitudinal case–control study was to further characterise the adaptive immune system in pregnancies complicated by GDM. More specifically, our aims were as follows: (1) to quantify pro- and anti-inflammatory $CD4^+$ T cell subsets in women with vs without GDM, during and after pregnancy and (2) to examine the relationship between inflammatory $CD4^+$ subsets and severity of GDM.

Methods

Cohort

This prospective longitudinal study was conducted at the Royal Hospital for Women, Sydney, between May 2013 and October 2015. Women were recruited in their third trimester of pregnancy. Women with GDM were recruited from the GDM clinic and the control group of women without GDM were recruited from the antenatal clinic.

GDM diagnostic criteria

GDM was diagnosed using the Australasian Diabetes in Pregnancy criteria at the time of study commencement: fasting plasma glucose ≥5.5 mmol/l and/or 2 h plasma glucose ≥8.0 mmol/l on a 2 h 75 g oral GTT. Women with a history of GDM in a prior pregnancy, polycystic ovarian syndrome, BMI ≥35 kg/m^2, maternal age ≥40 years or a first-degree relative with type 2 diabetes, had a 2 h 75 g GTT after their booking-in visit (15.5 ± 1.5 weeks gestation). All others were screened with a 1 h 50 g oral glucose challenge test (GCT) at 26–28 weeks of gestation. Women with a 1 h plasma glucose ≥7.8 mmol/l on the GCT proceeded to a 2 h 75 g GTT. During the study period, the diagnostic criteria for GDM changed at our institution. From January 2015 onwards, the IADPSG 2010 criteria were employed (fasting glucose ≥5.1 mmol/l, 1 h glucose ≥10.0 mmol/l and/or 2 h glucose ≥8.5 mmol/l), with universal GTT screening at 26–28 weeks. All women recruited from January 2015, and 44 of the 55 women with GDM, fulfilled both old and new criteria for GDM, ensuring a consistent GDM diagnostic criteria throughout recruitment. The women in the control group did not fulfil either new or old criteria for GDM.

Exclusions

Women with multiple pregnancy, autoimmune disease, type 1 or type 2 diabetes, hypertensive disorders (pre-eclampsia, pre-existing/new hypertension), those on immunosuppressive agents (including glucocorticoids) or who received intramuscular steroids for fetal lung maturation were ineligible for the study. Samples from women with a respiratory infection or inflammatory illness were excluded.

Demographic data

Data including maternal age, ethnicity, recalled pre-pregnancy BMI, booking-in BMI and blood pressure, parity, GTT results, mode of delivery, birthweight, birth length and gestational age at delivery were collected from medical records. Birth centiles were calculated using the Perinatal Institute's customised centile calculator (https://www.gestation.net/birthweight_centiles/birthweight_centiles.htm), which accounts for maternal height, weight, ethnicity and parity and for the sex and gestational age of the baby, for an Australian population. Large for gestational age (LGA) and small for gestational age (SGA) were defined as customised birthweight centiles ≥90% and ≤10%, respectively.

Timeline

At 36–38 weeks of gestation, maternal blood was collected for HbA_{1c}, fructosamine, full blood count and flow cytometry. Blood was collected 6–8 weeks postpartum for full blood count, flow cytometry and, in women with GDM, a 2 h 75 g GTT. After study commencement, the study protocol was amended to include maternal serum collection for islet autoantibody assay.

Assays

All assays were performed in the same laboratory, using automated systems. HbA_{1c} was measured by cation-exchange HPLC (Bio-Rad D-10 analyser; Bio-Rad, Sydney, NSW, Australia) and fructosamine was measured by colorimetric assay (Roche cobas analyser; Roche, Sydney, NSW, Australia). Maternal serum was assayed for GAD antibodies and islet antigen-2 (IA-2) antibodies (ElisaRSR, RSR, Pentwyn, Cardiff, UK).

Flow cytometry

Whole blood from women with and without GDM was obtained in EDTA tubes. Peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation using Ficoll-Hypaque (GE Healthcare, Chicago, IL, USA) and cryopreserved in liquid nitrogen. PBMCs were stained at 4°C for 30 min with saturating concentrations of the following anti-human monoclonal antibodies (used as per manufacturer's instructions) in the presence of the fixable viability stain ZombieUV (Biolegend, San Diego, CA USA): mouse anti-CD4 (1:100), rat anti-CXCR5 (1:20), mouse anti-CXCR3 (1:20), mouse anti- CD161 (1:10) (all from BD Biosciences, Franklin Lakes, NJ, USA), mouse anti-CCR6 (1:30; Biolegend,), mouse anti-CD127 (1:20; Biolegend), mouse anti-CD25 (1:20; eBiosciences/Thermo Fisher Scientific, Waltham, MA, USA) and mouse anti-CD45RA (1:50; eBiosciences). Acquisition of stained samples was performed on a five-laser Fortessa flow cytometer (BD Biosciences). Data was analysed on FlowJo software (version 9.8; https://s3-us-west-2.amazonaws.com/fjinstallers/FlowJo_9.9.4.zip). The gating strategy for identifying Th subsets based on surface markers [23, 24] is outlined in electronic supplementary material (ESM) Fig. 1.

