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Early transient suppression of immune checkpoint proteins T-cell immunoglobulin mucin-3 and programmed cell death-1 in peripheral blood lymphocytes after blastocyst transfer is associated with successful implantation

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Running title: Tim-3 and PD-1 during peri-implantation

Early transient suppression of immune checkpoint proteins

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2 **Capsule:**

3 Successful implantation is associated with a transient reduction of Tim-3 and

4 PD-1 expression in peripheral lymphocytes on days 3 and 6, but not on day 9,

5 after ET

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ABSTRACT

Objective: To compare the changing peripheral levels of immune checkpoint proteins Tim-3/Galectin-9 (Gal-9) and PD-1/PL-1 over a nine-day period after blastocyst transfer between women who did and did not conceive.

Design: Prospective observational study.

Setting: A university teaching hospital.

Patients(s): 51 infertile women who were undergoing day-5 blastocyst transfer.

Intervention(s): Serial blood samples were obtained on the day of embryo transfer (ET), and 3, 6 and 9 days afterwards for measurement of (1) membranous Tim-3 and PD-1 expression on various peripheral lymphocytes by flow cytometry; and (2) serum concentrations of ligands Gal-9 and PD-L1 by ELISA.

Main Outcome Measure(s): Membranous Tim-3 and PD-1 expression on lymphocytes and serum Gal-9 and PD-L1 concentrations and comparison of results between pregnant and non-pregnant women.

Result(s): In women who conceived, the measurements exhibited three different types of response: (1) a transient and significant reduction of Tim-3$^+$NK-like T cells, Tim-3$^+$/PD-1$^+$CD8$^+$ T cells and Tim-3$^+$/PD-1$^+$CD4$^+$ T cells which returned <u>back to baseline</u> level 9 days after ET; (2) a reduction followed by steady increase to <u>above baseline</u> level on day 9 (Tim-3$^+$CD56^{dim}NK cells); (3) a steady increase in expression after ET to reach a level significantly higher than that of the baseline by day 9 (Tim-3$^+$CD56^{bright}NK cells). Women who did not conceive showed no significant fluctuation in all the parameters measured across the 4 time points.

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2 Conclusion(s): Successful blastocyst implantation is associated with a
3 reduction of Tim-3 and PD-1 expression in peripheral lymphocytes on days 3
4 and 6, which is no longer apparent on day 9.

5 Key Words: implantation, immune checkpoint, Tim-3, PD-1, natural killer (NK)
6 cells

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INTRODUCTION

Immunological adaptation is a pre-requisite for endometrial preparation which is necessary for successful implantation (1). The uterus becomes receptive during the mid-secretory phase (days 19-23) of the menstrual cycle, commonly known as the window of implantation (WOI). There are considerable changes in endometrial immune cell numbers during WOI, especially the uterine natural killer (uNK) cells, which increase from 40% of the total leukocyte population during the proliferative phase to 60% during WOI and 70% during early pregnancy (2). The exact role of uNK cells is unknown but they may play a role in angiogenesis and decidualization of stromal cells to create a unique immunological microenvironment suitable for the attachment and invasion of embryo expressing paternal antigens (3, 4).

However, a limitation of immune cell studies at the maternal fetal interface is that obtaining suitable tissue is invasive and cannot be repeated at regular intervals to observe the dynamic changes which occur as the implantation process progresses. In contrast, peripheral immune parameters may be measured at repeated regular intervals to examine how peripheral immune responses change during the implantation process. Recently, it has been demonstrated that both peripheral and decidual NK cell cytotoxicity is inhibited by T-cell immunoglobulin mucin-3 (Tim-3) which has higher expression in pNK cells in the first trimester of pregnancy (5, 6). Tim-3 has been shown to be an immune checkpoint marker of immune suppression, similar to programmed death-1 (PD-1), the well-known target for immunotherapy in cancer (7). Previous studies have demonstrated that together with Tim-3, PD-1 modulates decidual CD4^{+}T and CD8^{+}T cells to produce Th2 type cytokines which may

lead to maternal-fetal immune tolerance after implantation (8). However, it is uncertain to what extent PD-1 and Tim-3 are involved in immunological adaptation at the very early stages of implantation.

