The immunological profile of maternal obesity at 28 weeks of gestation underpins common negative pregnancy outcomes

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Abstract

Healthy pregnancy is accompanied by various immunological and metabolic adaptations. Maternal obesity has been implicated in adverse pregnancy outcomes such as miscarriage, preeclampsia, and gestational diabetes mellitus (GDM), while posing a risk to the neonate. There is a lack of knowledge surrounding obesity and the maternal immune system. The objective of this study was to consider if immunological changes in pregnancy are sabotaged by maternal obesity.

Peripheral blood was collected from fasted GDM-negative pregnant women at 26-28 weeks of gestation. Analysis was done using immunoassay, flow cytometry, bioenergetics analysis and cell culture.

The plasma profile was significantly altered with increasing BMI, specifically leptin (r=0.7635), MCP-1 (r=0.3024) and IL-6 (r=0.4985). Circulating leukocyte populations were also affected with changes in the relative abundance of intermediate monocytes (r=-0.2394), CD4:CD8 T cell ratios (r=0.2789), and NKT cells (r=-0.2842). Monocytes analysed in more detail revealed elevated CCR2 expression and decreased mitochondrial content. However, LPS-stimulated cytokine production and bioenergetic profile of MNCs was not affected by maternal BMI. The Th profile skews towards Th17 with increasing BMI; Th2 (r=-0.3202) and Th9 (r=-0.3205) cells were diminished in maternal obesity, and CytoStim^{TM}-stimulation exacerbates IL-6 (r=0.4166), IL-17A (r=0.2753), IL-17F (r=0.2973) and IL-22 (r=0.2257) production with BMI, while decreasing IL-4 (r=-0.2806).

Maternal obesity during pregnancy creates an inflammatory microenvironment. Successful pregnancy requires Th2-biased responses yet increasing maternal BMI favours a Th17 response that could be detrimental to pregnancy. Further research should investigate key populations of cells identified here to further understand the immunological challenges that beset pregnant women with obesity.

Introduction

Maternal obesity during pregnancy is associated with adverse pregnancy outcomes such as miscarriage [1], preeclampsia [2] and gestational diabetes mellitus [3], and poses an increased risk to the offspring of fetal mortality and childhood obesity as a result of macrosomia and metabolic syndromes [4]. With the prevalence of obesity in women of reproductive age recently reported as between 20% and 28% in England [5], reflecting the increasing prevalence of obesity worldwide, maternal obesity and the health consequences for mother and child are an endemic problem. In the not pregnant general population, we have good mechanistic insight into the links between excessive fat accumulation, systemic low-grade inflammation, and obesity-associated health risks such as type 2 diabetes mellitus (T2DM), reproductive dysfunction and cardiovascular disease [6-8]. Elevated circulating inflammatory markers such as IL-6, TNF and C-reactive protein (CRP) characterise the systemic inflammation that typically occurs with increasing adiposity [9]. The current COVID-19 pandemic highlights the detrimental impact of obesity on inflammation, immune function, and risk from infectious disease with, for example, obesity and high CRP levels an indicator for severity of COVID-19 symptoms [10].

How obesity in pregnancy might mechanistically underpin the well documented adverse pregnancy and child health outcomes is relatively unknown. The effects of maternal obesity on inflammation and immune function in the term placenta [11] and first trimester uterus [12] have received some attention revealing impact on immune cell number and function that could contribute to adverse pregnancy outcomes. This includes depleted decidual macrophages [13] and increased numbers of placental macrophages in obesity [11], although there are some conflicting findings regarding the number of placental macrophages with maternal obesity [14]. Maternal obesity also diminishes the numbers of uterine resident NK cells

and alters their contribution to extracellular matrix remodelling and growth factor signalling to compromise trophoblast survival and spiral artery remodelling [12]. In contrast, the systemic effects of obesity in pregnant women are largely unknown. Like the general population, maternal IL-6, CRP and leptin levels are elevated in pregnant women with obesity compared to their lean counterparts [11, 15]. This suggests a common outcome of systemic inflammation in pregnant and not pregnant adults with obesity. It also highlights that obesity-related changes can occur over and above the systemic inflammatory alterations that are a normal feature of pregnancy, including reduced pro-inflammatory cytokines (e.g. IL-6, CCL2, CXCL10, IL-18, TNF) and increased immunomodulatory and anti-inflammatory mediators (e.g. soluble TNF-receptor I, sTNF-RII, IL-1 Receptor Agonist (RA))[16]. Pregnancy per se is also associated with cellular changes linked to inflammation and innate immune function such as increases in peripheral blood neutrophils and monocytes for example [17]; maternal obesity exacerbates the neutrophil count even further [18]. Functional effects have also been described and include evidence of monocyte activation such as increased expression of CD14, CD64 and CD11b and heightened production of oxygen free radicals [19]. In the general population, monocytes seem particularly susceptible to the effects of obesity including increases in the non-classical subset of monocytes [20], elevated expression of CCR2 by classical and intermediate monocytes and higher expression of CX_3CR1 by all three subsets likely leading to increased intrinsic migratory capacity in response to chemokines such as CX_3CL1 and CCL2 secreted by adipose tissue [21]. Beyond reported increased production of LPS-stimulated IL-1β and RANTES and ssRNA-stimulated TNF and IL-10 in monocytes of the general population with obesity [21] little is known about the effects of maternal obesity on myeloid effectors of innate immunity and inflammation. One recent study has shown that at term, monocytes of pregnant women with obesity appear to be disrupted in their ability to adapt to pregnancy, perhaps explaining their

increased susceptibility to infections [22]. With both atypical levels of circulating pro-inflammatory cytokines such as IL-6 [23] and exacerbated activation and maturation of monocytes to the non-classical subset [24] linked to preeclampsia for which obesity is a risk factor [25], there is real need to address this shortcoming.

Obesity in the general population is also recognised to negatively affect the function of multiple lymphocyte populations. This ranges from suppression of T and natural killer (NK) cell function – including reductions in cytotoxicity, IFNγ production and expression of perforin and granzymes [26] - and altered B cell activity that manifests as reduced class-switching and immunoglobulin activity [27]. A reduced CD8+ T cell count in peripheral blood with obesity in both the general population [28] and pregnant women [29] has been described and possibly links to their accumulation in adipose tissue that, from mouse models, precedes that of macrophages [28]. The cytokine producing capacity of T cells also changes with obesity in the general population and obesity-associated inflammation is in part driven by a shift to Th1 and Th17 which is thought to be mediated by leptin [30]. Th1 and Th17 cytokines such as TNF and IFNγ are detrimental to pregnancy [31]. Conversely, a Th2 and regulatory T cell (Treg) dominated environment is considered essential to pregnancy success [31]. Maladaptation of adaptive immune processes could very much underpin obesity-associated adverse obstetric outcomes with upregulation of Th1 described in GDM [32].

Here, we describe the changes in the immune environment of GDM-negative, pregnant women of differing pre-pregnancy BMI at 28 weeks of gestation and reveal a phenotype of systemic inflammation, monocyte activation and altered Th1/Th2/Th17 balance.

Materials and Methods

Human peripheral blood mononuclear cells (PBMCs) isolation

Human peripheral blood was collected from healthy, fasted pregnant women into one 9 ml heparinised Vacuette™ and one 4 ml EDTA Vacuette™ (Greiner Bio-one, Frickenhausen, Germany), and processed within 30 minutes of collection. Women were tested for gestational diabetes mellitus (GDM) and all participants obtained a negative result from the oral glucose tolerance test (OGTT). All samples were collected with informed written consent and ethical approval obtained from a Health Research Authority Research Ethic Committee (19/LO/0722). The demographics of the women whose samples were used are shown in table 1.

EDTA anti-coagulated blood was centrifuged at 1800 *x g* for 10 min at room temperature and the plasma removed and stored at -80°C for cytokine and chemokine analysis.

The heparinised blood was first diluted 1 in 4 with PBS before layering onto 15 ml of Lymphoprep ™ (Stem Cell Technologies, UK) and centrifugation at 400 *x g* for 40 min at room temperature. MNCs were extracted and washed with RPMI 1640 (Life Technologies, Paisley, UK) twice by centrifugation at 515 *x g*. MNCs were used directly for flow cytometry or stimulated with lipopolysaccharide (LPS; 10 ng/ml, Invitrogen) or CytoStim™ (Miltenyi Biotec, UK) in RPMI 1640, 10% fetal bovine serum (FBS; Hyclone, Cytiva) and 2-mercaptoethanol at 37°C in 5% CO_2-in-air for 24 h; an unstimulated control was included. Cell free supernatants were harvested and stored at -20°C for cytokine analysis.