In vitro stimulation and intracellular cytokine staining of PBMCs

Total PBMCs were cultured at 37°C, 5% CO_2 (RPMI medium, 10^5 cells per well) for 24 h in the presence of T cell activation and expansion beads (TAE, anti-CD3/CD28/CD2 mAb micro beads; Miltenyi Biotech, Bergisch Gladbach, Germany), providing polyclonal stimulation, or with culture media alone. Four hours prior to cell harvest, 2 μg Brefeldin A (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) was added to each culture. The cells were stained for extracellular markers with monoclonal antibodies (described above), followed by permeabilisation and intracellular staining using Transcription Buffer Set (BD Biosciences; intracellular staining protocol) and anti-human IL-17A antibody (Biolegend). Unstained and fluorescence minus one cells (stained with similar reagents but omitting the anti-IL-17 antibody) were used to confirm positive intracellular staining. Stained sample acquisition and data analysis are described above (ESM Fig. 1) with the addition of gating for IL-17^+ cells.

Ethics

This study was approved by the South Eastern Sydney Local Health District Northern Network Human Research Ethics Committee. Informed consent was obtained from study participants.

Statistical methods

Statistical analyses were conducted using SPSS software version 24.0 (SPSS, Chicago, IL, USA). With a power of 0.80, desired total sample size, based on the difference in Treg and Th17 cell percentages in pre-eclamptic vs normal pregnancies in Santner-Nanan et al [5], was 34 for Tregs and 56 for Th17, assuming equal numbers in the groups with and without GDM. Clinical characteristics were evaluated using unpaired Student's t test or χ^2 test. Flow cytometry results, which were non-parametrically distributed, were compared using the Mann–Whitney U test (for GDM vs control samples) and Wilcoxon signed-rank test (for antepartum vs postpartum samples). Simple linear regression and multiple regression were used to assess relationships between Th17 cell percentages and clinical variables. A p value of <0.05 was considered significant.

Results

A proinflammatory T cell phenotype in women with GDM during late pregnancy

Blood was collected from 55 women with GDM and 65 women without GDM at 36–38 weeks of gestation. Mean age, recalled pre-pregnancy BMI and booking-in BMI were similar between groups (Table 1). Figure 1a shows the BMI distribution.

The characteristics of pregnancies in the GDM and control groups of women are outlined in Table 1. Women with GDM had higher mean fasting, 1 and 2 h glucose levels on the diagnostic GTT. Mean HbA_{1c} and fructosamine did not differ at 36–38 weeks. The women with GDM delivered 4.6 days earlier than the women without GDM ($p < 0.001$). While mean birthweight and birth length were slightly lower in GDM pregnancies, the mean customised birthweight centile was similar between groups (45.6 ± 26.8 [GDM] vs 50.6 ± 27.5 [without GDM], not statistically significant).

Comparative $CD4^+$ subsets and ratios are shown in Fig. 1b–h and Table 2. Analysis of the proinflammatory $CD4^+$ population revealed that women with GDM had a significantly greater percentage of Th17 (median 2.49% [interquartile range 1.62–4.60] vs 1.85% [1.13–2.98], $p = 0.012$)