The aim of this study was to compare the serial changes, in the expression of Tim-3 and PD-1 in peripheral NK, T and NK-like T cells, and the serum levels of their soluble ligands Galectin-9 (Gal-9) and programmed death -ligand 1 (PD-L1), in the nine days after embryo transfer, between women who did and did not conceive.

MATERIALS AND METHODS

Subjects

Women undergoing embryo transfer after in-vitro-fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment at the Assisted Reproductive Technology Unit, Prince of Wales Hospital, Chinese University of Hong Kong between November 2018 and August 2019 were recruited into the study.

The inclusion criteria were women (1) having one or two day 5 blastocysts transferred in fresh or frozen cycles; (2) maternal age 20-40 years old. The exclusion criteria include: (1) hydrosalpinx; (2) structural uterine abnormalities; (3) antiphospholipid syndrome; (3) abnormal thyroid function; (4) polycystic ovarian syndrome; (5) hormonal or metabolic disorders; (6) known clinical autoimmune disease; (7) undergoing immunotherapy; (8) cancer or post-cancer status; (9) significant medical complications.

Patients recruitment

All subjects included in this study were recruited on the day of ET following informed, written consent. They had blood samples taken on the day of ET,

and 3, 6, 9 days afterwards. This study has been approved by our Institutional Review Board (CREC Ref: 2014.637).

IVF treatment

Ovarian stimulation was initiated by human menopausal gonadotrophins (HMG) (Pergonal, Serono, Switzerland) or recombinant follicle stimulating hormone (rFSH) (Gonad-F, Serono, Switzerland). The ovulation trigger used was 10,000 units HCG (Profasi, Serono, Switzerland) administered intramuscularly when there were three or more leading follicles reaching 16 mm or more in diameter on transvaginal ultrasound. Transvaginal oocyte retrieval was performed 36 h after hCG trigger. Luteal support was commenced in the evening of oocyte retrieval in the form of vaginal progesterone, either a 90 mg daily dose of Crinone (Merck, Germany) or Endometrin (Ferring, Saint-Prex, Switzerland) 100 mg TDS. Blastocysts were transferred on day 5.

In the cycle of frozen-thawed embryo transfer, natural cycle and hormonal replacement treatment cycle were monitored with endometrial thickness, ovarian activity and hormonal levels, as previously described (9). Blastocysts were thawed and transferred five days following the estimated day of ovulation or progesterone administration respectively.

Culture system

Cumulus cells were removed from oocytes with hyaluronidase (Vitrolife, Goteborg, Sweden). After denudation, all mature oocytes were incubated in 60 µl G-IVF medium for at least an hour in culture, and then fertilized by conventional IVF or inseminated by ICSI. Fertilized oocytes were cultured in G-1 medium (Vitrolife, Goteborg, Sweden) supplemented with 10% serum

substitute supplement, (SSS (Irvine Scientific, Santa Ana, USA)). On the morning of day 3 development, each embryo was cleaned and rinsed with G1 medium using a 170-μm pipette (Flexipets, Cook Medical, Bloomington, USA). Each embryo was individually cultured in G-2 medium (Vitrolife, Goteborg, Sweden) supplemented with 10% SSS until blastocyst stage. All embryos in this study were cultured using sequential culture media in 30-μl microdrops under oil, with incubation conditions of 37.0 °C and 6% CO_2/5% O_2 balanced in N2 as previously described by our group(10).

Embryo cryopreservation

All blastocysts included in the FET cycles of this study were cryopreserved by vitrification on day 5, using a commercial vitrification kit (Vit Kit® Freeze, 90133-SO, Irvine Scientific, Santa Ana, CA, USA) and commercial warming kit (Vit Kit® Thaw, 90137-SO, Irvine Scientific), following the manufacturer's instructions.

Confirmation of pregnancy

All subjects included in this study had serum β-hCG measurement on the blood samples obtained 9 days after ET, according to our routine clinical practice to verify if pregnancy has occurred. Women who had serum β-hCG (<10 mIU/L) were included into the non-conception group. Women who had serum β-hCG (>10 mIU/L) were offered ultrasonography 23 days and 30 days after ET to confirm viability and location of the pregnancy; those who had ongoing intrauterine pregnancy beyond 12 weeks of gestation were included into the conception group. For the purpose of this study, women with biochemical pregnancy loss and miscarriage were excluded because the main aim of this study was to examine the changes of peripheral Tim-3 and PD-1

1 levels in subjects with normal pregnancy as compared to subjects who did not

2 conceive. Biochemical pregnancy was defined as one in which the serum level

3 of β-hCG>10 mIU/L on 9th day after embryo transfer, but no ultrasound

4 evidence of intrauterine pregnancy at 6 weeks of gestation. Miscarriage was

5 defined as one in which there was ultrasound evidence of intrauterine

6 pregnancy but the pregnancy did not progress to beyond 12 weeks of

7 gestation.