Bioenergetic analysis

Bioenergetic analysis of MNCs was carried out using the Seahorse Extracellular Flux Analyser XF^{e}96 (Agilent Technologies). MNCs (2.0 x 10^5 cells/well) in XF assay media minimal DMEM (Agilent) supplemented with 5.5 mM glucose (Agilent), 1 mM pyruvate (Agilent) and 2 mM glutamine (Sigma) were seeded onto a Cell-Tak (Corning) coated microplate [33]. Parameters for oxidative phosphorylation (OXPHOS) and glycolysis were measured simultaneously via oxygen consumption rate (OCR; pmoles/min) and extracellular acidification rate (ECAR; mpH/min) respectively with use of injections: oligomycin (1 μM), FCCP (1 μM), antimycin A and rotenone (both 1 μM) and monensin (20 μM) (all from Sigma).

Cytokine analysis

LEGENDplex ™

Cytokine analysis was done via a multiplex approach using kits from BioLegend. The pre-defined panels used were: 13-plex human Inflammation 1 panel (MNC cultures with LPS), 12-plex human T-helper cytokine panel version (MNC cultures with CytoStim™), and a 4-plex human diabesity panel (plasma). These were performed according to the manufacturer's instructions, with cultures containing LPS diluted 1:3, and the plasma and CytoStim™ run neat.

ELISA

Plasma levels of IL-6, IL-8 TNFα and MCP-1 were measured using Human Quantikine® High Sensitivity ELISA kits (Quantikine, Bio-Techne) according to the manufacturer's guidelines.

Flow Cytometry

Whole blood populations were first analysed using an 8-colour immunophenotyping kit, human (Miltenyi Biotec, UK). This cocktail contains: anti-CD3 PE (IgG1, clone REA613), anti-

CD4 VioBright ™ 667 (IgG1, clone REA623), anti-CD8 APC-Vio ® 770 (IgG1, clone REA734), anti-CD14 VioBlue ® (IgG1, clone REA599), anti-CD16 VioBright 515 (IgG1, clone REA423), anti-CD19 PE-Vio 770 (IgG1, clone REA675), anti-CD45 VioGreen ™ (IgG1, clone REA747), anti-CD56 VioBright 515 (IgG1, clone REA196).

Monocytes within MNC preparations were characterised using anti-CD14 Alexa Fluor® 647 (IgG1, clone 63D3, BioLegend) and anti-CD16 VioBlue® (IgMκ, clone VEP13, Miltenyi). Mitochondrial content of monocytes was monitored using 2 nM MitoTracker Green (Life Technologies). Expression of phenotypic markers were assessed using anti-CD11b PE (IgG1, clone CBRM1/5), anti-CD38 PE (IgG1, clone HB-7), anti-CD36 PE (IgG2a, clone 5-271), from BioLegend, and anti-CD220 PE (IgG1, REA260), anti-CD98 PE (IgG1, clone REA387), anti-CD80 PE (IgG1, clone REA661), anti-CD86 PE (IgG1, clone REA968), anti-CD64 PE (IgG1, clone REA978), anti-CD163 PE (IgG1, clone REA812), anti-CD192 (CCR2) PE (IgG1, clone REA264), and anti-CX_3CR1 PE (IgG1, clone REA385) from Miltenyi Biotec.

CD4+ T cells were identified using anti-CD3 VioBlue® (IgG1, REA613) and anti-CD4 VioGreen™ (IgG1, REA623). CD4+ T cell subsets were then further analysed by chemokine receptor expression to define Th subsets (reference) using anti-CD194 (CCR4) APC (IgG1, REA279), anti-CD196 (CCR6) PE-Vio® 615 (IgG1, REA190), anti-CD183 (CXCR3) VioBright™ FITC (IgG1, REA232) and anti-CCR10 PE (IgG1, REA326), all of which were from Miltenyi Biotec.

Statistics

The data sets were first tested for normality using the Kolmogorov-Smirnov (K-S) one sample test, where a significant p value <0.05 indicated significant deviation from normality.

Depending on if the data reported as parametric or non-parametric, a Pearson or Spearman correlation test was used respectively. The r values are reported to indicate direction (negative values a downward trend; positive values upward trend) and weight of correlation, and a p value <0.05 determined the r value to be significant.

Results

Leptin and IL-6 levels are directly correlated to increasing BMI at 28 weeks of pregnancy. To evaluate systemic inflammation at 28 weeks of pregnancy in this population of fasted, GDM-negative women of varying pre-pregnancy BMI (n=80) we measured key inflammatory mediators present in plasma. Plasma rather than serum was chosen for analysis as this reflects the liquid phase of blood as it circulates in the body rather than after clotting has occurred. Leptin and IL-6 had a significant positive correlation with BMI (Figure 1A), as described previously [11], and we are the first to show that MCP-1 increases with BMI in pregnant women (Figure 1A) in keeping with the same relationship in not pregnant adults [34, 35]. Insulin, cortisol PAI-1, TNFα and IL-8 did not vary with BMI (Figure 1A).

Circulating leukocyte numbers are altered with increasing maternal BMI. Whilst obesity in general is associated with increased levels of neutrophils [36], B cells [37] and non-classical monocytes [20] but decreased levels of eosinophils [38], NK cells [26], and NKT cells [39], little is known about the effects on circulating leukocyte numbers in obese pregnant women. Using flow cytometry, we observed several changes in key blood immune cell populations (Supplementary Figure 1). Increasing BMI was associated with a decrease in the intermediate subset of monocytes (**Error! Reference source not found.**). BMI did not have any effect on the total T cell number, however, increasing BMI was correlated with an increasing CD4:CD8

ratio directly attributable to a significant increase in CD4+ T cells accompanied by a significant decrease in CD8+ T cells (**Error! Reference source not found.**). This decline in CD8+ T cells is in keeping with studies in both pregnant women [29] and the general population [28] with obesity. From animal studies it has been suggested that this decline in CD8+ T cells in the peripheral blood is attributable to infiltration of CD8+ T cells into adipose tissue that precedes macrophage accumulation [28]. Whilst other studies also have shown a reduction in the proportion of $CD3^+/CD8^+$ T cells in pregnancies with obesity [29], the question as to whether they have accumulated in the adipose tissue remains unanswered. NKT cells also showed a decrease with increasing BMI (**Error! Reference source not found.**) which is also in keeping with observations in the general population [39] and in pregnancy [29]. While neutrophils tended to increase with BMI and eosinophils tended to decrease with BMI this was not significant; other populations did not show any differences with maternal BMI.

LPS-stimulated cytokine production is unchanged with maternal obesity. Having confirmed systemic inflammation occurs with increasing BMI in pregnant women (Figure 1) and that there are differences in the relative abundance of some peripheral blood leukocytes with changing BMI in pregnancy (**Error! Reference source not found.**) we next considered whether the inflammatory response of blood mononuclear cells (MNCs) might differ with BMI. MNCs were isolated and then challenged with LPS as a prototypic inflammatory stimulus with thirteen cytokines (IL-1β, IFNα2, IFNγ, TNFα, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23 and IL-33) measured using a multiplex approach. IL-17A was not detectable in any sample and there was no correlation between the LPS-induced levels of any of the other cytokines and maternal BMI (Figure 3).

193 *Monocytes have an adapted phenotype in response to obesity at 28 weeks of gestation.* Given
194 the decline in intermediate monocytes with BMI and the well-recognised role of mononuclear
195 phagocytes in obesity-associated inflammation [20, 21], we used flow cytometry to further
196 phenotype the classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical
197 ($CD14^{+}CD16^{++}$) subsets of monocytes (see Supplementary Figure 2). The markers chosen for
198 study were those commonly used for phenotyping monocytes linked to various effector
199 functions (CD11b, CD64, CD80, CD86 and CD163), chemokine receptors (CCR2 and CX3CR1)
200 and metabolism associated transporters and receptors (CD36, CD38, CD98 and CD220)
201 including mitochondria. Examples of the histograms of these markers for each subset can be
202 visualised in Figure 4A.
203
204 CD163 (haemoglobin scavenger receptor) expression was increased in intermediate and non-
205 classical monocytes with increasing maternal BMI (Figure 4B). Several studies have shown a
206 correlation between soluble CD163 and BMI which might act as an indicator for risk of insulin
207 resistance [40]. Except for decreased expression of the co-stimulatory molecule CD86 on
208 intermediate monocytes the other markers in this group (CD64 Fcγ receptor 1, CD11b
209 activation marker, CD80 costimulatory molecules) were unchanged (Figure 4B).
210
211 Both CCR2 and CX3CR1 are commonly studied in obesity [21] and are also differentially
212 expressed on monocyte subsets, i.e., classical monocytes are $CCR2^{high}CX3CR1^{low}$,
213 intermediates $CCR2^{high}CX3CR1^{high}$, and non-classical $CCR2^{low}CX3CR1^{high}$. While CX3CR1 did not
214 differ on any of the monocyte subsets with maternal obesity (Figure 4C), CCR2 expression on
215 all subsets of monocytes was elevated with increasing BMI (Figure 4C). Combined with MCP-
216 1 levels that also increased with BMI (Figure 1A) this suggests that the CCL2/CCR2 axis that

contributes to obesity related inflammation in the general population is likely also activated in pregnant women with obesity and warrants further investigation.