Table 1 Characteristics of pregnancies with and without GDM

Characteristic	Antepartum cohort			Postpartum cohort		
	Without GDM	GDM	*p* value	Without GDM	GDM	*p* value
Maternal characteristics						
n	65	55		25	28	
Age, years	33.2 ± 4.5	33.9 ± 3.6	0.31	32.3 ± 4.4	33.6 ± 3.4	0.23
Recalled pre-pregnancy BMI, kg/m^2	24.4 ± 6.7	24.5 ± 5.4	0.90	25.0 ± 6.1	25.3 ± 4.6	0.87
Booking BMI, kg/m^2	25.1 ± 5.5	25.1 ± 6.8	0.96	25.8 ± 4.8	25.4 ± 6.0	0.78
Ethnicity, *n* (%)						
Europid	47 (72.3)	27 (49.1)	0.009[a]	18 (72.0)	13 (46.4)	0.06[a]
Non-Europid	18 (27.7)	28 (50.9)		7 (28.0)	15 (53.6)	
Nulliparous, *n* (%)	36 (55.4)	26 (47.3)	0.38	14 (56.0)	15 (53.6)	0.86
Booking systolic BP, mmHg	103 ± 11	105 ± 11	0.38	104 ± 10	107 ± 12	0.29
Booking diastolic BP, mmHg	64 ± 6	65 ± 8	0.73	65 ± 6	65 ± 10	0.89
Diagnostic GTT						
Fasting glucose, mmol/l[b]	4.3 ± 0.4	4.8 ± 0.6	<0.001	4.4 ± 0.5	4.9 ± 0.7	0.002
1 h glucose, mmol/l[c]	6.7 ± 1.4	9.7 ± 1.5	<0.001	6.8 ± 1.3	9.9 ± 1.3	<0.001
2 h glucose, mmol/l[d]	5.4 ± 1.0	8.5 ± 1.4	<0.001	5.6 ± 0.9	8.7 ± 1.2	<0.001
HbA_{1c} (mmol/mol)	33 ± 2	34 ± 2	0.15	33 ± 2	34 ± 2	0.15
HbA_{1c} (%)	5.2 ± 0.4	5.3 ± 0.4	0.15	5.2 ± 0.4	5.3 ± 0.3	0.45
Fructosamine (μmol/l)	191 ± 17	197 ± 21	0.06	190 ± 18	186 ± 43	0.61
Postpartum GTT						
Fasting glucose (mmol/l)					4.7 ± 0.5	
1 h glucose (mmol/l)					8.3 ± 2.0	
2 h glucose (mmol/l)					7.1 ± 2.0	
Fetal characteristics						
Delivery by Caesarean section, *n* (%)	38 (58.5)	21 (38.2)	0.03	8 (32.0)	9 (32.1)	0.92
Delivery by emergency Caesarean section, *n* (%)	8 (12.3)	6 (10.9)	0.07	3 (12.0)	4 (14.3)	0.96
Gestational age at birth, weeks	39.9 ± 0.9	39.2 ± 1.0	<0.001	40.2 ± 0.9	39.1 ± 0.8	<0.001
Customised birth centile, *n* (%)	50.6 ± 27.5	45.6 ± 26.8	0.32	47.7 ± 32.3	42.2 ± 26.3	0.50
Birthweight, g	3540 ± 399	3316 ± 376	0.002	3551 ± 435	3243 ± 407	0.01
Birth length, cm	51.9 ± 2.5	50.8 ± 2.9	0.02	52.0 ± 2.3	50.9 ± 3.0	0.15
SGA, *n* (%)	5 (7.7)	4 (7.3)	0.93	3 (12.0)	3 (10.7)	0.88
LGA, *n* (%)	5 (7.7)	5 (9.1)	0.78	3 (12.0)	1 (3.6)	0.25
Sex of baby, *n* (%)						
Male	36 (55.4)	32 (58.2)	0.76	14 (56.0)	15 (53.6)	0.86
Female	29 (44.6)	23 (41.8)		11 (44.0)	13 (46.4)	

Data are expressed as mean ± SD unless stated otherwise

p values are given for control vs GDM (unpaired Student's *t* test), except for ethnicity

[a] *p* values are given for the difference between the proportion of Europid/non-Europid women with GDM vs without GDM (χ^2 test)

[b] Antepartum: $n = 55$ women without GDM; $n = 52$ women with GDM

[c] Antepartum: $n = 51$ women without GDM; $n = 37$ women with GDM

[d] Antepartum: $n = 55$ women without GDM; $n = 53$ women with GDM

and Th17.1 (3.06% [1.30–4.33] vs 1.55% [0.65–3.13], $p = 0.006$) cells compared with the control group of women without GDM. In functional studies performed using polyclonal in vitro stimulation of PBMCs, the phenotypically characterised Th17 and Th17.1 cells displayed an increased propensity to produce IL-17 in the GDM vs control group; this increase was significant in Th17.1 cells (Fig. 1i–k). The median Th17:Treg, Th17.1:Treg and Th1:Treg ratios were also significantly higher in women with GDM, with the proportion of Tregs being similar in both groups.

