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RESEARCH ARTICLE

Decidual glycodelin-A polarizes human monocytes into a decidual macrophage-like phenotype through Siglec-7

Madhavi Vijayan[1,*], Cheuk-Lun Lee[1,2,*], Vera H. H. Wong[1], Xia Wang[1], Kungfeng Bai[1,2], Jian Wu[2], Hannu Koistinen[3], Markku Seppälä[3], Kai-Fai Lee[1,2], William S. B. Yeung[2], Ernest H. Y. Ng[1,2,‡] and Philip C. N. Chiu[1,2,‡]

ABSTRACT

Decidual macrophages constitute 20–30% of the total leukocytes in the uterus of pregnant women, regulating the maternal immune tolerance and placenta development. Abnormal number or activities of decidual macrophages (dMs) are associated with fetal loss and pregnancy complications, such as preeclampsia. Monocytes differentiate into dMs in a decidua-specific microenvironment. Despite their important roles in pregnancy, the exact factors that regulate the differentiation into dMs remain unclear. Glycodelin-A (PAEP, hereafter referred to as GdA) is a glycoprotein that is abundantly present in the decidua, and plays an important role in fetomaternal defense and placental development. It modulates the differentiation and activity of several immune cell types residing in the decidua. In this study, we demonstrated that GdA induces the differentiation of human monocytes into dM-like phenotypes in terms of transcriptome, cell surface marker expression, secretome, and regulation of trophoblast and endothelial cell functions. We found that Sialic acid-binding Ig-like lectin 7 (Siglec-7) mediates the binding and biological actions of GdA in a sialic acid-dependent manner. We, therefore, suggest that GdA, induces the polarization of monocytes into dMs to regulate fetomaternal tolerance and placental development.

KEY WORDS: Glycodelin, Progestagen-associated endometrial protein, PAEP, Macrophage, Trophoblast, Endocrine, Siglec, Decidua

INTRODUCTION

Decidual macrophages (dMs) comprise 20–30% of the decidual leukocytes during the pre-implantation stage and early first trimester of pregnancy (Erlebacher, 2013; Mor et al., 2011). They are the most-abundant leukocyte population in the spiral artery adventitia and are in close proximity to the trophoblast (Lash et al., 2016; Mor and Abrahams, 2003). They regulate maternal immune tolerance and vascular remodeling through their interaction with trophoblast and endothelial cells. Previous studies have found an increase in the number of dMs with an alternatively activated macrophage (M2) phenotype, but not in those with a classically activated macrophage (M1) phenotype, coexpressing CD163 and CD206 from first to second trimester (Gustafsson et al., 2008; Heikkinen et al., 2003; Kwan et al., 2014). However, whether dMs belong to M2 macrophages (Gustafsson et al., 2008) or a new subset of macrophages (Houser et al., 2011) is still controversial. An aberrant number of dMs is associated with fetal loss, intrauterine growth restriction and preeclampsia (Erlebacher, 2013; Mor and Abrahams, 2003; Ning et al., 2016). Monocytes differentiate into dMs in a decidua-specific microenvironment, but the factors and mechanisms that regulate the differentiation of dMs remain unclear despite their important roles in pregnancy.

An increasing number of studies demonstrate the importance of decidual microenvironment in development of tolerogenic leukocytes. The progestagen-associated endometrial protein (PAEP, hereafter referred to as GdA) is a glycoprotein synthesized in secretory and decidualized endometrium; during pregnancy it is abundant in the amniotic fluid (Lee et al., 2011b; Seppälä et al., 2002). The ortholog of glycodelin is found only in certain suborders of higher primates that have a menstrual cycle, implying that its role in reproduction has evolved only recently (Lee et al., 2016). The concentration of GdA in decidua increases during early pregnancy and peaks between 6–12 weeks of gestation (Seppälä et al., 2002), coinciding with early placental development. GdA is a modulator of immune cells at the fetomaternal interface that is crucial for the maintenance of pregnancy (Lee et al., 2011b, 2016). It suppresses proliferation and induces apoptosis of T-cells, modulates cytokine production from peripheral blood natural killer (NK) cells (Lee et al., 2010, 2019a) and macrophages (Lee et al., 2012), and induces a Th-2 shift in cytokines (Lee et al., 2011a). GdA also participates in the differentiation of endometrial, trophoblast, NK and T-cells (Lee et al., 2016, 2019a). These biological activities of GdA are mediated by binding of its unique sialic acid-rich carbohydrate side-chains to cell surface sialic acid receptors, such as selectins and sialic acid-binding Ig-type lectins (SIGLECs), on various cell types at the human fetomaternal interface (Chiu et al., 2007; Lam et al., 2011; Lee et al., 2012).

In this study, we hypothesize that GdA polarizes the differentiation of human monocytes into dMs. This action regulates, at least in part, the immunotolerance towards fetal antigens and placental remodeling at the fetomaternal interface. Our study investigates (1) the role of GdA in mediating dM differentiation, (2) the functions GdA-polarized macrophage in regulating fetomaternal tolerance and placental remodeling, and (3) Siglec-7 mediated binding of GdA and the mechanism involved in dM differentiation.

RESULTS

Similar transcriptomes between GdA-polarized macrophages and dMs

Peripheral blood monocytes were induced to differentiate into macrophages in the presence or absence of GdA. To gain insight into the properties of GdA-polarized macrophages, we performed

[1]Department of Obstetrics and Gynaecology, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong S.A.R. [2]The University of Hong Kong Shenzhen Key Laboratory of Fertility Regulation, The University of Hong Kong-Shenzhen Hospital, Shenzhen, China. [3]Department of Clinical Chemistry and Obstetrics and Gynecology, University of Helsinki and Helsinki University Hospital, 00029 HUS Helsinki, Finland.
*These authors contributed equally to the work

‡Authors for correspondence (pchiucn@hku.hk; nghye@hku.hk)

M.V., 0000-0002-3367-5990; C.-L.L., 0000-0003-1553-3478; V.H.H.W., 0000-0002-0140-6665; X.W., 0000-0003-0362-0179; K.B., 0000-0002-4888-9158; E.H.Y.N., 0000-0002-7688-4557; P.C.N.C., 0000-0001-6217-1970

Handling Editor: Daniel Billadeau
Received 28 January 2020; Accepted 28 May 2020

transcriptome profiling using RNA-seq (Table S2: Supplementary Data Set 1). Differentially expressed genes were identified between macrophages polarized with macrophage colony-stimulating factor 1 (CSF1, hereafter referred to as MCSF), MCSF+GdA-polarized or MCSF+GdA+IL-10-polarized macrophages on the basis of our selection criteria (Fig. 1A,B). Addition of IL-10 resulted in only minimal effect on the transcriptome of MCSF-polarized and MCSF+GdA-polarized macrophages. Principal component analysis (PCA) further demonstrated that dMs and the macrophages polarized by GdA are transcriptionally closer to each other than to those without GdA polarization (Fig. 1C). In addition to PCA, we studied 90 reported dM-specific genes (Gustafsson et al., 2008). Hierarchical clustering on the basis of these dM-specific genes clustered the MCSF+GdA+IL-10-polarized and/or MCSF+GdA-polarized macrophages in a branch adjacent to dMs (Fig. 1D). Comparison between MCSF-polarized macrophages, MCSF+GdA-polarized macrophages and dMs by soft clustering (Fig. 1E) further indicated that the gene signature of GdA-polarized macrophages resembled that of dMs. These results suggested that GdA treatment transformed monocytes to macrophages with dM gene signature, although the treatment failed to complete the dM differentiation process.

The differentially expressed genes under the experimental conditions with and without GdA were subjected to gene ontology enrichment analyses. The top 30 enriched terms for categories are shown in Fig. 1F (adjusted *P*-value <0.05). Many of the enriched biological processes were related to the immune response. Other enriched biological processes included phagocytosis, apoptosis, cell adhesion, chemotaxis and angiogenesis. Kyoto encyclopedia of genes and genomes (KEGG) database pathway analyses identified different signaling pathways related to TNF-alpha, Rap1, PI3K–AKT–NFκB, MAPK and JAK–STAT signaling pathways (Fig. 1G). Gene set enrichment analysis (GSEA) identified some differentially expressed pathways, such as cytokine–cytokine receptor interactions and the chemokine signaling pathway, when MCSF-polarized macrophages were compared with MCSF+GdA-polarized macrophages and dMs (Fig. 1H).