8 **Blood sampling**

9 Peripheral blood was collected for serum and lymphocyte isolation. 3 ml whole

10 blood sample was obtained and placed into a tube with serum separator clot

11 activator and EDTA coated tube respectively on the day of blastocyst transfer,

12 and repeated 3, 6 and 9 days after, abbreviated as D0, D3, D6 and D9. Serum

13 was separated after centrifuged at 3000 rpm. at 4℃ for 30 min. and stored at

14 -80℃ for later analysis.

15 **Laboratory measurements**

16   **1. Peripheral blood mononuclear cell (PBMC) separation**

17 Peripheral blood was diluted 1:1 with 0.9% saline and lymphocytes were

18 isolated by Lymphopure Density Gradient Medium (Biolegend, USA) with

19 centrifuged at 800g for 30 min. After centrifugation, PBMCs formed a defined

20 cell layer at the plasma Lymphopure interface. The layer of PBMCs were

21 collected and washed in 1% BSA/PBS. The cells were centrifuged,

22 resuspended and counted for flow cytometry.

23   **2. Labeling of PBMCs and flow cytometric analysis**

24 1×10^{6} PBMCs were resuspended in 100 µl 1% BSA/PBS per tube and

1   incubated with anti-human CD56-BV605, CD3-FITC, CD4-APC/Cy7,

2   CD8-PE/Cy7 and PD-1-PE or Tim-3-PE purchased from BioLegend (San

3   Diego, CA, USA) for 30 min. in the dark at 4℃. In parallel, each sample was

4   incubated with matched isotype control antibodies. Then cells were washed

5   twice with cold 1% BSA/PBS. The expression of Tim-3 and PD-1 on

6    $CD3^+CD4^+/CD8^+$T cells, NK-like T ($CD3^+CD56^+$) cells, and $CD3^-CD56^{dim/bright}$

7   NK cells were assayed by FACS (BD LSRFortessa, USA) and analyzed by

8   FlowJo version 10 software (Tree Star). A minimum of 100 000 events per

9   sample were collected for phenotypic analysis. As regards flow cytometry

10   gating strategy, total lymphocytes were first gated on a forward scatter (FSC)

11   and side scatter (SSC) and then gated on the $CD56^{bright/dim}CD3^-$ population as

12   NK cells, $CD56^+CD3^+$ population as NK-like T cells, $CD56^-CD3^+$ population as

13   T cells which was further gated on the $CD8^+$ or $CD4^+$ population as $CD8^+$ T

14   cells and $CD4^+$ T cells. These cells were further gated on Tim-3 and PD-1 for

15   the subsets of interest, namely, $Tim3^+$NK cells, $Tim-3^+$NK-like T cells, $Tim-3^+$

16   $CD8^+$ T cells, $Tim-3^+CD4^+$ T cells, $PD-1^+$ NK-like T cells, $PD-1^+CD8^+$ T cells

17   and $PD-1^+CD4^+$ T cells.

18   3. **Quantification of PD-L1, Gal-9 in serum**

19   The levels of soluble ligands PD-L1 and Gal-9 in serum collected at the same

20   time points were measured by commercial ELISA kits (R and D Systems,

21   Minneapolis, USA) according to the manufacturer's protocols.

22   **Statistical analysis**

23   The observed immune parameters were expressed as mean±standard error of

24   the mean (SEM). Paired sample t tests or Wilcoxon signed-rank tests were

25   used to compare the change in percentage of lymphocyte subsets and Tim-3,

PD-1 levels in lymphocyte subsets at different time-points. The comparison of results between the pregnant and nonpregnant groups was made by using independent sample *t* tests for parametric data or Mann-Whitney *U* test for nonparametric data. The difference in qualitative data was compared by chi-square test. P-values < 0.05 were considered statistically significant in all statistical tests. The statistical analyses were performed using SPSS (Version 22; SPSS Inc., New York, NK, USA).