To ascertain whether the proinflammatory phenotype was restricted to ethnicity type, Europid and non-Europid women

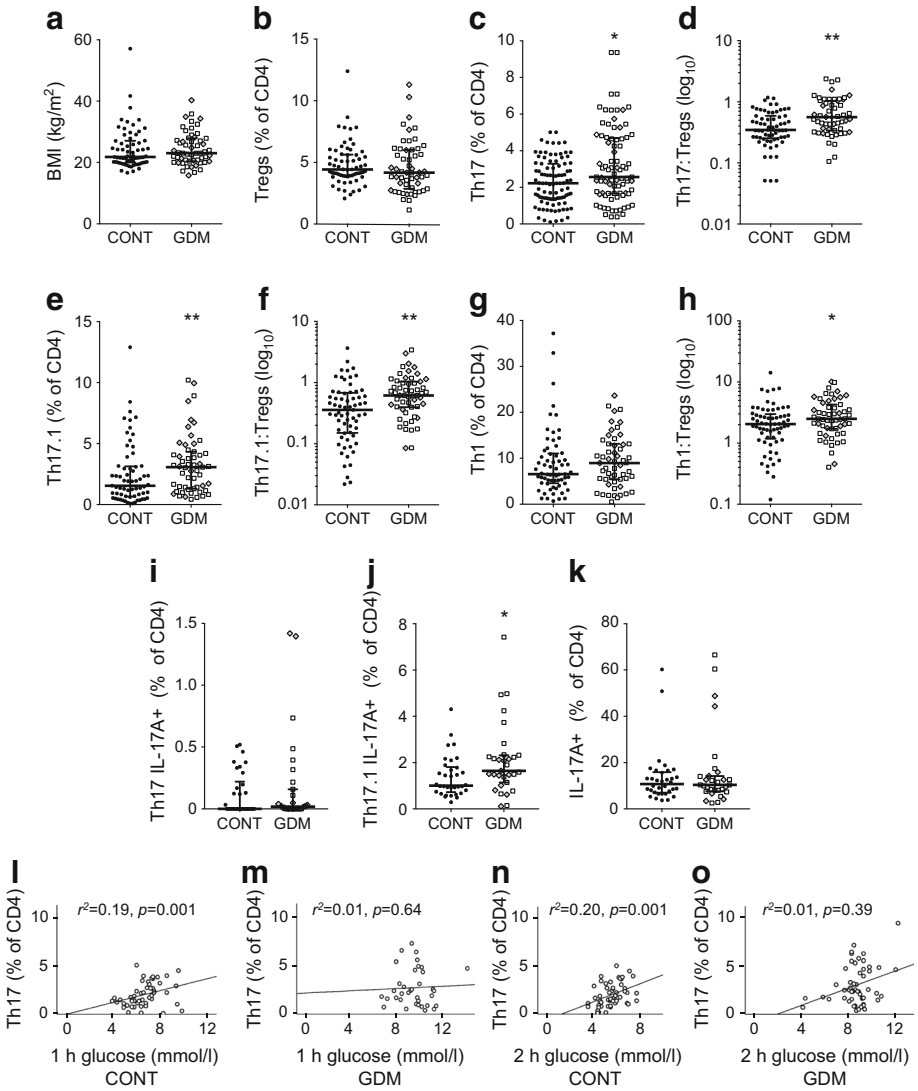

Fig. 1 $CD4^+$ T cell phenotype in pregnant women with and without (CONT) GDM. (**a**) BMI distribution. (**b**) Flow cytometric analysis of Treg percentages. (**c**–**h**) Flow cytometric analysis of Th17 (**c**), Th17.1 (**e**) and Th1 (**g**) percentages along with their respective Treg ratios (**d**, **f**, **h**). (**i**–**k**) Flow cytometric quantification of IL-17A-producing $CD4^+$ T cell subsets, after polyclonal stimulation. Black circles, control; white squares, GDM; grey diamonds, women with GDM not tested for islet autoantibodies; horizontal lines represent median values. (**l**–**o**) Linear relationship between 1 h GTT glucose (**l**, **m**) or 2 h GTT glucose (**n**, **o**) and Th17 percentages in women with (**m**, **o**) and without (**l**, **n**) GDM. $*p < 0.05$, $**p < 0.01$

were analysed separately. BMI and age were similar in women with and without GDM within each ethnicity group. A similar pattern of increased Th17 and Th17.1, as well as Th17:Treg and Th17.1:Treg ratios, was seen in GDM vs control groups, regardless of ethnicity (Table 2).

Maternal serum was collected in the last 38 of the 55 women with GDM recruited. Titres of GAD and IA-2 antibodies were undetectable in all 38 samples. There were no differences in age, BMI, HbA_{1c} or GTT values in women who did or did not have serum collected. Women with or without antibody testing are demarcated in all figures.