GdA induces the expression of dM markers

Immunostaining experiments showed the presence of dMs in frozen sections of first trimester decidual tissue expressing GdA (Fig. S1B). To confirm the ability of GdA to polarize macrophages into the dM phenotype, qPCR and flow cytometry were used to determine the expression of macrophages lineage markers upon GdA treatment. The treatment enhanced the expression of the dM markers, indoleamine 2,3-dioxygenase 1 (IDO-1) and CD209 antigen (CD209), in the differentiated macrophages when compared to the control (Fig. 2A, B). Simultaneous addition of GdA potentiated the stimulatory effect of IL-10 on expression of another dM marker, neuropilin-1 (NRP-1) (Fig. 2A). The stimulatory effect of GdA was specific for dM markers, and the treatment did not affect the expression of markers for pan and M2 macrophages (Fig. 2A). There was no detectable expression of M1 macrophage markers, i.e. CD80 and CD86.

GdA-polarized macrophages exhibit immunoregulatory features

IDO-1 catalyzes the oxidative cleavage of tryptophan into kynurenine an immuno-suppressive enzyme that prevents maternal T-cell activation and regulates maternal immunotolerence (Munn et al., 1998). We observed a significantly higher tryptophan catabolic activity in GdA-polarized macrophages compared with that in control macrophages (15.9±0.7 µM versus 11.8±1.3 µM; $P<0.05$; Fig. 2C), consistent with their higher expression IDO-1 (Fig. 2A).

Both membrane-based cytokine array (Fig. 3A,B) and ELISA (Fig. 3E) showed that the GdA-polarized macrophages produce more of the anti-inflammatory cytokines IL-10 and IL-13, than control macrophages. GdA induced the secretion of pro-inflammatory TNF-alpha, consistent with high expression of TNF-alpha in dMs (Renaud et al., 2005). GdA also stimulated the differentiated macrophages to produce the C-C motif chemokine 2 (CCL2) (Fig. 3B,E), which had been suggested to be involved in recruiting monocytes/macrophages to the decidua (Gustafsson et al., 2008). Both GdA and IL-10 treatment induced expression of insulin-like growth factor-binding protein 1 (IGFBP-1), an important regulator of trophoblast functions and angiogenesis in pregnancy (Giudice et al., 1998) (Fig. 3C–E).

dMs were isolated from human decidual tissue (Fig. 4A). To further compare control macrophages, GdA-polarized macrophages and dMs, mRNA expression of previously identified dM markers IDO-1, CD209 and NRP-1 (Fig. 2A,B), transcript factors IRF4, IRF5 and ARG1, and surface markers CD163, CD11c and HLADR were determined. In dMs, expression of IDO-1, CD209, NRP-1, ARG1, IRF4, IRF5, CD11c and HLA-DR was increased, whereas in GdA-polarized macrophages, it was that of IDO-1, CD209, ARG1, IRF4, CD11C and HLA-DR (Fig. 4B,C). High expression levels of IL-10, IL-13, CCL2 and IGFBP-1 were seen in both GdA-polarized macrophages and isolated dMs (Fig. 4D).

The secretome of GdA-polarized macrophages induces angiogenesis, and trophoblast invasion and integration

dMs lie in close proximity with the spiral arterioles and trophoblast, and regulate vascular remodeling and placental development (Lash et al., 2016). Therefore, we assessed the effects of serum-free conditioned (i.e. spent) medium of GdA-polarized macrophages regarding endothelial cell angiogenesis, and trophoblast invasion and integration. We demonstrated that the secretome of GdA-polarized macrophages have increased capability in promoting human umbilical vein endothelial cells (HUVECs) to form a tube-like endothelial network (i.e. HUVEC tubes) compared to spent medium derived from GdA-polarized macrophages. The secretome of GdA-polarized macrophages also promoted trophoblasts to invade through a basement membrane of the culture insert and encouraged trophoblast integration into the HUVEC network, when compared to spent medium derived from control macrophages (Fig. 5A). Similar stimulatory activities were observed with dMs, i.e. endothelial cell angiogenesis and trophoblast invasion (Fig. 5A), indicating the physiological relevance of our findings. The results were also in line with (1) gene ontology enrichment analysis, showing enriched cell adhesion, chemotaxis and angiogenesis processes in macrophages after GdA polarization (Fig. 1) and; (2) upregulation of angiogenic factors, such as endostatin, IGFBP-1 and human platelet-derived growth factor-BB homodimer (PDGF-BB), in the GdA-polarized macrophages (Fig. 3). Our data also suggest that the stimulatory effect on trophoblast invasion were mediated by the stimulatory activities of GdA on the production of IGFBP-1 by the polarized macrophage (Fig. 5B). However, treatment with MCSF alone or with MCSF+IL-10 did not affect angiogenesis and trophoblast functions (Fig. 5A). None of these treatments had an effect on viability of HUVECs and primary trophoblast and/or human choriocarcinoma (JEG-3) cells (data not shown).

GdA propagates its functions by binding to Siglec-7 on monocytes

Siglec-7 is a trans-membrane receptor on monocytes (Nicoll et al., 1999). It regulates immune cell function upon engagement with

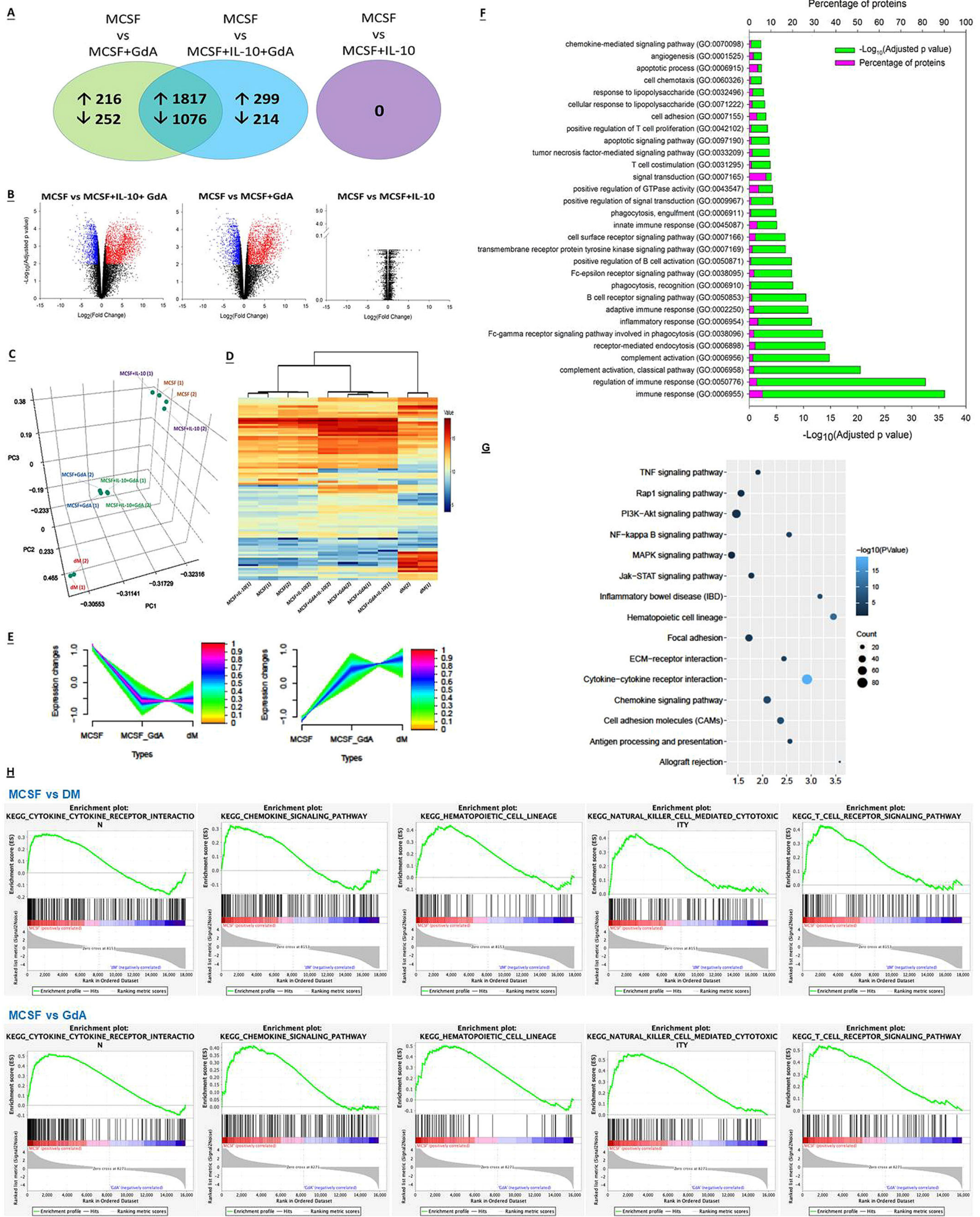

Fig. 1. See next page for legend.