With growing interest in the role of immunometabolism in determining cell fate and function we also considered the expression of key metabolic transporters CD36 (fatty acid translocator), CD98 (long-chain neutral amino acid transporter) and CD220 (insulin receptor); we also quantified mitochondrial content (MitoTracker Green™). No differences were found in the CD36, CD98 or insulin receptor with increasing BMI (Figure 4D). However, all three subsets of monocytes had decreased mitochondrial content as BMI increased. While deficient and dysfunctional mitochondria have been linked with obesity [41], we are the first to show this occurs in pregnancies with obesity and in leukocytes specifically. CD38 (cyclic ADP ribose hydrolase that metabolises NAD+) has been suggested to play a vital role in pregnancy and here we show on classical and intermediate monocytes at 28 weeks of gestation that CD38 expression was significantly decreased with increasing BMI (Figure 4D).

Cellular metabolism is not altered by maternal BMI. Given decreased mitochondrial content was common to all monocyte subsets (Figure 4C) and adipocytes in obesity have shown downregulated OXPHOS proteins, lowered mitochondrial oxidative capabilities and reduced mitochondrial biogenesis [42] we then considered whether OXPHOS might be altered with increasing BMI. Summary data for oxidative phosphorylation as oxygen consumption rate (OCR) and glycolysis as extracellular acidification rate (ECAR) are shown as grouped BMIs (BMI $\leq$ 29.9 versus BMI $\geq$ 30; Figure 5A). OXPHOS and glycolysis parameters were calculated (ATP production, bioenergetic scope, bioenergetic capacity, glycolytic index, supply flexibility index, spare respiratory capacity) and compared by BMI (Figure 5B). There were no significant

differences in any OXPHOS or glycolysis parameters with BMI at 28 weeks of gestation. MNCs in pregnancy at term have previously been found to have decreased basal glycolysis and glycolytic capacity in conjunction with increased bioenergetic health index [33]. Very little otherwise is known about specific immune cell bioenergetics in obesity or pregnancy.

Pregnant women with obesity show altered Th1/Th2/Th17. Given the dramatic effect of maternal obesity on T cell numbers (Figure 1) and the role of immune plasticity related to Th1/Th2/Th17 in pregnancy success [43] we also considered the effect of BMI on CD4+ T cell subsets. The relative abundance of different Th subsets was determined based on their chemokine expression profile – CXCR3, CX3CR1, CCR4, CCR6 and CCR10 - to identify Th1 (CXCR3-CCR4-CCR10-CCR6-), Th2 (CXCR3-CCR4+CCR10-CCR6-), Th9 (CCR4-CCR6+), Th17 (CXCR3-CCR4+CCR10-CCR6+), Th17/1 (CXCR3-CCR4-CCR10-CCR6+) and Th22 (CCR4+CCR6+CCR10+CXCR3-) subsets [44] (Figure 6A). The percentage of Th2 cells as well as closely related Th9 cells decreased with increasing maternal BMI (Figure 6B) but other subsets were unaffected. When we examined the cytokine profile induced in response to the TCR activator CytoStim™ and measured using a multiplex approach, we found that IL-4 production was also decreased with increasing BMI (Figure 6C). This decrease in IL-4 was accompanied by increases in IL-6, IL-17A, IL-17F and IL-22 (Figure 6C). Overall, this suggests a decrease in Th2 accompanied by an increase in Th17 that could underpin adverse obstetric outcomes in pregnant women with obesity.

Discussion

Using a cohort of women of varying BMI but of very similar gestation and confirmed as negative for GDM by glucose tolerance testing, in contrast to many other studies of obesity in pregnancy, we have been able to establish the effects of obesity on systemic maternal immunity early in the third trimester. We confirm that maternal obesity is associated with systemic inflammation and monocyte activation and extend this to suggest activation of the CCL2/CCR2 axis, as in the general population, with obesity. We also show a profound effect of increasing BMI on loss of mitochondrial content; while this did not seem to affect oxidative phosphorylation capacity this measure was made on total mononuclear cells rather than isolated monocytes as would have been ideal. Finally, using both phenotypic and functional analysis we show for the first time that maternal obesity causes downregulation of Th2 cells and responses favouring heightened Th17 in particular.

Increased leptin and IL-6 in pregnant women with obesity has been described previously [11] and we extend this phenotype of systemic inflammation to include CCL2/MCP-1. Importantly, we confirm increasing leptin with increasing maternal BMI in a cohort that does not include women with either current or a history of hyperglycaemia and/or GDM/T2DM. Together with Wang et al [43], we support that this relationship likely occurs throughout pregnancy. Similarly, we confirm systemic elevation of IL-6 but not TNF with increasing maternal BMI [11]. While CCL2/MCP-1, a pro-inflammatory chemokine, is reported to be decreased in healthy pregnancy [45], herein obesity in pregnant women was associated with increased CCL2/MCP-1 as has been observed in the general population [15]. Given maternal CCL2/MCP-1 levels have been suggested to be a marker of labour [45], levels with maternal obesity could contribute to increased risk of early labour or miscarriage [46]. Combined with our observation of elevated CCR2 on all monocyte subsets with increasing maternal BMI, as in the general population [21],

it is likely that the CCL2/CCR2 axis is also active in pregnant women and manifests as increased intrinsic migratory capacity of monocytes but this remains to be formally investigated. The suspected increased intrinsic migratory capacity of monocytes in pregnancies with obesity would suggest altered macrophage phenotype in adipose and placental tissue. Macrophages have been reported to accumulate in the placenta of pregnant women with obesity [11] but whether this is CCL2/CCR2 mediated recruitment of maternal monocytes remains to be determined.

The many obesity-associated changes in monocytes prompted our closer scrutiny of this population. In contrast to the increase in the non-classical subpopulation seen in the general population [20], we observed diminished intermediate monocytes with increasing maternal BMI. Other highlights of the effect of increasing maternal BMI include changes in CD163, CD86 and CD38 which are all novel findings for maternal obesity. An increased expression of CD163 is typical of monocytes and macrophages in response to inflammation [47] and was seen on intermediate and non-classical monocytes with increasing maternal BMI. We did not measure soluble CD163 but this is elevated in sepsis [48] and other inflammatory conditions and would be worth considering in further studies. CD86 was decreased with increasing maternal BMI on intermediate monocytes – in contrast to the general population, where CD86 expression has been found to be elevated on non-classical monocytes in obesity [49]. CD38 expression on monocytes and macrophages is induced in inflammatory conditions [50] and a decrease in the expression of CD38 correlates with suppression of adipogenesis and lipogenesis in adipose tissue in mouse models [51]. Our data shows a decrease in CD38 expression on classical and intermediate monocytes suggesting that the monocytes might be attempting to counter the exacerbated inflammatory state of maternal obesity. CD38 also has

312 a role in metabolism, with the ability to produce cyclic ADP-ribose and nicotinic acid adenine
313 dinucleotide phosphate (NAADP) from NAD+ and NADP+ respectively. Inhibitors of CD38,
314 such as the flavonoid apigenin from foods such as parsley, have shown beneficial effects in
315 tackling obesity in animal models [52]. In these models elevated cellular levels of NAD^+ are
316 beneficial, and CD38 knockout increases the NAD^+ levels and protects against obesity [52].
317
318 Metabolically, while our data on fatty acid and amino acid transporters revealed no
319 differences related to maternal BMI, all three subsets of monocytes had reduced
320 mitochondrial content suggesting that monocyte metabolism – especially OXPHOS - is
321 compromised in maternal obesity. Therefore, we also considered the bioenergetic
322 capabilities of MNCs. It would have been ideal to undertake this analysis on isolated
323 monocytes to better match the flow cytometry finding but this was not possible – this was a
324 study of MNCs and only flow cytometry allowed delineation of effects of maternal obesity on
325 discrete cell types within this heterogenous mix. There appears to be very little research
326 surrounding specific immune cell bioenergetics in obesity or pregnancy although the spare
327 respiratory capacity of monocytes has been shown to be negatively correlated with
328 percentage body fat [53]. A study investigating the effect of the bioenergetic function of
329 peripheral monocytes in women with HIV illustrated that monocytes of infected women with
330 obesity had impaired bioenergetic health (reduced basal and maximal oxygen consumption
331 rate as well as decreased bioenergetic health index) in comparison to lean infected women
332 [54]. A recent study by Sureshchandra et al. has shown that at term, monocytes from pregnant
333 women with obesity have reduced ECAR at baseline and following LPS and glucose injections,
334 in comparison to lean pregnant women that might support their maladaptive phenotype [22].
335 There are no other studies of the effects of obesity on the bioenergetic profile of MNCs in

pregnancy or in obesity in general. While our results show no effect of maternal BMI, using a similar approach we have seen that MNCs of pregnant women with GDM have reduced oxidative phosphorylation compared to their GDM-negative counterparts (unpublished data). Given the absence of any difference in cellular bioenergetics with maternal obesity it is perhaps not surprising that we did not see any difference in LPS-stimulated cytokine production despite differences in such responses in the general population with obesity such as increased production of IL-1β and RANTES upon LPS stimulation of classical monocytes [21] and heightened LPS-stimulated TNF, IL-2, and IFNγ and decreased IL-10 production from LPS-stimulated MNCs [55]. However, cytokine production by monocytes is underpinned by glycolysis [56] which does not depend on mitochondria and was also unchanged with maternal BMI. It would be worthwhile investigating the effects of maternal BMI on monocyte effector functions supported by the mitochondria including ROS production and fatty acid oxidation. All of this does suggest however that it is vital to further investigate phenotypic and functional adaptation of monocytes to both obesity and GDM. This is especially so as pregnancy-associated monocyte activation is exacerbated in for example preeclampsia [24], and obesity is a risk factor for preeclampsia [25].