Postpartum phenotype

Twenty-eight GDM and 25 control postpartum samples were available for flow cytometry. There was no difference in maternal age or BMI between the groups. The characteristics of these pregnancies are outlined in Table 1. At the time of sample collection (6–8 weeks postpartum), of the women whose pregnancy had been complicated by GDM, none had impaired fasting glucose (fasting glucose 6.1–6.9 mmol/l), seven had impaired glucose tolerance (2 h glucose 7.8–11 mmol/l) and one had type 2 diabetes (2 h glucose >11.0 mmol/l) according to the WHO criteria. No differences in maximum insulin dose, home blood glucose levels, BMI, age, antepartum GTT, need for insulin, HbA_{1c}, fructosamine, birthweight centile or gestational age at delivery were observed when comparing women with normal and abnormal postpartum GTT. Inflammatory cell percentages and cell ratios during and after pregnancy were similar between those with normal and abnormal postpartum GTT in women who had experienced GDM.

In the postpartum period, the Th17:Treg ratio was still significantly higher in the GDM vs control group (0.55 [interquartile range 0.34–0.78] vs 0.34 [0.18–0.55], $p < 0.05$; Fig. 2c). Overall, the GDM group had a higher percentage of Th17.1 and Th17 cells and a higher Th17.1:Treg ratio, although these differences did not reach statistical significance (Fig. 2a–g).

Table 2 Proinflammatory $CD4^+$ phenotype in women with and without GDM at 36–38 weeks of gestation

Cell type	Total cohort							Europid			Non-Europid		
	Without GDM	GDM	*p* value	Insulin-treated	*p* value	Non-insulin treated	*p* value	Without GDM	GDM	*p* value	Without GDM	GDM	*p* value
N	65	55		36		19		47	27		18	28	
Th1 (% of $CD4^+$ cells)	6.56 (4.57–11.00)	8.95 (5.42–13.13)	0.3	6.6 (2.81–13.1)	0.87	10.28 (7.28–16.18)	0.019	6.56 (4.62–-10.93)	8.95 (5.42–13.96)	0.45	6.59 (3.87–11.27)	8.43 (5.05–13.10)	0.44
Th17 (% of $CD4^+$ cells)	1.85 (1.13–2.98)	2.49 (1.62–4.60)	0.012	2.5 (1.66–4.34)	0.02	2.37 (1.46–4.44)	0.08	1.66 (0.84–2.71)	2.35 (1.55–3.42)	0.08	2.44 (1.46–3.42)	2.73 (1.91–4.80)	0.19
Th17.1 (% of $CD4^+$ cells)	1.55 (0.65–3.13)	3.06 (1.30–4.33)	0.006	2.32 (1.16–4.3)	0.06	3.28 (2.75–4.48)	0.005	1.91 (0.69–3.16)	3.02 (1.42–3.94)	0.06	1.42 (0.55–2.57)	3.17 (1.18–4.80)	0.05
Th1:Treg ratio	2.04 (1.18–2.97)	2.5 (1.72–4.27)	0.045	2.24 (1.34–3.13)	0.46	3.52 (2.33–5.72)	0.003	2.19 (1.48–3.46)	3.04 (2.04–4.56)	0.05	1.75 (0.68–2.75)	2.35 (1.42–4.00)	0.13
Th17:Treg ratio	0.35 (0.25–0.59)	0.56 (0.34–1.04)	0.001	0.54 (0.37–1.1)	0.002	0.57 (0.31–1.04)	0.02	0.35 (0.23–0.59)	0.61 (0.36–1.10)	0.003	0.34 (0.27–0.69)	0.49 (0.33–0.93)	0.07
Th17.1:Treg ratio	0.36 (0.15–0.68)	0.62 (0.40–1.03)	0.001	0.58 (0.29–0.94)	0.02	0.81 (0.52–1.14)	0.001	0.41 (0.17–0.71)	0.72 (0.4–1.15)	0.007	0.30 (0.09–0.50)	0.59 (0.30–1.00)	0.02

Data are expressed as median (interquartile range)