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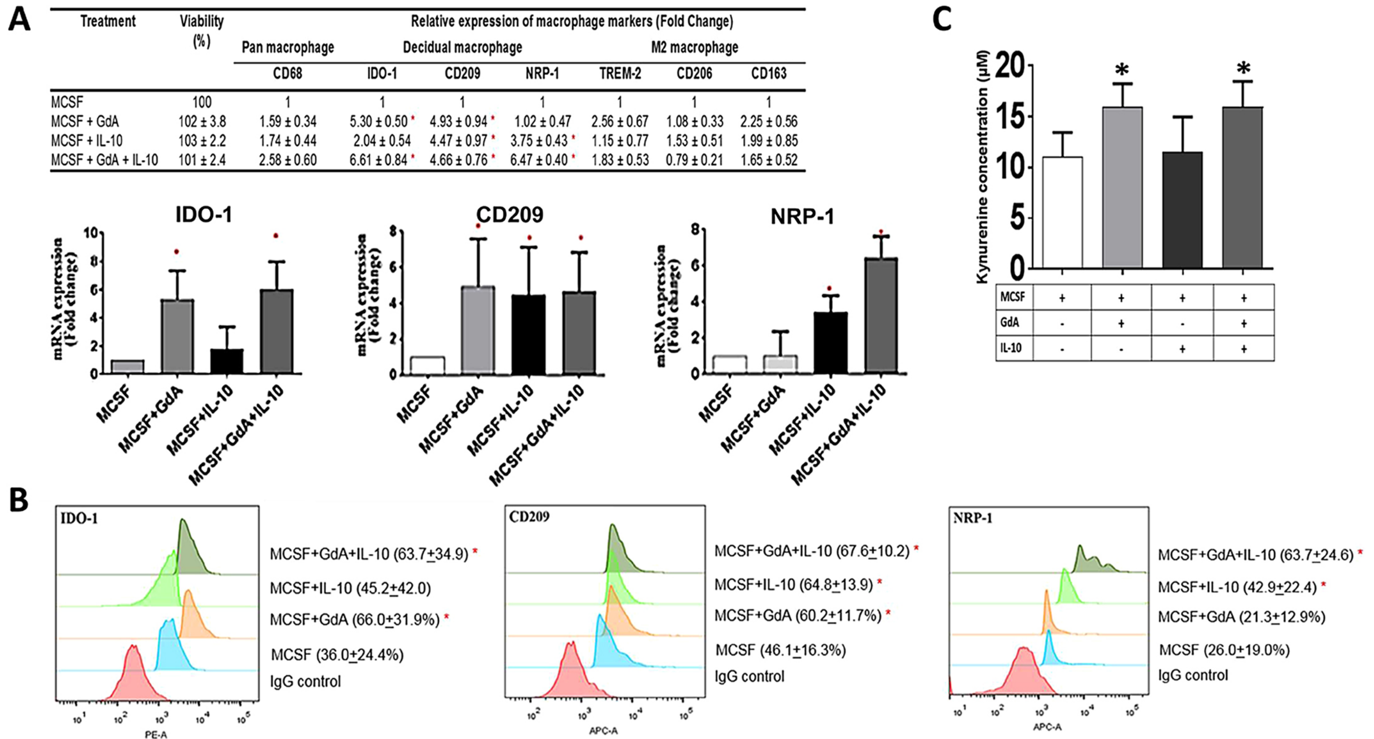
Fig. 1. Characterization of GdA-polarized macrophages by transcriptome profiling. Macrophages were prepared by treating human monocytes with 50 ng/ml MCSF for 9 days in the presence or absence of IL-10 (5 ng/ml) and/or GdA (10 µg/ml), and their transcriptome profiles were compared by mRNA sequencing. (A,B) Venn diagram (A) and volcano plots (B) of differentially expressed genes of different macrophage groups. Selection criteria were: adjusted *P*-value <0.01: $-\log_{10}$ (adjusted *P*-value) >2 and fold-change difference >2: $\log_2$ (fold-change) >1 or <−1. (C) Unsupervised principal component analysis of genes differentially expressed in different macrophage groups. (D) Heatmap and unsupervised hierarchical clustering of different macrophage groups on the basis of the 90 reported dM-specific genes. (E) Plotting results of MCSF-polarized macrophages, GdA-polarized macrophages and dMs by soft clustering (Fig. S2) (F) Functional characterization of differentially expressed genes. (G) KEGG signaling pathways analysis of differentially expressed genes. (H) GSEA identified the same differentially expressed pathways, such as cytokine–cytokine receptor interaction and chemokine signaling pathway, when MCSF-polarized macrophages were compared with MCSF+GdA-polarized macrophages and dM. *n*=2.

sialic acid-rich glycans on glycoproteins (Angata and Varki, 2000), such as GdA. The interaction between purified GdA and Siglec-7 in monocyte membrane protein extract was studied by co-immunoprecipitation with anti-GdA antibody followed by western blot analysis (Fig. 6A). Siglec-7 immunoreactivity was not observed when GdA was omitted during co-immunoprecipitation (Fig. 6A, lane 2). Interaction between GdA and Siglec-7 was also demonstrated by co-immunoprecipitation assays, using Siglec-7 chimera protein (Fig. 6B, lane 3); i.e. the band of immunoreactive desialylated GdA (Fig. 6B, lane 2) less strong compared with that of native GdA (Fig. 6B, lane 3), indicating that interaction between GdA and Siglec-7 is sialic acid dependent.

Flow cytometry analysis showed that the antibody against Siglec-7, but not other SIGLECs and isotype antibodies, reduced GdA binding onto monocytes (Fig. 6C). Addition of anti-Siglec-7 antibody during the differentiation process suppressed the stimulatory effects of GdA on the dM markers mRNA and protein expression (Fig. 6D), tryptophan catabolic activity (Fig. 6E), and cytokine and/or angiogenic factor production (Fig. 6F). In addition, the effect of GdA in promoting expression of IDO-1 and CD209, activity of IDO-1 and cytokine secretion were impaired when the GdA sialic acid had been enzymatically removed (Fig. 6D,F,G).

DISCUSSION

Monocytes differentiate into macrophages within the tissue they reside. The microenvironment of the tissues modulates the differentiation, enabling the resulting macrophages to response appropriately to local stimuli (Stout and Suttles, 2004). The decidual microenvironment in which dMs mature and differentiate during early pregnancy contains a high local concentration of M2 macrophage polarization factors, such as IL-10 (Chaouat et al., 1999) and MCSF (Svensson et al., 2011). However, studies have shown that the transcriptional profile of dMs is different from that of M1 and M2 macrophages (Houser et al., 2011). dMs express genes associated with immune activation, and secrete pro-inflammatory cytokines, such as TNF, in addition to potent anti-inflammatory cytokines (Lidström et al., 2003; Renaud et al., 2005). dMs also show a high expression of IDO-1 (Heikkinen et al., 2003; Houser



Treatment	Viability (%)	Relative expression of macrophage markers (Fold Change)						
		Pan macrophage	Decidual macrophage			M2 macrophage		
		CD68	IDO-1	CD209	NRP-1	TREM-2	CD206	CD163
MCSF	100	1	1	1	1	1	1	1
MCSF + GdA	102 ± 3.8	1.59 ± 0.34	5.30 ± 0.50 *	4.93 ± 0.94 *	1.02 ± 0.47	2.56 ± 0.67	1.08 ± 0.33	2.25 ± 0.56
MCSF + IL-10	103 ± 2.2	1.74 ± 0.44	2.04 ± 0.54	4.47 ± 0.97 *	3.75 ± 0.43 *	1.15 ± 0.77	1.53 ± 0.51	1.99 ± 0.85
MCSF + GdA + IL-10	101 ± 2.4	2.58 ± 0.60	6.61 ± 0.84 *	4.66 ± 0.76 *	6.47 ± 0.40 *	1.83 ± 0.53	0.79 ± 0.21	1.65 ± 0.52

Fig. 2. Expression of macrophage markers of polarized macrophages. Macrophages were prepared by treating monocytes with 50 ng/ml MCSF for 9 days in the presence or absence of IL-10 (5 ng/ml) and/or GdA (10 µg/ml). (A) qPCR analysis of the following markers for different macrophages lineages were performed on the differentiated macrophages: pan macrophages (CD68), dMs (IDO-1, CD209 and NRP-1), M1 macrophages (CD80 and CD86) and M2 macrophages (TREM-2, CD163 and CD206). Expression of CD80 and CD86 was undetectable. *n*=6. (B) Flow cytometric analysis of the dM markers IDO-1, CD209 and NRP-1. *n*=5. (C) Kynurenine production of differentiated macrophages was determined by colorimetric assay. *n*=5. Data are expressed as mean±s.d.; **P*<0.05 versus control (MCSF-polarized macrophages). Non-parametric ANOVA on Rank test followed by Mann–Whitney *U*-test.