RESULTS

Demographics

From November 2018 to August 2019, 60 subjects were recruited. Nine subjects were excluded because of biochemical pregnancy loss, miscarriage and ectopic pregnancy. In total, 51 subjects were included in the study, with 33 patients in conception group (all singleton) and 18 in non-conception group. The demographic details of these two groups are compared in table 1. The mean (±SD) age of women was 34.9 (±3.2) in the conception group and 37.3 (±1.8) years (P<0.05) in the non-conception group. Otherwise, these was no difference in baseline FSH, body mass index, duration of infertility, type of infertility, endometrial thickness on the day of hCG trigger and the number of blastocysts transferred between the two groups.

Phenotype analysis of peripheral lymphocytes

We investigated the relative proportion of NK cells (CD3$^-$CD56$^+$), T cells (CD3$^+$CD56$^-$) and NK-like T cells (CD3$^+$CD56$^+$), and the proportion of two NK cell subtypes (CD56^{dim}NK cells and CD56^{bright} NK cells) and two T cell subsets (CD4$^+$ and CD8$^+$ T cells) in peripheral blood lymphocytes on the day of blastocyst transfer, and three, six and nine days after blastocyst transfer in

women who did and did not conceive. The proportions of lymphocyte subsets did not alter at any of the time points and there was no significant difference in the results between women who did or did not conceive for all the time points. (Table 2).

Levels of Tim-3 expression after ET

The changing levels of Tim-3 expression in women who conceived are shown in Table 2. On the day of ET, Tim-3 was expressed in 34.82 % of pNK cell ($CD56^{dim}$NK cells (35.91%) and $CD56^{bright}$NK cells (25.58%)), 3.37% of T cells ($CD8^+$T cells (4.14%) and $CD4^+$ T cells (2.91%)) and 3.96% of NK-like T cells. On day 3 after ET, a significant ($p<0.05$) drop in Tim-3 expression was observed in 4 out of 5 lymphocyte subsets ($CD56^{dim}$NK cells, $CD8^+$T cells, $CD4^+$T cells and NK-like T cells). By day 6 after ET, there was a noticeable reversal of the earlier trend, resulting in a minor increase of Tim-3 expression in these 4 lymphocyte subsets, although at this stage the expression level was still significantly different to the baseline (ET). By day 9 after ET, the increased expression of Tim-3 had continued in all cell types, with Tim-3 expression being significantly higher than the baseline in $CD56^{dim}$NK cells and $CD56^{brihgt}$NK cells.

The changing levels of Tim-3 in women who did not conceive across the four time points are also summarized in table 2. There was no significant change in Tim-3 expression among all 5 lymphocyte subsets on all the time points examined with the exception of $CD56^{dim}$NK and $CD56^{bright}$ NK cells on day 9 after ET, which were significantly (42.17 ± 3.24, 28.11 ± 3.20) higher than the baseline level (34.49 ± 1.97, 22.02 ± 1.67).

Comparison of Tim-3 expression between women who did and did not conceive

On the day of ET, the percentage of Tim-3$^+$pNK cells, Tim-3$^+$T cells and Tim-3$^+$NK-like T cells were not significantly different between women who did and did not conceive. On day 3 after ET, Tim-3 expression in four of the 5 lymphocytes subsets (CD56^{dim}NK cells, CD8$^+$T cells, CD4$^+$T cells and NK-like T cells) was significantly lower in women who conceived compared to women who did not. There was no difference in CD56^{bright}NK cells. On day 6, a significant decrease in Tim-3 positive cell numbers was seen in CD56^{dim}NK cells, CD4$^+$T cells and NK-like T cells, with no difference in CD8$^+$T cells and CD56^{bright}NK cells. On day 9, there was no significant difference in Tim-3 expression in all lymphocyte subsets (Figure 2A-E).