p values are given for women with vs women without GDM

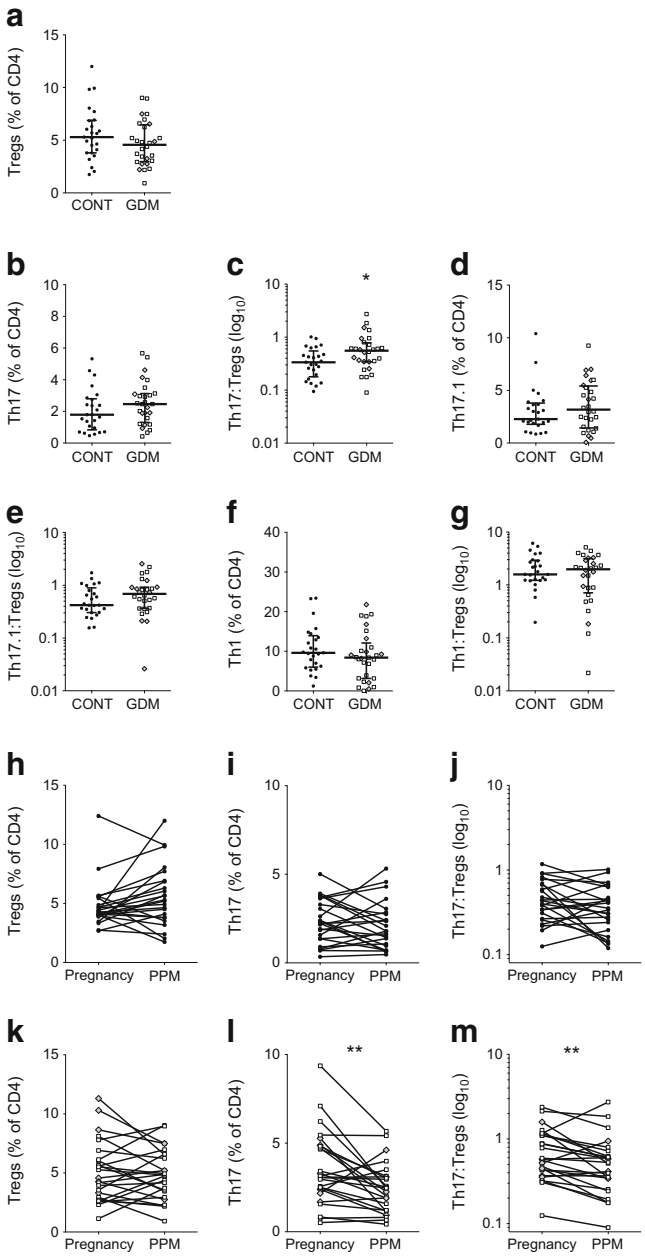

Fig. 2 $CD4^+$ T cell phenotype in the postpartum period in women whose pregnancies had been complicated by GDM or had not been complicated by GDM (CONT). (**a**) Flow cytometric analysis of Treg percentages. (**b**–**g**) Flow cytometric analysis of Th17 (**b**), Th17.1 (**d**) and Th1 (**f**) percentages along with their respective Treg ratios (**c**, **e**, **g**). Horizontal lines represent median values. (**h**–**m**) Change in Tregs, Th17 cells and Th17:Treg ratio from pregnancy (37 weeks of gestation) to 6–8 weeks postpartum (PPM) in women without (**h**–**j**) and with (**k**–**m**) GDM. Black circles, control; white squares, GDM; grey diamonds, women with GDM not tested for islet autoantibodies; $*p < 0.05$ and $**p < 0.01$

Examining the changes from pregnancy to the postpartum period, the percentage of Th17 cells declined significantly after delivery in women with GDM but not in women without GDM (Fig. 2h–m). The Th17:Treg ratio also declined after delivery in women with GDM but not in the control group. Tregs did not change significantly after delivery in women either with or without GDM.

Proinflammatory phenotype and severity of GDM

GDM treatment Of the 55 women with GDM, 36 were treated with insulin, with a mean dose of 32.7 ± 27.9 U by the end of pregnancy. These women had a similar BMI to those who did not require insulin (25.5 ± 5.4 vs 22.6 ± 4.6 kg/m^2). They had higher fasting glucose levels on the diagnostic GTT (5.0 ± 0.6 vs 4.5 ± 0.4 mmol/l, $p = 0.001$) and a higher HbA_{1c} (36 ± 4 mmol/mol [5.4 ± 0.4%] vs 32 ± 2 mmol/mol [5.1 ± 0.2%], $p = 0.003$). The insulin-treated women had a lower Th1:Treg ratio than the untreated women (2.24 [interquartile range 1.34–3.13] vs 3.51 [2.33–5.72], $p = 0.019$), with no statistical differences in Th17, Th17.1, Th1 or Treg percentages. Similarly, the trend towards a more marked proinflammatory phenotype remained when comparing either the insulin-treated or non-treated women with GDM vs the control group of women without GDM (Table 2). The Th1:Treg ratio was an exception, being similar in insulin-treated women and in the control group.

GTT glucose values and T cell subsets

Women without GDM In women without GDM, there was a positive relationship between 1 h glucose levels and percentage of Th17 cells ($p = 0.01$), with 1 h glucose accounting for 12% of the explained variability in Th17 (Fig. 1l). There was also a positive relationship between 2 h glucose and percentage of Th17 cells ($p = 0.003$), with 2 h glucose accounting for 16% of the explained variability in Th17 (Fig. 1n).