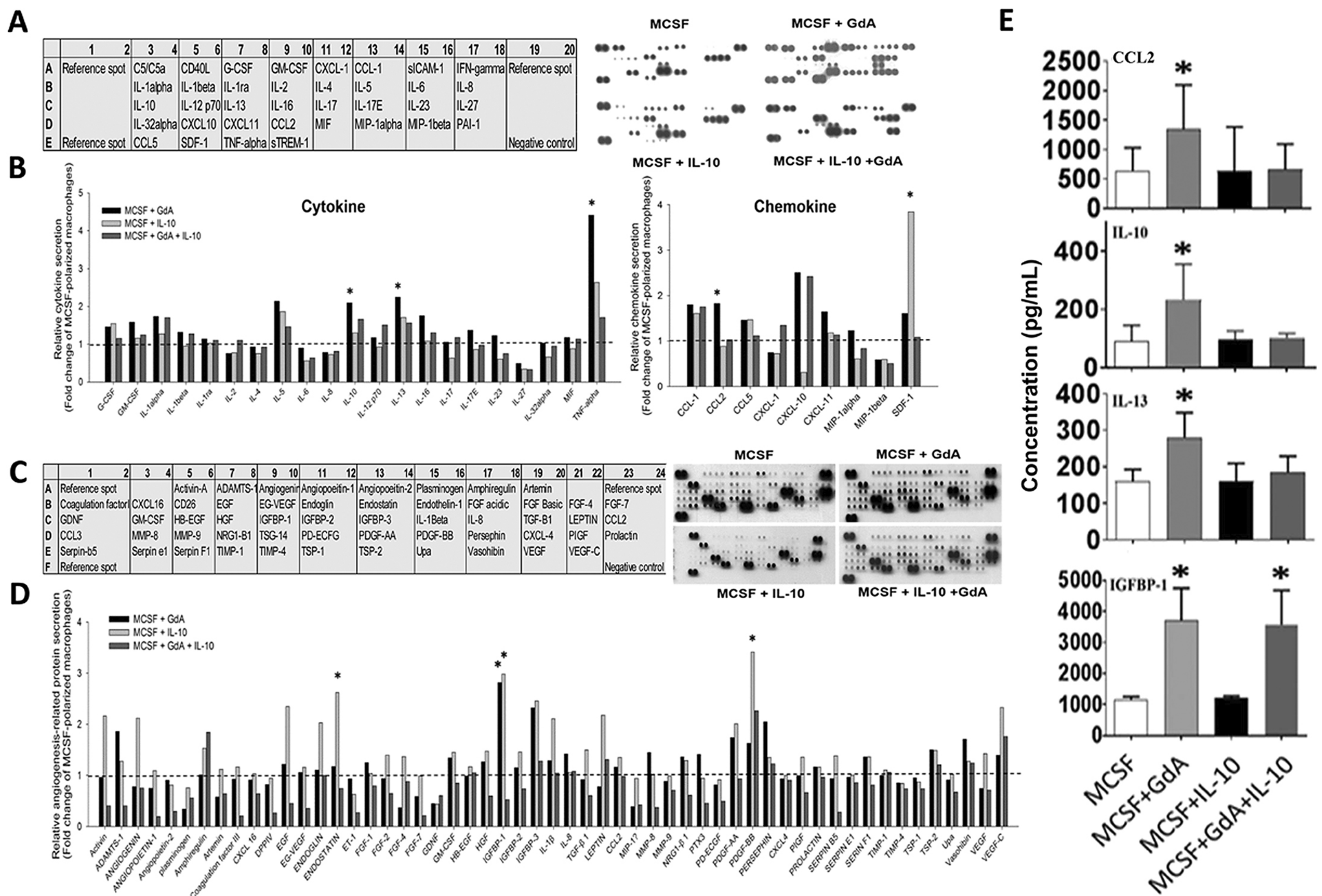


Fig. 3. Cytokine and angiogenic factor analysis of polarized macrophages. (A–C) Macrophages were prepared by treating human monocytes with 50 ng/ml MCSF for 9 days in the presence or absence of IL-10 (5 ng/ml) and/or GdA (10 µg/ml). Macrophages were cultured in serum-free medium for 24 h and the spent medium was collected for (A,B) cytokine or (C,D) angiogenic factor arrays. The results shown are representative of four replicate experiments. Cytokine or angiogenic factor secretion into the culture medium were analyzed by densitometry. The results are expressed as the fold change relative to the control. (E) Macrophages were cultured in serum-free medium for 24 h and the spent medium was collected for IL-10, IL-13, CCL2 and angiogenic protein IGFBP-1 determination by ELISA. *n*=6. Data are expressed as mean±s.d; *$P<0.05$ versus control (MCSF-polarized macrophages). Non-parametric ANOVA on Rank test followed by Mann–Whitney *U*-test.

et al., 2011), which is more highly expressed in M1 compared with M2 macrophages (Rani et al., 2012; Wang et al., 2014).

GdA is a pleiotropic decidual secretory glycoprotein occurring during early human pregnancy. dMs are differentiated in an environment with a high concentration of GdA that modulates functions and differentiation of several cell types at the fetomaternal interface, including endometrial cells, trophoblasts and T-cells (Lee et al., 2011b, 2016). By exposing monocytes to GdA during polarization by MCSF (with or without IL-10), we found that GdA is a potent inducer of macrophages that have characteristics of dMs but are different to MCSF/MCSF+IL-10-polarized macrophages. Unbiased hierarchical clustering of the dM-specific gene profile showed that the gene expression pattern of MCSF+GdA-/MCSF+IL-10+GdA-polarized macrophages is more similar to that of dMs than that of MCSF-/MCSF+IL-10-polarized macrophages. Compared to MCSF- or MCSF+IL-10-polarized macrophages, corresponding GdA-polarized macrophages showed higher expression of dM-specific markers, including cell-surface markers (IDO-1, CD209, NRP-1, CD11c, HLA-DR), transcription factors (IRF4, ARG1), anti-inflammatory cytokines (IL-10, IL-13), pro-inflammatory cytokines (TNF-alpha), chemokine (CCL2) and angiogenic factors (IGFBP-1). These results demonstrate that GdA, together with MCSF, is capable to polarize monocyte differentiation towards dMs. Notice, however, that MCSF- and MCSF+IL-10-polarized macrophages shared a similar transcriptome (Fig. 1A,C).

One important function of dMs is the ability to amend the vascular remodeling activity of trophoblasts (Ning et al., 2016). However, the potential functions of dMs are mainly deduced from gene expression, cell surface phenotyping and cytokine secretion analyses (Gustafsson et al., 2008; Svensson et al., 2011). To demonstrate the physiological relevance of GdA-induced dMs, we studied the biological activities of these macrophages *in vitro*. Our results demonstrated that secretion of MCSF+GdA or MCSF+IL-10+GdA-polarized macrophages towards enhanced angiogenesis, trophoblast invasion and integration into endothelial cell network, compared with macrophages that had not been GdA polarized. These observations support vascular remodeling-promoting activities of dMs (Lash et al., 2016; Ning et al., 2016).