Monocytes are not the only cell type affected by maternal obesity. The immunophenotyping performed on MNCs revealed that T cells are particularly susceptible to the effects of maternal BMI. As already reported [29], we too found a negative correlation between maternal obesity and the abundance of iNKT cells. In the general population, iNKT cells are depleted in adipose tissue of people with obesity [41] and the addition of iNKT cells resolves increased body fat, leptin and insulin sensitivity [39]. For conventional T cells, there was no change in total T cells but there was a significant increase in CD4+ T cells accompanied by a decrease in CD8+

T cells which significantly impacted the CD4:CD8 T cell ratio. This decline in peripheral CD8+ T cell counts with obesity in both the general population [28] and in pregnant women [29] appears to be a common finding. While animal studies suggest that this might be explained by the accumulation of CD8+ T cells in adipose tissue [28], little is known about the effects of pregnancy - either with or without obesity - on adipose tissue immune profiles. While there are recent studies exploring the impact of obesity on adipocyte hypertrophy and adipose tissue macrophage populations in visceral adipose tissue from pregnant women with and without obesity [57] there are few studies of the effects of maternal obesity on adipose tissue with most focusing on GDM [58]. Our and other findings in changes to the abundance of some circulating immune cell populations certainly warrant further effort to understand what is happening within adipose tissues in pregnancy and the interrelationship of blood, adipose tissue and placenta. It also is unfortunate that we did not include regulatory T cells in our analysis but clearly investigation of these and other important minor cell subsets such as ILCs and MAIT cells is needed.

The effect of maternal BMI on the relative abundance of CD4 and CD8 T cells, combined with the recognised importance of the Th1/Th2/Th17/Treg axis in pregnancy success [59] prompted us to consider the impact of obesity on this phenotype. Using a flow cytometry based approach based on patterns of chemokine expression by CD4+ T cells [44] we found that maternal obesity was associated with a decline in Th2 and Th9 cells. This decline in Th2 cells was accompanied by decreased production of IL-4 upon stimulation of MNCs revealing a negative effect of maternal obesity on Th2 responsiveness in particular. This appears to be accompanied by increased Th1 and Th17 cytokine production suggesting disruption of the Th1/Th2/Th17 axis in pregnant women with obesity. Our findings are consistent with the

obesity-associated shift to Th1 and Th17 in the general population [30, 60]. In the setting of pregnancy such a shift could lead to recurrent pregnancy loss [61] and pre-term birth [62] for which obesity is a recognised risk factor. Obesity also has been shown to be an indicator for severity of COVID-19 symptoms [10] including in pregnancy [63] and the altered Th1/Th2/Th17 profile shown here could account for this obesity-associated increased risk of severe disease in pregnant women.

Conclusions

BMI is strongly correlated to several differences in pregnant woman at 28 weeks of gestation. These changes offer explanations for increased risk of adverse obstetric outcomes, and some may offer targets for therapy. Further investigation into isolated cell populations as well as adipose tissue and placenta is required to further our understanding of the influence of obesity on pregnancy outcome.

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Competing Interests

The authors declare no competing interests.

Contributions

A.R. and O.R. performed experiments. A.R., O.R., N.J. and C.A.T. designed the experiments and provided insight into the discussion. A.R. analysed the data, with N.J. playing an important role in interpretation. A.R. and C.A.T. wrote the manuscript. All authors critically revised and approved the manuscript.

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	BMI ≤ 29.9		BMI ≥ 30.0		p-value
	Mean	± SEM	Mean	± SEM	
Age (years)	30.06	0.7304	29.74	0.6182	0.7157
Gestation (weeks)	27.46	0.2143	27.05	0.1259	0.0593
Gravidity	2.50	0.2289	2.65	0.1753	0.5281
Parity	0.98	0.1466	1.01	0.1104	0.8893
Fasting glucose (mmol)	4.44	0.0456	4.52	0.0464	0.2288
2 hr glucose (mmol)	5.57	0.1359	5.40	0.1119	0.3850
BMI	24.44	0.4203	35.63	0.5291	<0.0001

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591 **Table 1. Summary data for study participants. pants.** All women were GDM-negative on oral glucose tolerance test the only

592 significant difference between the lean/overweight (BMI ≤ 29.9) and obese/morbidly obese (BMI ≥ 30.0) groups was BMI.

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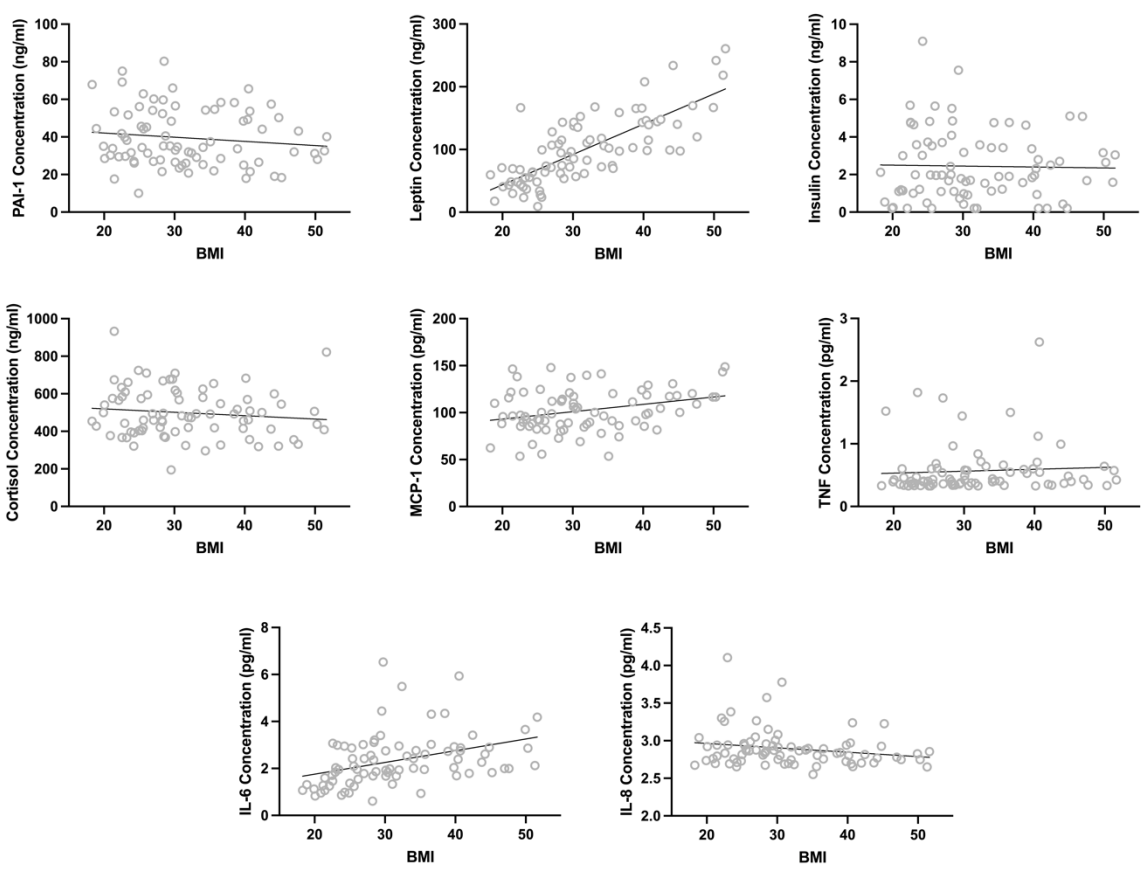

Figure 1: Plasma levels of inflammatory mediators in GDM-negative women of varying pre-pregnant BMI at 28 weeks of gestation. Plasma was available from fasted pregnant women of approximately 28 weeks of gestation (n=80) and was used for analysis as described in the Materials and Methods and correlated to BMI. Statistics were determined using either a Pearson r or Spearman r test dependent on their K-S test result, where $p < 0.05$ was determined significant. Analytes measured were: PAI-1 ($r = -0.1242$; $p = 0.2722$), leptin ($r = 0.7635$; $p < 0.0001$), insulin ($r = 0.0017$; $p = 0.9882$), cortisol ($r = -0.1196$; $p = 0.2937$), MCP-1 ($r = 0.3024$; $p = 0.0064$), TNF ($r = 0.1931$; $p = 0.0861$), IL-6 ($r = 0.4895$; $p < 0.0001$) and IL-8 ($r = -0.1923$; $p = 0.0875$).