On multiple regression, including maternal pre-pregnancy BMI and ethnicity group (Europid vs non-Europid) in the linear model, 1 h glucose remained a significant predictor of Th17 percentage (β 0.28, $p < 0.05$). In a similar regression model using 2 h instead of 1 h glucose, this factor also remained the only significant predictor of Th17 (β 0.347, $p = 0.01$).

Women with GDM While women with GDM had significantly higher median percentages of proinflammatory T cell subsets, no correlation was found between inflammatory cells and glucose levels on the GTT (Fig. 1m, o), or with mean home-monitored fasting or postprandial blood glucose. In both control and GDM groups, postpartum GTT values were not correlated with postpartum inflammatory cell percentages or cell ratios.

HbA_{1c} and fructosamine were similar in women with and without GDM (Table 1), suggesting good glycaemic control in the GDM group. There was no correlation between HbA_{1c} or fructosamine and inflammatory cell percentages or cell ratios in women with or without GDM.

Birthweight

There was no correlation between inflammatory cell subsets or their ratios and customised birthweight centile. $CD4^+$ subsets were also similar in pregnancies with LGA, SGA and normal-weight babies.

Discussion

While there is growing interest in the role of inflammation in the pathogenesis of miscarriage and pre-eclampsia, the T cell inflammatory phenotype has not been well elucidated in GDM. Characterising adaptive immunity in GDM contributes to our understanding of its pathogenesis and associated long-term maternal risks. This study finds an increase in proinflammatory $CD4^+$ T cell subsets in the peripheral blood of women with GDM compared with women of similar age and BMI without GDM. This suggests that GDM, like pre-eclampsia, is a disorder of adaptive immunity.

While causality cannot be implied directly, there are plausible mechanisms by which a proinflammatory T cell phenotype could result in glucose intolerance in pregnancy. Prospective studies in non-pregnant individuals support an association between chronic, low-grade systemic inflammation and the development of type 2 diabetes, with an increase in innate inflammatory markers such as C-reactive protein, IL-6 and TNF-α [11, 12]. Mechanistically, this has been linked to impaired insulin signalling mediated by proinflammatory cytokines, most of which are lymphocyte-derived [25]. More recently, alterations in adaptive immunity have been detected in type 2 diabetes and include increased serum IL-17 [13, 14] and increased percentage of Th17 cells [13, 14]. Increased IL-17 levels reported in individuals with type 2 diabetes, compared with BMI-matched healthy individuals, are positively associated with HbA_{1c} [26].

Th17 cells have the potential to cause diabetes through several mechanisms. They secrete the proinflammatory cytokine IL-17A, which triggers activation of the intracellular NF-κB pathway with consequent proinflammatory cytokine production by monocytes, fibroblasts, stromal, epithelial and endothelial cells and adipocytes [27]. IL-17A impairs preadipocyte differentiation, promotes lipolysis and impairs glucose uptake in adipocytes [28].

Th17 cells in maternal blood have not been previously quantified in GDM, nor their function tested. This is the first study to show a proinflammatory $CD4^+$ T cell phenotype in women with GDM, mainly driven by an increased percentage of Th17 and Th17.1 cells. Our finding was supported by functional T cell studies showing an increased proportion of IL-17-producing Th17.1 cells in women with vs without GDM. The lack of difference between total percentages of PBMC IL-17-staining cells in women with and without GDM could be explained by the supraphysiological effects of polyclonal stimulation on other subsets. A greater proportion of polyclonally activated Th17.1 cells produced IL-17 when compared with Th17 cells. Our results may reflect the potential importance of Th17.1 cells, a very proinflammatory $CD4^+$ subset, in GDM. As the women with and without GDM had similar BMI, this suggests that the increase in Th17 and Th17.1 cells in GDM is independent of maternal BMI. This is akin to recent studies showing that Th17 cells and their cytokines distinguish diabetic/insulin-resistant obese individuals from metabolically healthy obese individuals [26, 29].

There was an independent relationship between glucose levels from the diagnostic GTT and the proinflammatory phenotype in pregnant women without GDM but not those with GDM; higher glucose was associated with a higher percentage of proinflammatory cells. One possible explanation is that treating GDM modulates the inflammatory phenotype. For example, acute exercise alters the Th1:Th2 balance [30] and insulin has well-documented anti-inflammatory effects [31–33]. This could also explain why insulin-treated women with GDM had a comparable or better inflammatory profile than those on diet control alone, despite higher GTT results. After treatment, the women with GDM attained similar HbA_{1c} and fructosamine levels compared with their control counterparts without GDM. Despite this, the group of women with GDM still had higher percentages of proinflammatory cells at the end of pregnancy.