Signaling pathway analysis suggested that several signaling pathways, such as those of NFκB, PI3K–AKT and JAK–STAT, can be involved in GdA-mediated monocyte polarization. Consistently, macrophage differentiation is associated with PI3K–AKT (Busca et al., 2014; Lu et al., 2017) and NFκB signaling activation (Busca

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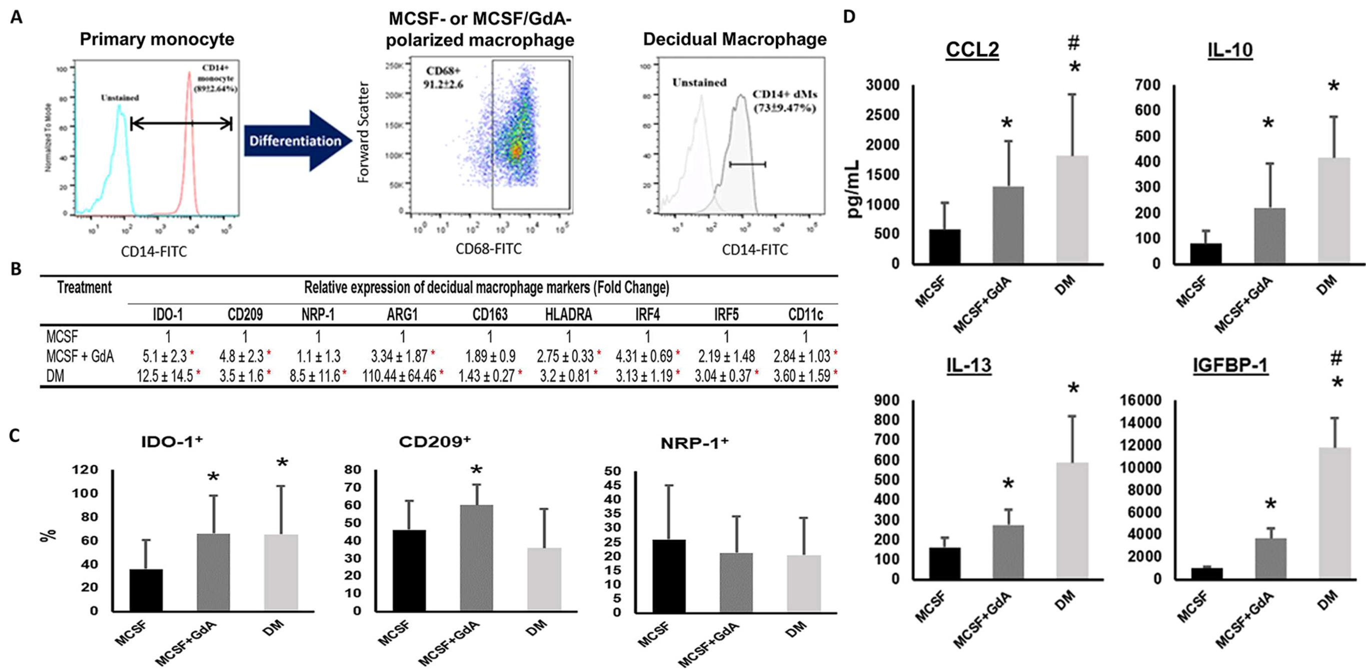

Treatment	Relative expression of decidual macrophage markers (Fold Change)								
	IDO-1	CD209	NRP-1	ARG1	CD163	HLADRA	IRF4	IRF5	CD11c
MCSF	1	1	1	1	1	1	1	1	1
MCSF + GdA	5.1 ± 2.3 *	4.8 ± 2.3 *	1.1 ± 1.3	3.34 ± 1.87 *	1.89 ± 0.9	2.75 ± 0.33 *	4.31 ± 0.69 *	2.19 ± 1.48	2.84 ± 1.03 *
DM	12.5 ± 14.5 *	3.5 ± 1.6 *	8.5 ± 11.6 *	110.44 ± 64.46 *	1.43 ± 0.27 *	3.2 ± 0.81 *	3.13 ± 1.19 *	3.04 ± 0.37 *	3.60 ± 1.59 *

Fig. 4. Comparison of control macrophages, GdA-polarized macrophages and dMs. Isolation of dMs was from first trimester human decidual tissues. Macrophages were prepared by treating human monocytes with 50 ng/ml MCSF for 9 days in the presence or absence of GdA (10 µg/ml). (A) The purity of the differentiated macrophages and dMs was determined by flow cytometry. (B) mRNA expression of dM markers IDO-1, CD209, NRP-1, ARG1, CD163, HLADRA, IRF4, IRF5 and CD11c was determined by qPCR. *n*=5. (C) Protein expression of key the dM markers IDO-1, CD209 and NRP-1 was determined by flow cytometry. *n*=5. (D) Secretion of CCL2, IL-10, IL-13 and IGFBP-1 from macrophages was then determined by ELISA. Data are expressed as mean±s.d.; **P*<0.05 versus control (MCSF-polarized macrophages); #*P*<0.05 versus MCSF+GdA-polarized macrophages. Non-parametric ANOVA on Rank test followed by Mann–Whitney *U*-test.

et al., 2014), and STAT is an upstream regulator of IDO-1 (Cheng et al., 2010). Their involvement in the biological activities of GdA is worth further validation.

Apart from GdA and MCSF/IL-10, a panel of placenta- and decidua-derived factors, including soluble human leukocyte antigen G5 (Lee et al., 2015) and IL-34 (Lindau et al., 2018), are also involved in the differentiation of dMs. Lack of these factors during differentiation might explain the difference in transcriptome and dM-specific gene profiles between the MCSF+GdA/MCSF+IL-10+GdA-polarized macrophages and the dMs. In addition, macrophages are capable of repolarization in response to changes in cytokine levels (Davis et al., 2013), highlighting the complexity and plasticity of macrophage differentiation in response to their microenvironment.

N-linked glycosylation, especially of terminal sialic acids, mediates binding and biological activities of GdA on immune cells (Lee et al., 2011b, 2016). Sialic acid-binding proteins, such as SIGLECs and selectins are expressed on monocytes. We demonstrated here, for the first time, that Siglec-7 serves as a GdA-binding protein on monocytes. This conclusion was drawn from three observations. First, GdA bound to recombinant and native Siglec-7 in co-immunoprecipitation experiments (Fig. 6A). Second, anti-Siglec-7 antibody abolished the binding and biological activities of GdA on monocytes (Fig. 6C-G). Third, desialylated GdA showed reduced affinity towards the recombinant Siglec-7 chimeric protein with an IgG Fc region (Fig. 6B). Moreover, SIGLECs present on monocytes/macrophages are known to perform several important cellular functions (Crocker et al., 2007), such as, attenuation of innate immune responses, and dampening of inflammation (Pillai et al., 2012) and endocytosis (Crocker et al., 2007).

Even though our data suggested that desialylated GdA and anti-Siglec-7-blocking antibody exhibit decreased binding and/or functions when compared with native GdA, other receptors might be involved in GdA binding on monocytes. Our pervious study demonstrated that GdA induces IL-6 secretion by monocytes and/or macrophages via L-selectin in a sialic acid-independent manner (Lee et al., 2012). However, in this study, we found that monocyte differentiation is modulated through a sialic acid-dependent interaction between GdA and Siglec-7. These observations suggest that L-selectin and Siglec-7 are responsible for the different biological activities of GdA, depending on treatment or differentiation condition(s), which is consistent with the phenotypic and functional plasticity of monocytes/macrophages (Guilliams et al., 2018).