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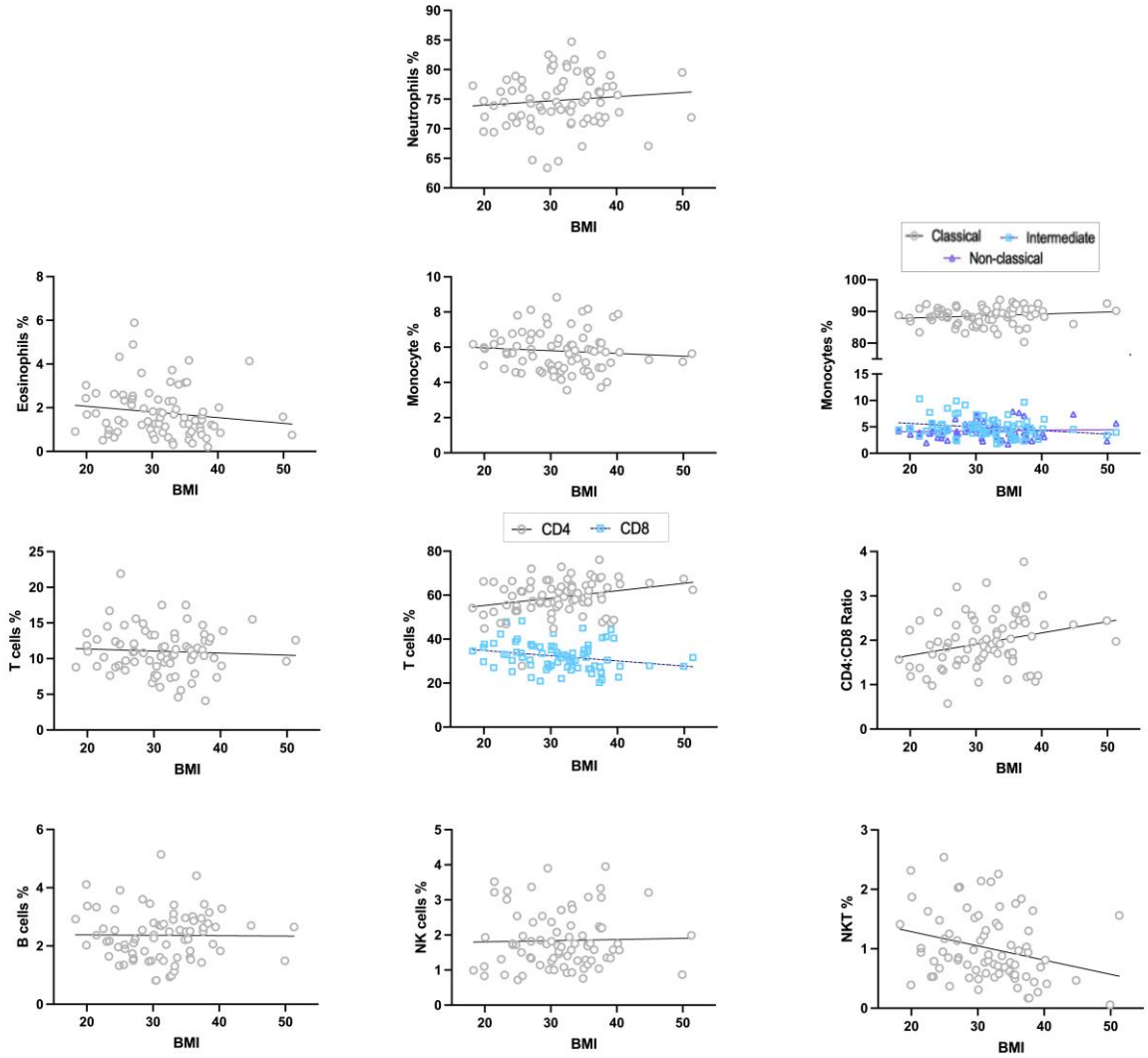

Figure 2: The impact of BMI on leukocyte populations in pregnancy. Whole blood (n=77) from fasted pregnant women of approximately 28 weeks of gestation was used for leukocyte phenotyping as described in the materials and methods. They were correlated to BMI. Statistics were determined using either a Pearson r or Spearman r test dependent on their K-S test result, where $p < 0.05$ was determined significant. Leukocyte populations which were determined where: neutrophils ($r = 0.1053$; $p = 0.3654$), eosinophils ($r = -0.2034$; $p = 0.0760$), total monocytes ($r = -0.898$; $p = 0.4375$), classical monocytes ($r = 0.2138$; $p = 0.0637$), intermediate monocytes ($r = -0.2394$; $p = 0.0372$), non-classical monocytes ($r = -0.0354$; $p = 0.7614$), total T cells ($r = -0.0606$; $p = 0.6004$), CD4 T cells ($r = 0.2798$; $p = 0.0137$), CD8 T cells ($r = -0.2476$; $p = 0.0299$), CD4:CD8 ratio ($r = 0.2789$; $p = 0.0140$), B cells ($r = -0.0105$; $p = 0.9276$), NK cells ($r = 0.0628$; $p = 0.5877$) and NKT cells ($r = -0.2842$; $p = 0.0123$).

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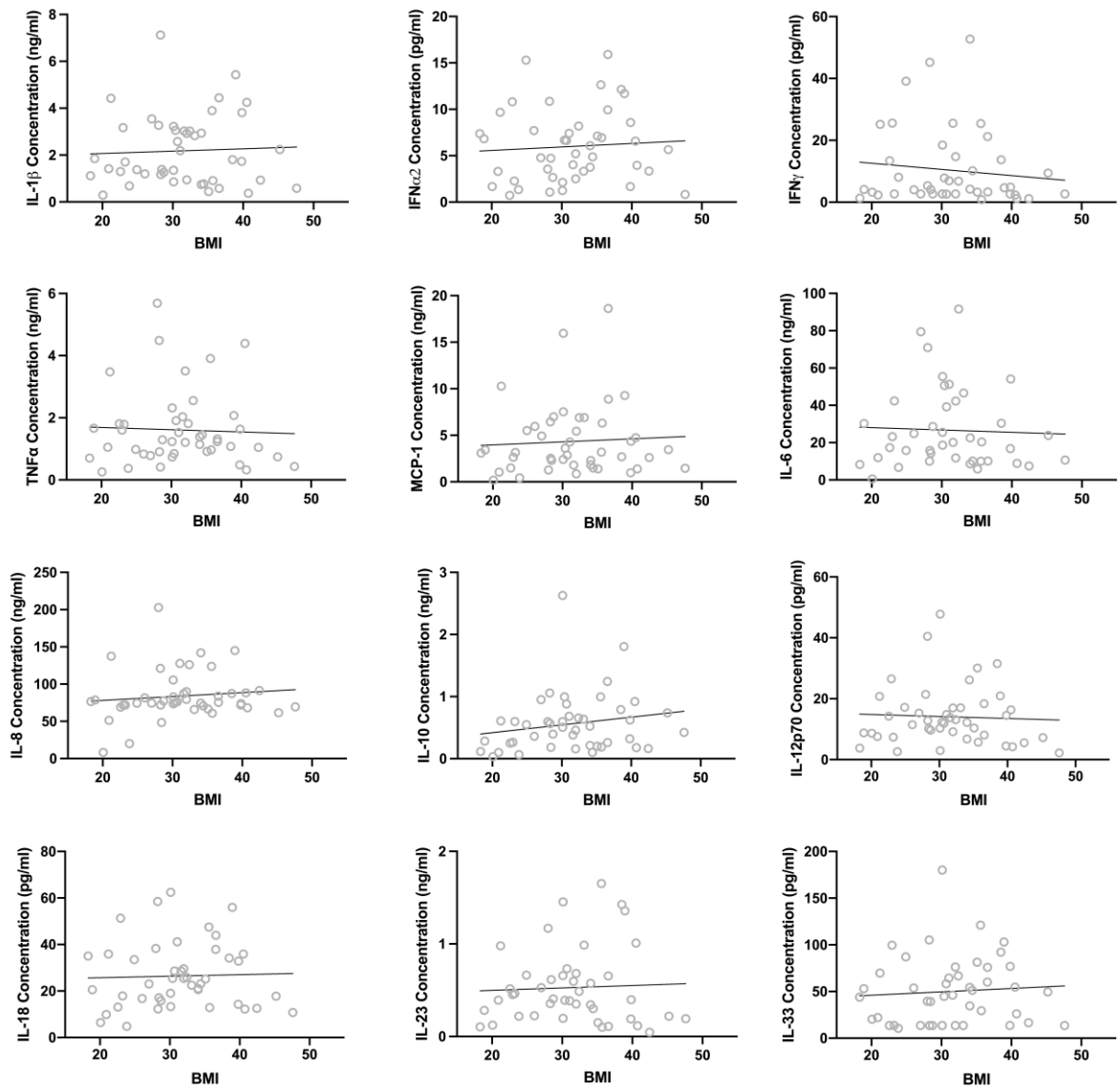