Levels of PD-1 expression after ET

The levels of PD-1 expression in women who conceived after ET are shown in Table 2. On the day of ET, PD-1 was not expressed in either type of pNK cells, but in 26.83% of T cells and 31.11% of NK-like T cells. There were similar proportions of cells expressing PD-1 in the 2 T-cell subtypes: 26.53% of CD8$^+$T cells and 26.88% of CD4$^+$T cells. On day 3 after ET, a significant (p<0.05) drop in PD-1 expression was observed only in CD4$^+$T cells. By day 6 after ET, PD-1 expression was significantly lower in both CD4$^+$T cells and CD8$^+$T cells. By day 9 after ET, there was a reversal of the decreasing trend, resulting in an increase of PD-1 expression in both CD4$^+$T cells and CD8$^+$T cells to the baseline level. The levels of PD-1 expression in NK-like T cells were not significantly different at any time point.

In the non-pregnant group, there were no significant changes in the percentage of PD-1$^+$T cells and PD-1$^+$ NK-like T cells at any of the time points examined.

14

Comparison of PD-1 expression between women who did and did not conceive

The percentages of PD-1$^+$T cells and PD-1$^+$NK-like T cells were not significantly different between women who did and did not conceive at all time points after ET (Figure 2F-H).

Concentration of serum Gal-9 and PD-L1

Figure 2I and 2G show the levels of Gal-9 and PD-L1 in serum of pregnant and non-pregnant women on days 0-9 after ET. There was no difference in levels of either ligand in women who conceived and those who did not.

DISCUSSION

Main findings

In the present study, we found that there is a transient and significant reduction of Tim-3 and PD-1 expression on the cell surface of peripheral lymphocytes within 9 days after blastocyst transfer. The findings suggest that the Tim-3 and PD-1 may be useful immunological biomarkers of successful embryo implantation. In addition, the study results may also help to elucidate immunological mechanisms that facilitate implantation.

Proportion of peripheral NK and T cells after ET

We found that the relative proportion of subsets of peripheral NK and T cells were not changed after ET, regardless of whether conception occurred or not. Earlier studies in the first trimester of pregnancy also showed that the proportion of peripheral NK subsets prior to conception is similar to those in the first trimester of pregnancy (11). Our observation further suggested that there

1 is no alteration in numbers of pNK cell and T cell after ET. Taken together, it

2 suggests that the process of implantation does not significantly alter the

3 proportion of lymphocyte subsets in peripheral blood.

4 **Changing levels of Tim-3 after ET**

5 We observed a significant but transient decrease in Tim-3 expression in

6 CD56^{dim}NK cells, T cells and NK-like T cells in the nine days after ET.

7 Measurement beyond day 9 after ET were not carried out in our study, but

8 earlier reports suggested that the level of expression of Tim-3 in the peripheral

9 NK cells remained elevated in the first trimester of pregnancy (6). In addition,

10 Tim-3 was strongly expressed in decidual NK and T cells (5, 8). Tim-3 was

11 originally identified as a surface marker for interferon-γ (IFN-γ)-producing

12 CD4$^+$ T helper 1 (Th1) specific cell and CD8$^+$ T cytotoxic (Tc) T cell whose role

13 is to suppress inflammatory responses and to facilitate the development of

14 immunological tolerance through Gal-9 engagement (12, 13). Later studies

15 have shown that Tim-3 is expressed on other innate immune cells, such as NK

16 cells, monocyte-macrophages, and dendritic cells and may regulate their

17 differentiation and functions (14). The abundance of Tim-3 at the site of

18 embryo implantation has led to the hypothesis that the anti-inflammatory

19 environment mediated by Tim-3 is a pre-requisite for the establishment of a

20 successful pregnancy.

21 The unexpected finding in our study is a significant and transient reduction

22 in Tim-3 expression after ET. The exact biological mechanism for such a

23 biphasic response is not clear, but supports the hypothesis that the initial

24 phase of embryo implantation is associated with a pro-inflammatory response

25 (1, 15). The implantation site is characterized by the presence of inflammatory

26 cytokines interleukin-6 (IL-6), IL-8, IL-15, granulocyte-macrophage

colony-stimulating factor (GM-CSF), CXC chemokine ligand 1(CXCL1), CC-chemokine ligand 4 (CCL4), osteopontin and tumor necrosis factor (TNF) which are derived from both endometrial stromal cells and infiltrated immune cells (15). Precisely coordinated interplay between immune cells, cytokines and hormones is crucial for the tissue remodeling required for successful embryo implantation (16). The transient reduction of Tim-3 may indicate that in peripheral blood the pro-inflammatory response occurs after ET followed by anti-inflammatory response associated with the increase in Tim-3.