Immune maladaptation contributes to inadequate trophoblast invasion and defects in vascular remodeling, leading to insufficient placental development and perfusion (Redman and Sargent, 2005; Young et al., 2010), followed by complications in pregnancy, such as preeclampsia, fetal growth restriction, preterm labor and late spontaneous miscarriage (Burton et al., 2019; Redman and Sargent, 2005; Young et al., 2010). These pregnancy-associated complications are the leading cause of maternal and neonatal morbidity and mortality, and may have long-term health consequences to mother and child (Burton et al., 2019). On the basis of GdA action on differentiation and function of macrophages and other decidual immune cells in early pregnancy (Lee et al., 2011a,b), it is reasonable to associate the deficiency of GdA bioactivity resulting from either abnormal concentration or improper glycosylation – with the pathophysiology of pregnancy-associated complications. Indeed, abnormal serum levels of GdA are associated with unexplained infertility, early pregnancy loss, recurrent miscarriages and preeclampsia (Dundar et al., 2018;

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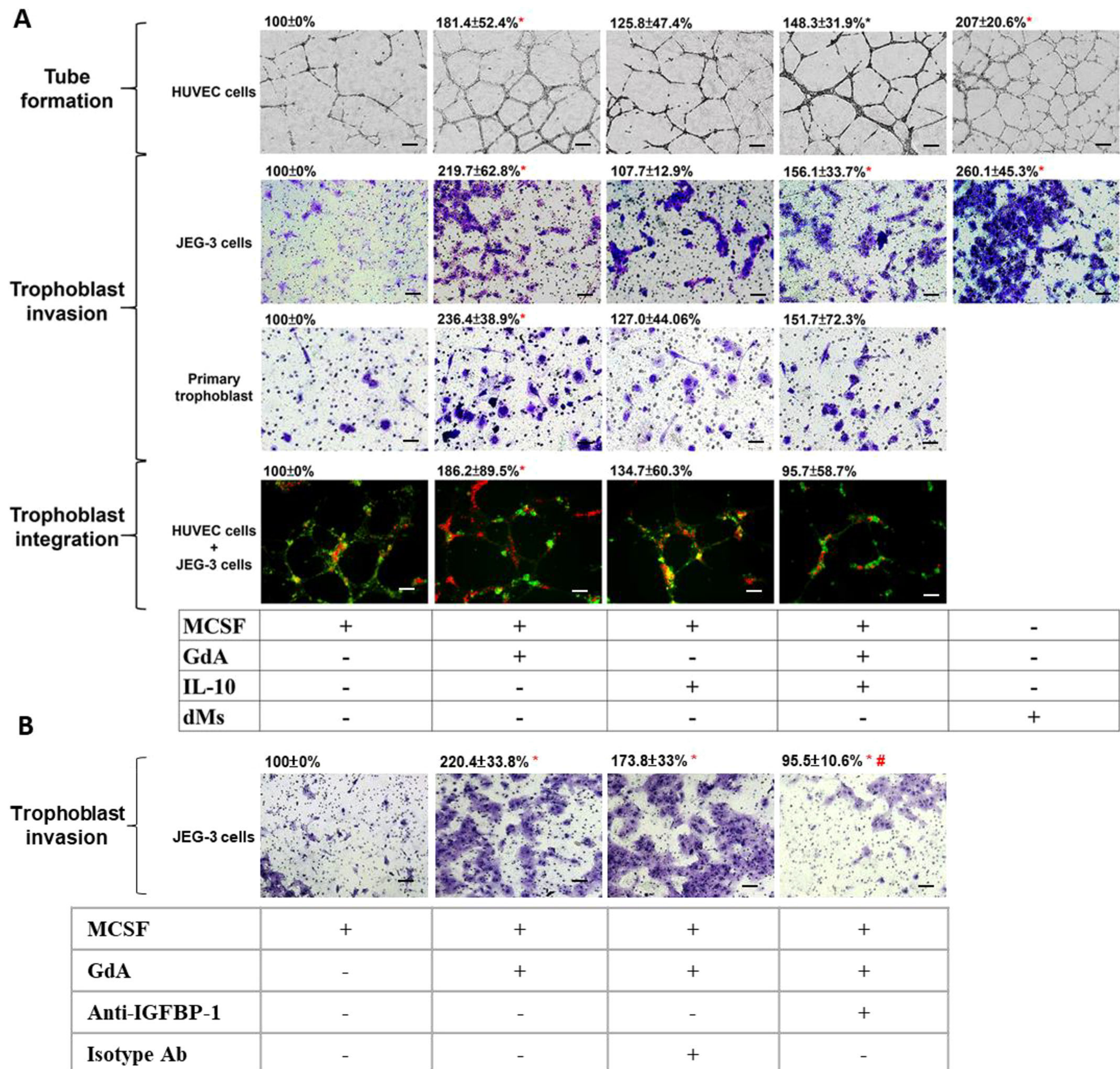

Fig. 5. The secretome of GdA-polarized macrophages induces endothelial cell angiogenesis, trophoblast invasion and trophoblast integration. Macrophages were prepared by treating human monocytes with 50 ng/ml MCSF for 9 days in the presence or absence of IL-10 (5 ng/ml) and/or GdA (10 µg/ml). dMs were isolated from human decidual tissue. Macrophages were cultured in serum-free medium for 24 h and the spent medium was collected. (A) Tube formation assay: HUVECs ($0.5×10^5$ cells/well) formed a tube-like endothelial network in macrophage-spent medium. *n*=5. Trophoblast invasion assay: Trophoblasts in macrophage-spent medium (1:1) with or without isotype, or anti-IGFBP-1 antibody (5 µg/ml) were invaded through a basement membrane for 24 h. *n*=6. Trophoblast integration assay: Red fluorescence-labelled JEG-3 cells with macrophage-spent medium integrated to the tube-like structures containing Green fluorescence-labelled HUVECs. *n*=5. (B) Macrophages were prepared by treating human monocytes with 50 ng/ml MCSF with/without GdA for 9 days in the presence or absence of isotype and anti-IGFBP-1 antibody (5 µg/ml). *n*=5. Scale bars: 100 µm. All the data are expressed as a the ratio (in %) between the treatment groups and control group (MCSF alone) in form of mean±s.d., **P*<0.05 versus control (MCSF-polarized macrophages). #*P*<0.05 versus MCSF+GdA+isotype antibody group. Non-parametric ANOVA on Rank test followed by Mann–Whitney *U*-test.

Koistinen et al., 2009; Lee et al., 2016; Seppälä et al., 2007, 2002), and the trophoblast of preeclampsia patients have abnormal expression level of GdA silaic acid receptor, Siglec-6 (Rumer et al., 2013; Winn et al., 2009).

The main limitation of the present study was that the leukocytes were isolated from non-pregnant women rather than pregnant women, as the decidua of the latter group of women produces other pregnancy-associated factors that mediate dM differentiation. In addition, the hormonal levels of the decidua donors were unknown. Steroid hormones, such as estrogen and progesterone, can regulate macrophage activation and differentiation (Pepe et al., 2017; Routley and Ashcroft, 2009). Therefore, the possible role of