Figure 3: LPS-stimulated cytokine production by peripheral blood mononuclear cells from GDM-negative women of varying pre-pregnant BMI at 28 weeks of gestation. MNCs (n=45) were stimulated with LPS and then levels of cytokines (ng/ml or pg/ml) measured using a multiplex bead array for flow cytometry. Statistics were determined using either a Pearson r or Spearman r test dependent on their K-S test result, where $p < 0.05$ was determined significant. IL-17A was not detectable and there was no significant correlation between BMI and any of the other cytokines measured: IL-1β ($r = -0.0040$; $p = 0.9791$), IFNα2 ($r = 0.0687$; $p = 0.6579$), IFNγ ($r = -0.1182$; $p = 0.4503$), TNF ($r = -0.0144$; $p = 0.9254$), MCP-1 ($r = 0.0651$; $p = 0.6710$), IL-6 ($r = -0.0602$; $p = 0.7085$), IL-8 ($r = 0.0083$; $p = 0.5642$), IL-10 ($r = 0.2085$; $p = 0.1694$), IL-12p70 ($r = -0.0596$; $p = 0.6972$), IL-18 ($r = 0.0331$; $p = 0.8290$), IL-23 ($r = -0.0791$; $p = 0.6099$) and IL-33 ($r = 0.1555$; $p = 0.3078$).

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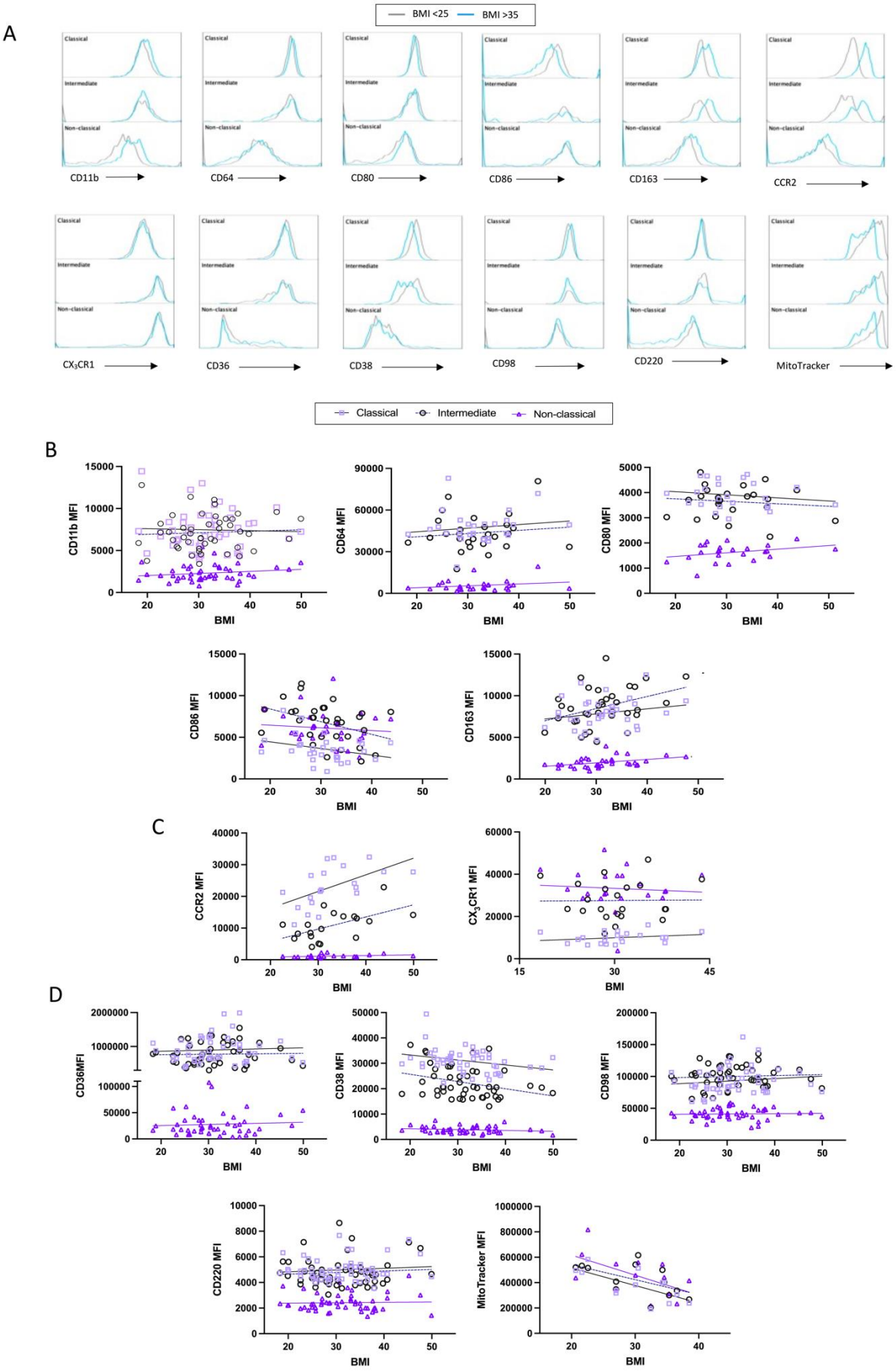
A
BMI <25
BMI >35
Classical
Intermediate
Non-classical
CD11b
CD64
CD80
CD86
CD163
CCR2
CX3CR1
CD36
CD38
CD98
CD220
MitoTracker
Classical
Intermediate
Non-classical
B
CD11b MFI
CD64 MFI
CD80 MFI
CD86 MFI
CD163 MFI
BMI
C
CCR2 MFI
CX3CR1 MFI
BMI
D
CD36MFI
CD38 MFI
CD98 MFI
CD220 MFI
MitoTracker MFI
BMI

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Figure 4: Phenotype of classical, intermediate, and non-classical peripheral blood monocytes of GDM-negative women of varying pre-pregnant BMI at 28 weeks of gestation. CD14 and CD16 expression were used to define classical (C; CD14++/CD16-), intermediate (I; CD14++, CD16+) and non-classical (NC; CD14+, CD16++) monocytes for further analysis of key surface antigens; MFI values are reported for correlation with BMI for each subset. Statistics were determined using either a Pearson r or Spearman r test dependent on their K-S test result, where $p < 0.05$ was determined significant. **(A)** Example histograms dictating obese (blue) and non-obese (grey) for each marker on each monocyte subset. **(B)** CD11b (n=46; C r = -0.0339, p = 0.8228; I r = 0.0592, p = 0.6960; NC r = 0.1953, p = 0.1934), CD64 (n=28; C r = 0.1248, p = 0.5269; I r = 0.1201, p = 0.5427; NC r = 0.1880, p = 0.5499), CD80 (n=24; C r = -0.1713, p = 0.4235; I r = -0.1178, p = 0.5834; NC r = 0.3106, p = 0.1396), CD86 (n=30; C r = -0.2969, p = 0.1110; I r = -0.3924, p = 0.0263; NC r = -0.1029, p = 0.5753), CD163 (n=35; C r = 0.2085, p = 0.2293; I r = 0.3806, p = 0.0241; NC r = 0.4034, p = 0.0163). **(C)** CCR2 (n=19; C r = 0.5596, p = 0.0083; I r = 0.5627, p = 0.0121; NC r = 0.4696, p = 0.0317), CX_3CR1 (n=22; C r = 0.2466, p = 0.2685; I r = -0.0515, p = 0.8199; NC r = -0.0587, p = 0.7951). **(D)** Expression CD36 (n=47; C r = 0.0761, p = 0.6073; I r = 0.0274, p = 0.8533; NC r = 0.0372, p = 0.8040), CD38 (n=47; C r = -0.2872, p = 0.0478; I r = -0.3195, p = 0.0269; NC r = -0.1711, p = 0.2501), CD98 (n=47; C r = 0.1307, p = 0.3791; I r = 0.0612, p = 0.6828; NC r = 0.0382, p = 0.7966), CD220 (n=53; C r = 0.0316, p = 0.8222; I r = -0.0105, p = 0.9407; NC r = -0.0233, p = 0.8682) and MitoTracker™ (n=13; C r = -0.6818, p = 0.0103; I r = 0.5597, p = 0.0467; NC r = -0.5659, p = 0.0438).