An increased proportion of Th17 cells and a decreased Th17:Treg ratio is also described in pre-eclampsia, along with a blunted expansion of the Treg compartment [5, 6]. Pre-eclampsia and GDM are clinically related disorders: the presence of one condition increasing the risk of the other [34]; treating GDM lowers the risk of pre-eclampsia [35, 36] and both conditions are associated with placental dysfunction [2, 37]. Our findings extend their clinical proximity to a shared inflammatory phenotype.

This is the first study to examine the T cell inflammatory phenotype in women with GDM in the postpartum period and to follow inflammatory status longitudinally from pre- to post-delivery. We found that the Th17:Treg ratio remained significantly higher in the postpartum period in women whose pregnancies had been complicated by GDM. The increase in other inflammatory cells did not reach statistical significance, perhaps due to the smaller number of postpartum samples. Altogether, these data suggest that women with GDM retain a propensity to a proinflammatory state after delivery. This is consistent with the increased risk of developing type 2 diabetes and cardiovascular disease in women with a history of GDM [38, 39].

An interesting observation was the decrease in Th17 and the Th17:Treg ratio from pregnancy to postpartum in women with GDM, while there was no decrease in women without GDM. Thus, the difference in inflammatory status in women with vs without GDM was magnified during pregnancy

compared with the postpartum period. This is different from normal pregnancy, where Th17 levels were reported as being slightly decreased [5] or similar [40] when compared with the non-pregnant state. It is also different from the course of autoimmune diseases associated with increased Th17 in pregnancy (e.g. rheumatoid arthritis, psoriatic arthritis and multiple sclerosis), where there is a shift to a less-inflammatory $CD4^+$ phenotype [41]. So, while many inflammatory conditions are associated with a decrease in Th17 in pregnancy, the opposite occurs in GDM, suggesting a defect in immune tolerance to the fetus rather than autoimmunity.

Understanding the adaptive immune system during and after GDM may provide a more targeted approach to reducing immune activation in order to prevent or treat GDM and reduce the risk of type 2 diabetes. For example, the drug metformin, increasingly used to treat GDM, has been shown in animal models to promote an immune-tolerant phenotype by upregulating the Treg:Th17 ratio [42]. One plausible mechanism is through the recently reported effects of metformin on the gut microbiome [43].

Defective immune tolerance has implications on fetal wellbeing, including altered fetal growth and placental dysfunction, as classically seen in pre-eclampsia [5, 15]. A dysregulated maternal immune response is a novel potential mechanism through which GDM results in complications such as stillbirth and macrosomia and long-term complications such as metabolic programming of the fetus. Rodent studies show that maternal immunostimulation in pregnancy has proinflammatory effects on the immune system of offspring, with accelerated development and heightened responsiveness of Th1, Th17 and cytotoxic effector T cells to immune stimuli [44–46].

Our study has some limitations. Maternal blood was collected near the end of pregnancy, not at the time of GDM diagnosis. A more pronounced difference in inflammatory phenotype may have been observed prior to treatment of GDM, as Treg numbers peak in the second trimester and start to decrease by late gestation [16]. Serum from 70% of the women with GDM was available for islet autoantibody testing, excluding an autoimmune cause of their diabetes. It is possible that some untested women may have been autoantibody positive, although the likelihood of a large proportion of them being positive is small given that the selection of women for islet autoantibody testing was based on study chronology rather than clinical characteristics. The smaller number of postpartum samples may have contributed to decreased statistical power. Ethnicity was slightly different in the groups, with a greater proportion of non-Europids in the GDM group (50.9% vs 27.7%). We thus analysed Europid and non-Europid women separately and found similar trends in inflammatory T cell status. Reassuringly, within each ethnicity group, women with GDM still had an increased percentage of proinflammatory T cells compared with women without GDM.

One of the strengths of this study was careful participant selection, with the comparator group being similar in age and BMI. We excluded women who received antenatal steroids and those with a history of autoimmune or hypertensive disease. These factors have not been considered in the majority of previous studies of inflammation in GDM. We were able to correlate percentage of inflammatory cells with the diagnostic GTT data and customised birthweight centiles. The longitudinal nature of our study allowed follow-up of women with GDM into the postpartum period.

This is the first study to examine $CD4^+$ Th subsets in GDM and the postpartum period. We show that GDM is an inflammatory condition involving T cell-mediated immunity and resolves, somewhat incompletely, in the postpartum period. This supports a novel paradigm for GDM, aligning it with other 'inflammatory' conditions in pregnancy, such as pre-eclampsia, that are also detrimental to the fetus. Current treatments for GDM address raised blood glucose but are not specifically targeted at modulating the inflammatory environment. Further studies should explore the effects of maternal inflammation on the short- and long-term phenotype of offspring and should examine ways in which adaptive immunity can be modulated for the benefit of the mother and fetus.

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