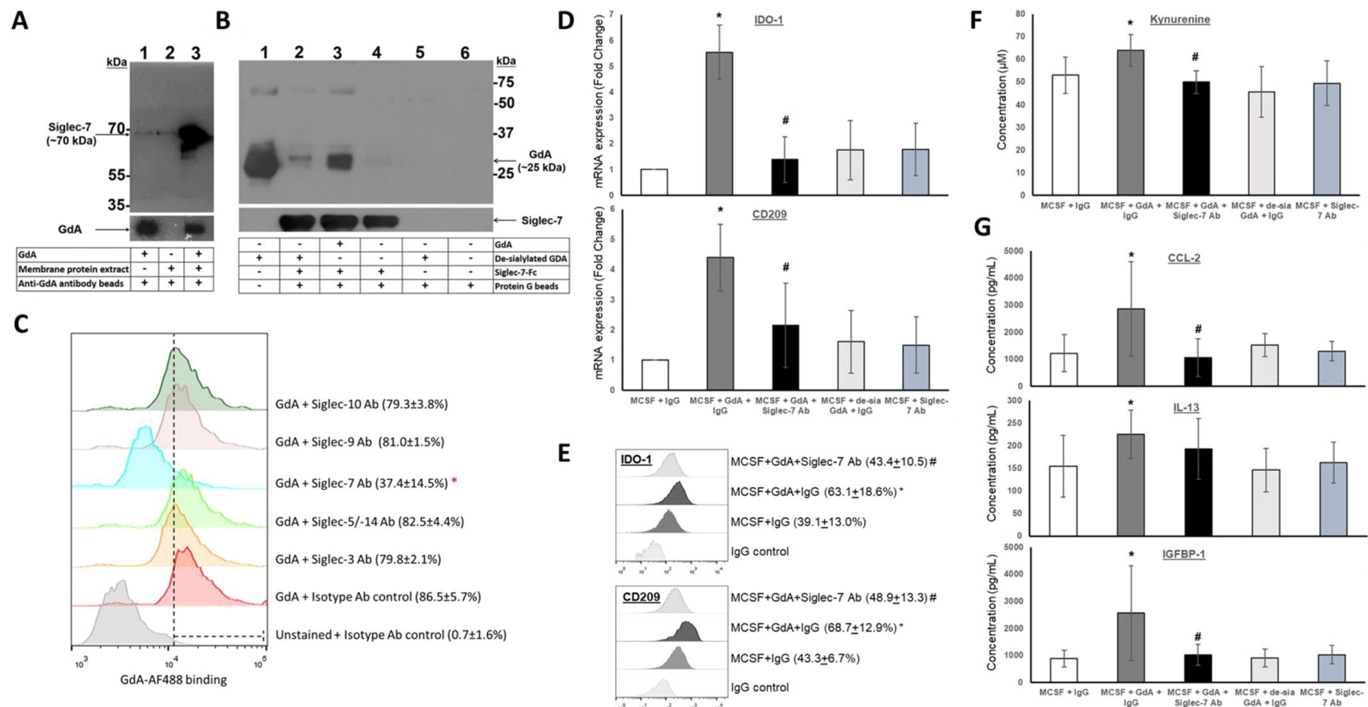

Fig. 6. GdA propagates its functions via Siglec-7 on monocytes. (A) Transmembrane proteins of human monocytes (2×10^{7}) were extracted and incubated with GdA in PBS at 4°C. After overnight incubation with gentle shaking, anti-glycodelin antibody (clone F43-7F9)-conjugated Sepharose beads (GE Healthcare) were used to precipitate the GdA-interacting protein complexes. The captured complex was analyzed by western blotting using anti-Siglec-7 antibody. *n*=5. (B) Immuno-precipitation was also done using recombinant Siglec-7 chimeric protein with an IgG Fc region. De-sialylated GdA was used as control. *n*=5. (C) Monocytes were incubated with fluorescence-conjugated GdA in the presence of anti-Siglec-3, -5/-14, -7, -9, -10 or control isotype antibodies followed by flow cytometric analysis. *n*=5. All the data are expressed as mean±s.d.; **P*<0.05 versus control (GdA+isotype Ab control). (D–G) Macrophages were prepared by treating monocytes with MCSF (50 ng/ml) and GdA (10 µg/ml) for 9 days in the presence of anti-Siglec-7/isotype antibody (5 µg/ml). mRNA (D), protein expression of the key dM markers (E), kynurenine production (F) and cytokine/angiogenic factor production (G) of the differentiated macrophages were then determined by qPCR, flow cytometry, colorimetric assay and ELISA, respectively. *n*=5. All the data are expressed as mean±s.d.; **P*<0.05 versus control (MCSF+IgG). #*P*<0.05 versus MCSF+GdA+IgG group. Non-parametric ANOVA on Rank test was followed by Mann–Whitney *U*-test.

hormones and other pregnancy-associated factors on GdA-induced polarization of monocytes is worth exploring in future studies. Furthermore, the ortholog of glycodelin is found only in certain suborders of higher primates whose females have a menstrual cycle, implying that a mouse model is unsuitable to study GdA functions. Instead, future clinical studies that aim to correlate serum GdA levels with the expression of dM markers in decidua tissue obtained from women going through a normal pregnancy as well as those experiencing one of the above listed pregnancy-associated complications, might help to design GdA-based diagnostic assays for the prediction of these complications.

To conclude, by binding to Siglec-7, GdA induces the differentiation of human monocytes towards macrophages that resemble dMs in terms of transcriptome, cell surface marker expression, production of cytokines, chemokines and/or angiogenic factors, and regulatory functions on placental development. dMs and MCSF+GdA/MCSF+IL-10+GdA-polarized macrophages have characteristics that are distinct from those of MCSF/MCSF+IL-10-polarized macrophages. Aberrant macrophage activity (Mor and Abrahams, 2003; Ning et al., 2016) and reduced GdA expression (Lee et al., 2016) are associated with fetal loss and pregnancy complications. Elucidating the biological functions of GdA, particularly its mechanisms of action on monocytes and macrophages, will further our understanding regarding the pathophysiology of fetal loss and pregnancy complications. To determine expression levels of GdA in the maternal serum might aid to develop a test for early prediction of these complications.

MATERIALS AND METHODS

Purification of GdA

The Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster approved the protocol of this study. Affinity chromatography using anti-glycodelin monoclonal antibody (clone F43-7F9) was used to purify GdA from amniotic fluid as described (Lee et al., 2009; Riittinen et al., 1989). In brief, amniotic fluid samples were diluted at 1:2 with 0.1% Triton X-100 in Tris-buffered saline (TBS pH 7.4). The diluted amniotic fluid was loaded onto an affinity column and washed successively by 15 column volumes of TBS, 1% isopropanol in 1 M NaCl, 0.1% isopropanol in 10 mM ammonium acetate (pH 5) and TBS. GdA was eluted by 0.1% trifluoroacetic acid in 20 mM $CaCl_2$. The purified GdA was concentrated and the buffer was exchanged into PBS by using a10 kDa cut-off centrifugal filter (Millipore, Darmstadt, Germany). Endotoxins were removed from the purified glycoprotein by using spin columns (Thermo Fisher Scientific, Massachusetts, MN). The glycoprotein was confirmed to be glycodelin by western blotting and mass spectrometry (Fig. S1A). Desialylation of GdA was done by incubation with sialidase-coated agarose and confirmed by decreased binding to wheat germ agglutinin lectin as described (Lee et al., 2012).

Primary monocyte/macrophage isolation and macrophage differentiation

Human female peripheral blood was obtained from the Hong Kong Red Cross. Primary monocytes were isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare, Buckinghamshire, UK), followed by negative immunomagnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany). Since IL-10 and MCSF are highly expressed in decidua and promote dM polarization (Chaouat et al., 1999; Svensson et al.,

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2011), macrophages were prepared by treating monocytes with 50 ng/ml MCSF (BioLegend, San Diego, CA) for 9 days in the presence or absence of IL-10 (5 ng/ml, BioLegend) and/or GdA (10 µg/ml). The dosage of GdA used was consistent with the reported decidual GdA level in early gestation (Seppälä et al., 2009). In some experiments, macrophages were washed twice in PBS and cultured for one additional day in serum-free medium to collect the spent medium.

Fourteen first trimester human decidual tissues (and placental tissues, see below), were obtained (with written consent in accordance with the Declaration of Helsinki) from women that had undergone surgical termination of pregnancy because of psychosocial reasons (gestational age at termination of pregnancy: 10.8±1.0 week; maternal age: 36.3±3.3 year; gravidity/parity: 4.4±1.7/2.2±0.8). Tissues were digested with collagenase type V and DNase I. dMs were isolated using Ficoll-Paque density gradient medium (GE Healthcare) and immunomagnetic separation using anti-CD14 beads (Miltenyi Biotec) as described (Duriez et al., 2014; Gustafsson et al., 2008). Purity and phenotypes of the isolated dMs (Fig. 4A) was determined by flow cytometry (Cytoflex, Beckman Coulter, IN). Cells were cultured in 10% FBS-supplemented RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO).

Primary human extravillous trophoblast and trophoblast cell lines

First trimester placental villi were obtained with a written consent from women undergoing surgical termination of pregnancy as stated above. Primary human extravillous trophoblasts (EVTs) were isolated as described (Trundley et al., 2006). Briefly, the placental villi were digested with 0.25% trypsin, filtered through 100-µm and 40-µm filters (BD Bioscience, San Jose, CA), and centrifuged through Ficoll-Paque density gradient medium. The isolated cells were then incubated in a culture dish for 20 min to remove adherent leukocytes. Non-adherent cells were seeded onto fibronectin-coated plates and incubated overnight. The adherent EVTs were collected for experiments. In some experiments, we used cells of the human choriocarcinoma cell line JEG-3 (American Type Culture Collection, Manassas, VA) that expresses surface marker proteins similar to those in EVTs (Apps et al., 2009). The cells were recently authenticated and tested for contamination. The primary trophoblast and the JEG-3 cells were cultured in DMEM/F12 (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich).

RNA isolation and sequencing

Total RNA from macrophages was isolated using the mirVANA PARIS RNA isolation kit (Merck Millipore) and quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). RNA quality was assessed using a Bioanalyzer (Agilent Technologies, Santa Clara, CA). For library preparation, 150–300 ng total RNA per sample was used. cDNA libraries were prepared using the KAPA stranded mRNA-seq kit (Roche, Pleasanton, CA), PCR amplified for ten cycles, and sequenced using the Illumina HI-seq 500 sytem with the HiSeq PE cluster kit V4 and the HiSeq SBS kit v4 at the Centre for PanorOmic Sciences (The University of Hong Kong, HKSAR).