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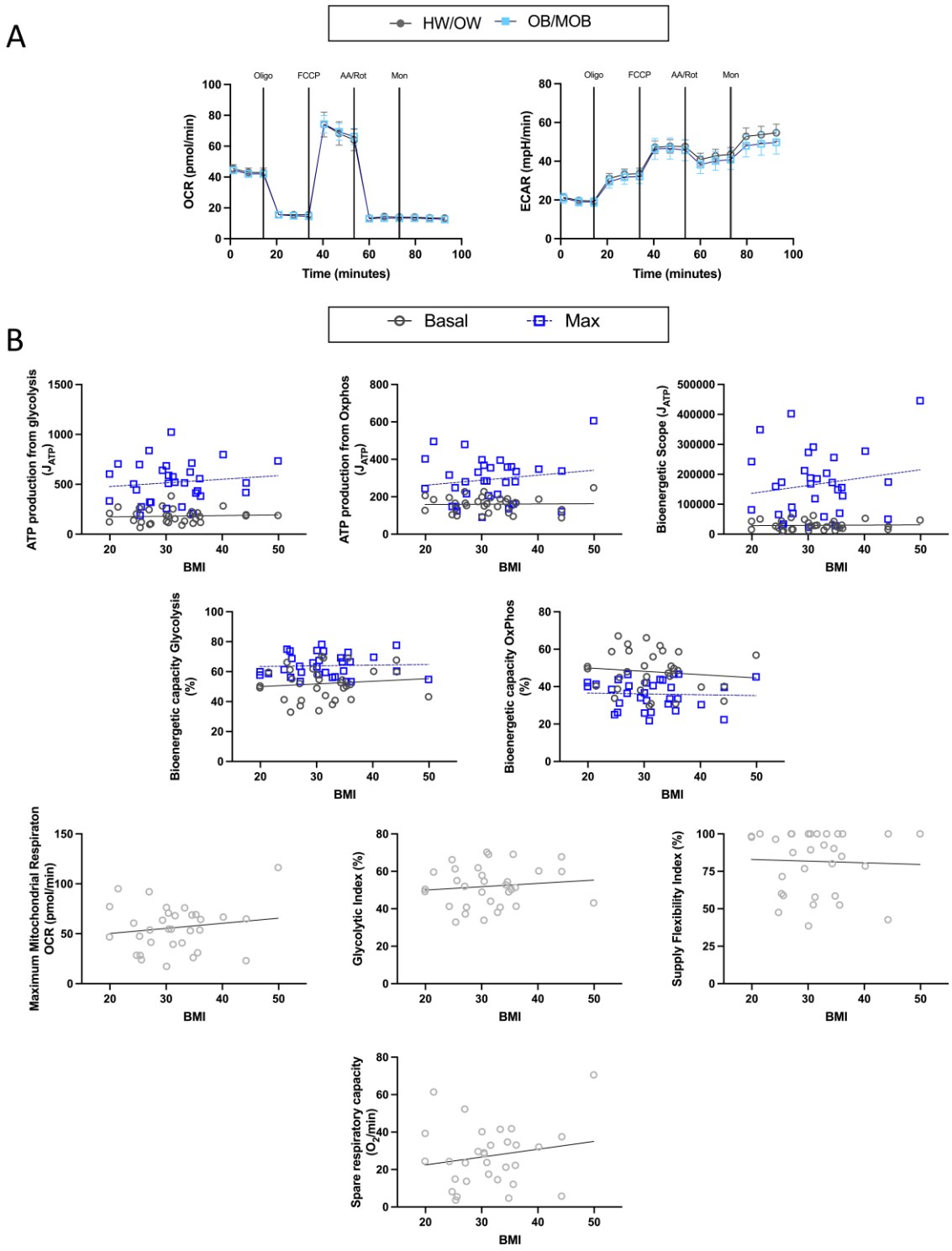

Figure 5: The bioenergetic capacity of mononuclear cells from GDM-negative women of varying pre-pregnant BMI at 28 weeks of gestation. OCR and ECAR of MNCs from pregnant women at approximately 28 weeks (n=32) were measured using the Seahorse extracellular flux analyser as described in the Materials and Methods and correlated to maternal obesity. **(A)** The trace of OCR and ECAR against time for grouped not-obese vs obese. **(B)** Specific glycolytic or oxidative phosphorylation parameters were extracted and correlated against BMI. Statistics were determined using either a Pearson r or Spearman r test dependent on their K-S test result, where $p < 0.05$ was determined significant. These parameters included: ATP production from glycolysis (basal r = 0.0650, p = 0.7239; max r = 0.1268, p = 0.4894) and OXPHOS (basal r = 0.0226, p = 0.9025; max r = 0.1549, p = 0.3972), bioenergetic scope (basal r = 0.0386, p = 0.8339; max r = 0.1668, p = 0.3617), bioenergetic capacity for glycolysis (basal r = 0.1155, p = 0.5289; max r = 0.0405, p = 0.8259) and OXPHOS (basal r = -0.1155, p = 0.5289; max r = -0.0405, p = 0.8259), maximum mitochondrial respiration (r = 0.1549; p = 0.3972), supply flexibility index (r = 0.0168; p = 0.9271), glycolytic index (r = 0.1156; p = 0.5288) and spare respiratory capacity (r = 0.1814; p = 0.3286).

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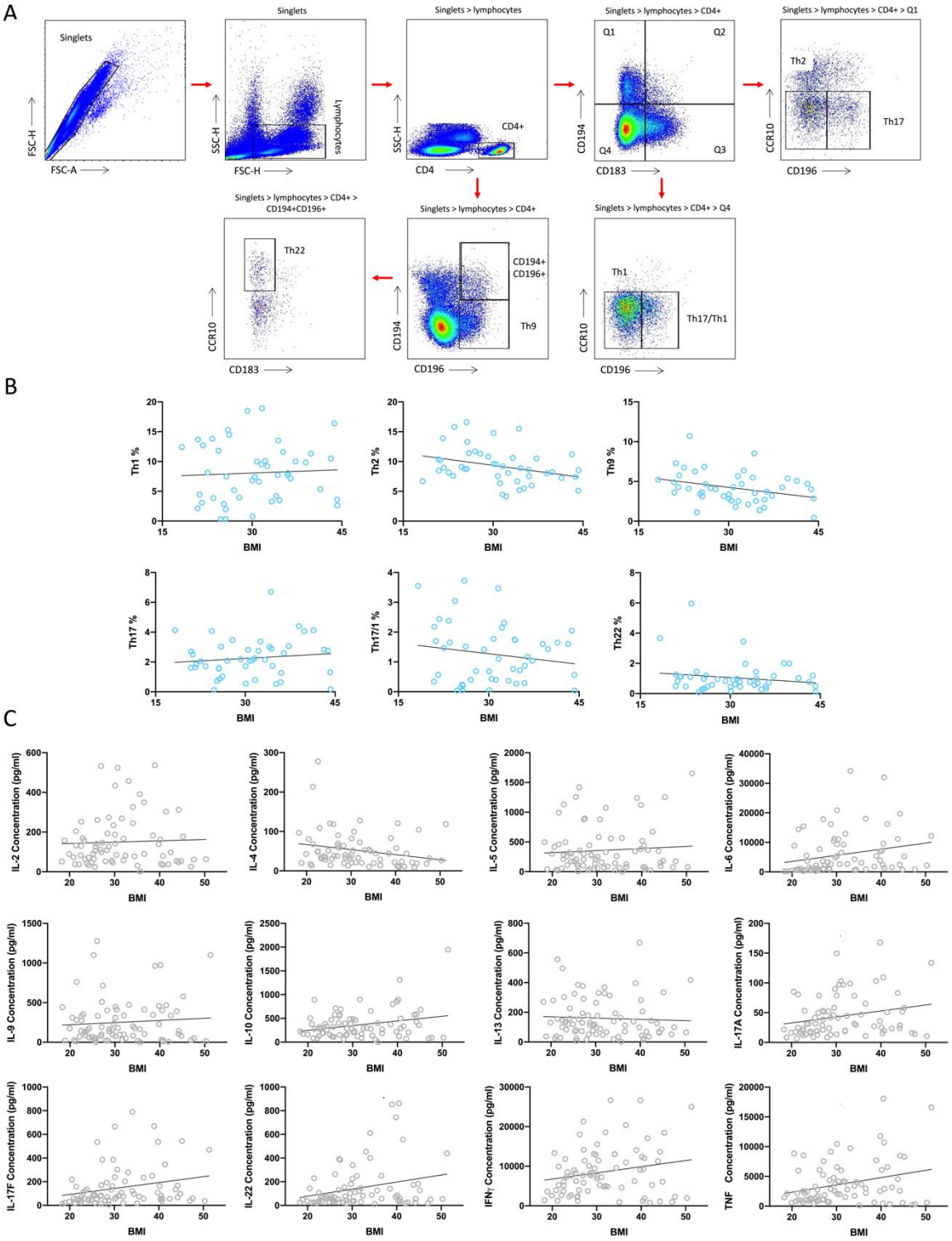
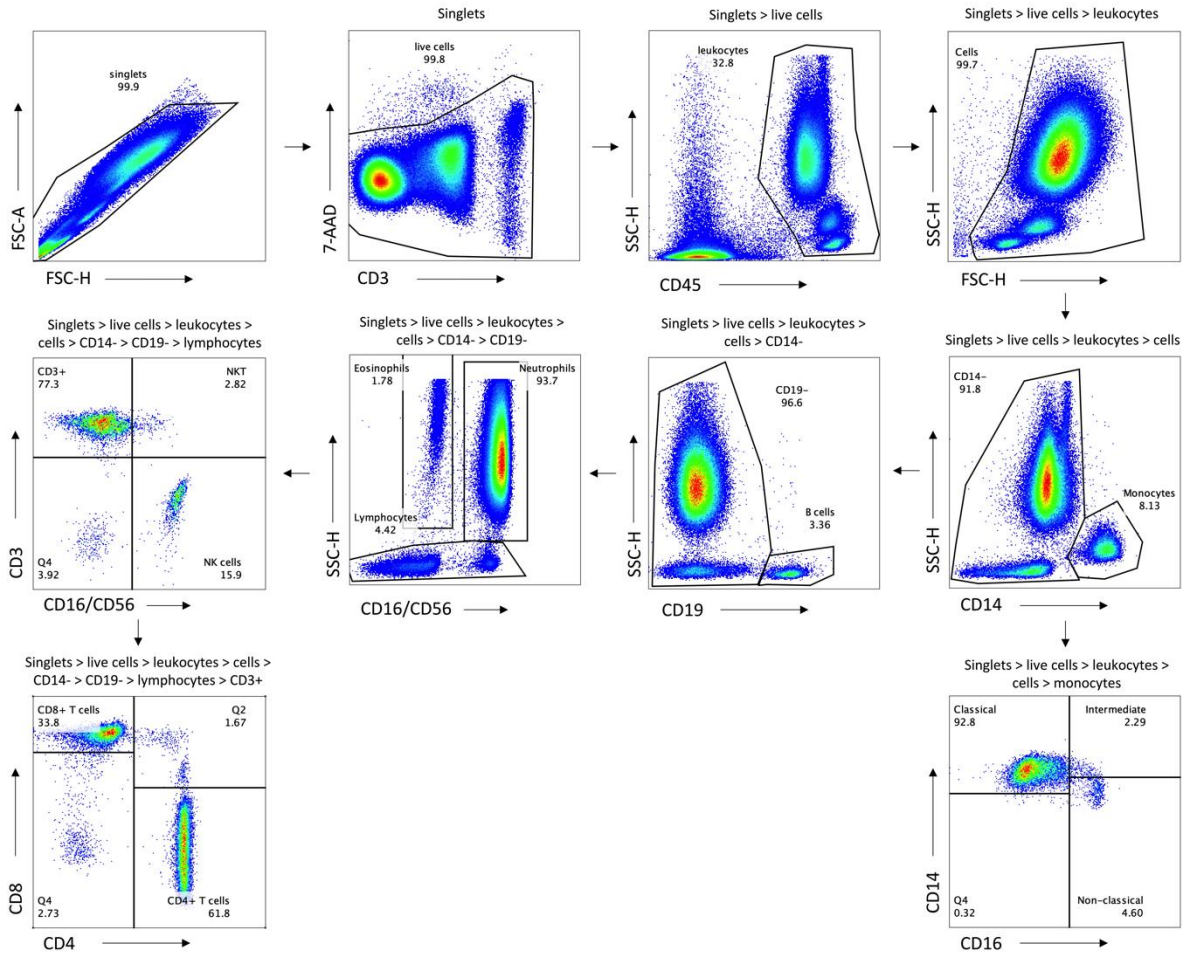


Figure 6: The Th profile of GDM-negative women of varying pre-pregnant BMI at 28 weeks of gestation. (A) Gating strategy for identifying the Th subsets using flow cytometry and chemokine receptor profile – CCR4-CXCR3-CCR10-CCR6- Th1; CCR4+CXCR3-CCR10-CCR6- Th2; CCR4-CCR6+ Th9; CCR4+CXCR3-CCR10-CCR6+ Th17; CCR4-CXCR3-CCR10-CCR6+ Th17/1; CCR4+CCR6+CCR10+ Th22. Statistics were determined using either a Pearson r or Spearman r test dependent on their K-S test result, where $p < 0.05$ was determined significant. **(B; blue)** The populations identified were (n=46): Th1 ($r = 0.0538$; $p = 0.7225$), Th2 ($r = -0.3202$; $p = 0.0341$), Th9 ($r = -0.3205$; $p = 0.0319$), Th17 ($r = 0.1202$; $p = 0.4315$), Th17/1 ($r = -0.1662$; $p = 0.2696$) and Th22 ($r = -0.0705$; $p = 0.6415$). **(C; grey)** Multiplex analysis of cytokines from CytoStim™-stimulated MNCs (n=74): IL-2 ($r = 0.0295$; $p = 0.8046$), IL-4 ($r = -0.2806$; $p = 0.0162$), IL-5 ($r = -0.0158$; $p = 0.8898$), IL-6 ($r = 0.4166$; $p = 0.0002$), IL-9 ($r = 0.0240$; $p = 0.8339$), IL-10 ($r = 0.1193$; $p = 0.2983$), IL-13 ($r = -0.1013$; $p = 0.3745$), IL-17A ($r = 0.2753$; $p = 0.0168$), IL-17F ($r = 0.2973$; $p = 0.0091$), IL-22 ($r = 0.2257$; $p = 0.0484$), IFNγ ($r = 0.1762$; $p = 0.1228$) and TNF ($r = 0.1979$; $p = 0.0824$).



Supplementary Figure 1: Gating strategy for the 8-colour immunophenotyping. Singlets are first selected via a forward scatter (FSC) height (H) vs area (A). Dead cells (7-AAD positive) are excluded. Only leukocytes (CD45+) are then selected, before debris is removed from the gating strategy on an FSC-H. vs side-scatter (SSC)-H plot. Monocytes are isolated by being CD14+ and further separated into its subsets based on their CD14+ and CD16+ expression. From the CD14-negative cells, B cells (CD19+) are isolated. It is possible to identify eosinophils (CD16/CD56-negative), neutrophils (CD16/CD56-positive) and lymphocytes (low SSC profile). In the lymphocyte profile, T cells (CD3+), NKT cells (CD3+CD16/CD56+) and NK cells (CD3-CD16/CD56+) are identified. T cells can be segregated into by their CD4 and CD8 expression.

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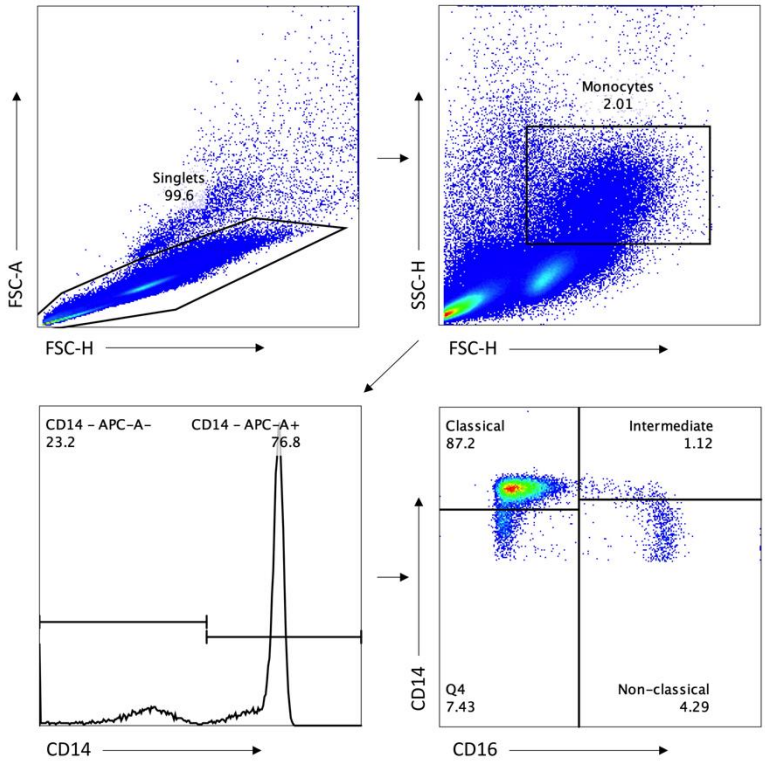

Supplementary Figure 2: Gating strategy for the selection of monocytes and their subsets in MNCs. Singlets were first selected using a FSC-H vs FSC-A dot plot. Using a FSC-H vs SSC-H dot plot, monocytes were identified according to their relative size and granularity. A histogram visualising the CD14 expression enabled gating on the CD14-positive peak. Monocyte subsets were then identified according to their CD14/CD16 profile.