Analysis of macrophage markers

To validate the RNA sequencing results, quantitative PCR (qPCR) analysis and flow cytometric analysis of different macrophages lineages were performed for the following markers proteins: CD68 (pan macrophages); CD80 and CD86 (M1); CD163, CD206 and TREM-2 (M2); and IDO-1, CD209 and NRP-1 (dMs) (Table S1). Subset identification and detailed phenotyping of $CD68^+$ cells were conducted within the macrophage forward/size scatter gate.

Determination of macrophage functions

The cytokines and angiogenic factors of macrophages secreted into the spent medium were determined semi-quantitatively using a membrane-based antibody array (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. The intensity of the cytokine spots was analyzed by densitometry using Quantity One software (Bio-Rad, Hercules, CA). The levels of IL-10, IL-13, CCL2 and IGFBP-1 in the macrophage-derived spent medium were confirmed by ELISA-based assays (IL-10: BD Biosciences; IL-13: eBiosciences; CCL2 and IGFBP-1: Life Technologies, CA) according to the manufacturer's protocol. IDO-1 activity in macrophages was determined by measuring the concentration of kynurenine, the IDO-1 catabolic product, in the macrophage-derived spent medium as described (Lee et al., 2015).

Effects of macrophage-derived secretory factors on trophoblast and endothelial cell functions

Trophoblast invasion was studied by using a transwell invasion assay (Corning, NY) (Lam et al., 2011). In brief, JEG-3 cells or primary EVTs ($5×10^5$) were cultured in serum-free DMEM and serum-free macrophage-spent medium (1:1), and seeded onto a basement membrane for 24 h to analyze their invasive ability. The invading cells on the membrane were stained with 0.1% Crystal Violet dye and quantified by measuring the absorbance at 595 nm after dissolution of the dye from the membrane with 10% acetic acid.

Human umbilical vein endothelial cells (HUVECs; American Type Culture Collection, Manassas, VA) were cultured in DMEM/F-12 (Sigma-Aldrich) supplemented with 10% FBS, 1% endothelial cell growth supplement (Sigma-Aldrich) and 0.1% heparin (Sigma-Aldrich). Tube formation assay was performed by seeding HUVECs ($0.5×10^5$ cells) onto Matrigel in the presence of serum-free macrophage-spent medium (1:1) for 15 h as described (Donovan et al., 2001). Tube formation was quantified by analysis of digitized images using the ImageJ software (National Institutes of Health, USA) and the Angiogenesis Analyzer plugin.

An *in vitro* co-culture model of EVTs and endothelial cells was employed to study the ability of EVTs to integrate into the vascular endothelium (Xu et al., 2011). In brief, HUVECs ($0.5×10^5$) labeled with CellTracker Green CMFDA dye (Thermo Fisher Scientific) were allowed to form tube-like structures for 15 h. Thereafter, JEG-3 cells ($0.5×10^5$) labelled with CellTracker Red CMTPX (Thermo Fisher Scientific) were added together with serum-free macrophage-spent medium and co-cultured with HUVEC tubes for 10 h. EVT integration was then quantified by confocal microscopy and the Image-Pro Plus software (Media Cybernetics, Rockville Pike, MD).

Interaction between GdA and Siglec-7 on monocytes

Transmembrane proteins of $2×10^7$ monocytes were extracted by the ProteoExtract transmembrane protein extraction kit (Novagen, Sacramento, CA) according to the manufacturer's instructions. The extracted membrane protein fractions were incubated with native or desialylated GdA in PBS at 4°C overnight. Anti-glycodelin antibody (clone F43-7F9)-conjugated Sepharose beads (GE Healthcare) were used to precipitate the GdA-interacting protein complexes. The captured complex was washed with PBS, resolved in 12% SDS-PAGE, and analyzed by western blotting using anti-glycodelin (Abcam, Cambridge, MA) or anti-Siglec-7 (MAB1138, R&D Systems) antibodies. Co-immunoprecipitation was performed as described (Lam et al., 2011) using recombinant Siglec-7 chimeric protein with an IgG Fc region (1138-SL, R&D Systems).

GdA were labeled with AF488 fluorescence dye as described (Thermo Fisher Scientific) as described (Lee et al., 2019b). Monocytes ($0.5×10^6$) were fixed with 4% paraformaldehyde solution in PBS for 5 min. Then the cells were incubated with 1 µg/ml labeled GdA/desialylated GdA and FC blocking reagent (Miltenyi Biotec) with or without antibodies against Siglec-3, -5/-14, -7, -9 and -10 (Table S1) for 1.5 h. The cells were then washed with PBS twice and analyzed by flow cytometry. We studied the effects of GdA, desialylated GdA and GdA with or without anti-Siglec-7 blocking antibody (AF1138, R&D Systems) (Varchetta et al., 2012) or of control isotype antibody on macrophage differentiation and functions.

Statistical and bioinformatic analyses

Data are expressed as mean±standard deviation (±s.d.), unless stated otherwise. All results were analyzed using the Kolmogrov–Smirnov normality test. Non-parametric ANOVA on Rank test was used to test the statistical differences between groups, followed by either non-parametric Mann–Whitney *U*-test or parametric *t*-test as the post-test, using the Graph Pad Prism software (Graph pad software Inc., San Diego, CA).

For transcriptomic data, statistical analysis was performed in the R version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria). Raw counts were normalized using the TMM (EdgeR, R version 3.4.0) (Robinson et al., 2010) for genes with one count per million (cpm) in at least one sample. Log transformation was applied on the normalized counts by using the Voom function (limma package, R version 3.18.2) (Law et al., 2014). Differential expression was computed with limma, and the moderated *t*-test was used for each comparison. False discovery rate (FDR)-adjusted *P*-values were calculated with the Benjamini–Hochberg method. We considered transcripts as differentially expressed when the adjusted *P*-value was <0.01 and the fold-change difference between groups was ≥ 2. Principle component analysis (PCA) and heatmap analysis with hierarchical clustering were performed using the BioVinci software (BioTuring Inc., San Diego, CA). Hierarchical clustering was done using the Euclidean distance and the Ward's criterion (Zhang et al., 2017). Soft clustering based on fuzzy c-means was conducted using the software package Mfuzz (developed by the Institute of Medical Informatics and Biometry, Charité, Humboldt-University, Berlin, Germany (Kumar and Futschik, 2007)). Differentially expressed genes under experimental conditions with and without GdA were subjected to functional enrichment analyses – KEGG signaling pathway analysis and GSEA to illustrate their functional characteristics and mechanisms, respectively – by using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool with cut-off values of adjusted *P*-values <0.05.

Acknowledgements
The authors thank the Hong Kong Red Cross Blood Transfusion Service for their help in obtaining the buffy coat samples, and staff of the Faculty Core Facility, LKS Faculty of Medicine, the University of Hong Kong for their technical support.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.-L.L., H.K., M.S., K.-F.L., W.S.B.Y.; Methodology: C.-L.L., V.H.H.W., X.W., K.B., J.W., H.K.; Software: J.W.; Formal analysis: M.V., C.-L.L., H.K.; Investigation: C.-L.L., V.H.H.W., X.W., K.B., P.C.N.C.; Resources: C.-L.L.; Data curation: M.V., C.-L.L., V.H.H.W., X.W., K.B., J.W.; Writing - original draft: M.V., C.-L.L.; Writing - review & editing: C.-L.L., H.K., M.S., K.-F.L., W.S.Y., E.H.Y.N., P.C.N.C.; Supervision: C.-L.L., M.S., K.-F.L., W.S.B.Y., E.H.Y.N., P.C.N.C.; Project administration: C.-L.L., M.S., K.-F.L., W.S.B.Y., E.H.Y.N., P.C.N.C.; Funding acquisition: C.-L.L., E.H.Y.N., P.C.N.C.

Funding
This work was supported in part by the Hong Kong Research Grant Council Grant (grant numbers: 17115619 and 17118917), Sanming Project of Medicine in Shenzhen, Syöpäjärjestöt (Finnish Cancer Foundation), Sigrid Juséliuksen Säätiö (Sigrid Jusélius Foundation) and the Finnish Society of Clinical Chemistry.

Data availability
RNA-seq data supporting the findings of this study have been deposited with the Gene Expression Omnibus (GEO) under accession codes GSE153606.

Supplementary information
Supplementary information available online at
https://jcs.biologists.org/lookup/doi/10.1242/jcs.244400.supplemental

Peer review history
The peer review history is available online at https://jcs.biologists.org/lookup/doi/10.1242/jcs.244400.reviewer-comments.pdf

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