Changing levels of PD-1 after ET

PD-1 expression was not seen in NK cell, but was expressed by T cells and NK-like T cells. The changing pattern of PD-1 expression after ET in T-cell and NK like T cells was similar to that of Tim-3, but with subtle differences. There was a reduction in expression among all three lymphocytes subsets 3-days after ET, but the reduction was significant in only CD4$^+$T cells. On day 6 after ET there was a significant reduction of PD-1 expressed in both CD4$^+$T cells and CD8$^+$T cells. By day 9 after ET, the expression level had returned back to baseline level in all three subsets. PD-1 in decidual T cells plays a critical role in the maintenance of maternal tolerance by interacting with PD-L1 expressed on stromal cells (17), trophoblast cells (18), and antigen presenting cells (19). Moreover, PD-1 is involved in the T-cell homeostasis, namely the Th1/Th2/Th17/Tregs immune balance at the maternal fetal interface (20, 21). Given that both Tim-3 and PD-1 are recognized to be anti-inflammatory mediators (13, 22); the similar changing pattern after ET supports the hypothesis that a pro-inflammatory response may be a pre-requisite of early embryo implantation. However, whether this is also reflected at the fetal endometrial interface is unknown.

Tim-3 and PD-1 results in women who did not conceive

In contrast to women who conceived, the expression of Tim-3 and PD-1 in various lymphocyte subsets in women who did not conceive did not show any significant changes 3, 6 and 9 days after ET, with the exception of increased Tim-3 expression on CD56^{dim}NK cells 9 days after ET. This observed increase in CD56^{dim}NK cells may be a consequence of progesterone therapy used for luteal support, as it has been reported that physiological concentrations of progesterone induces Tim-3 expression on pNK cells via the IL-4-STAT6 pathway, either directly or indirectly (6).

There was no difference in expression of PD-1 between subjects who did and did not conceive in all lymphocyte subsets on all the days examined. The observation suggests the change in the expression of Tim-3 associated with successful implantation is more pronounced than that of PD1.

Levels of ligands (Gal-9 & PD-L1)

In contrast to the changes in immune inhibitory receptors Tim-3 and PD-1 expression in lymphocyte subsets, there were no observable changes in the serum concentration of their respective ligands Gal-9 and PD-L1 3, 6 and 9 days after ET, and no difference in the levels between women who did or did not conceive. The findings suggest that the _initial phase_ of immune modulation during pregnancy is primarily, if not entirely, mediated via immune inhibitory receptors but not their ligands. This is in contrast to the finding of an earlier study which showed that maternal blood level of Gal-9 and PD-L1 were significantly elevated after confirmation of pregnancy (equivalent to nine days after ET), which might be caused by their secretion from trophoblasts (23). The later observation represents the second phase of immunomodulation in pregnancy. Gal-9 and PD-L1 are expressed by both haemopoietic and

1 non-haemopoietic cells (24, 25). The source of these ligands during pregnancy

2 might be primarily from trophoblasts since high level of both proteins are

3 expressed in the placenta (23). Based on animal studies, the function of these

4 two ligands are proposed to mediate maternal immune tolerance though

5 binding to immune cells expressing Tim-3 or PD-1(8). Prospectively planned

6 clinical studies are required to confirm their roles in humans.

7 **Strength of current study**

8 A particular strength of this study is that serial measurements were obtained at

9 several precisely timed points after ET in a population undergoing the same

10 treatment. Many earlier studies on immune-modulation of pregnancy involved

11 a single time point (26, 27), not as precisely timed, usually in terms of

12 gestational weeks rather than days (23, 28). In addition, the observations in

13 this study were made in the very early stage of embryo implantation, well

14 before it is known whether or not the implantation is successful, which enabled

15 us to study the initial, transient phase of immunomodulation of pregnancy.

16 Thirdly, we have included in our study two cohorts, one cohort with successful

17 implantation leading to ongoing pregnancy and the other cohort with failure of

18 implantation leading to non-conception. The latter cohort serves as a

19 comparison group with which the observations in the conception cohort could

20 be meaningfully compared. Finally, we have also obtained baseline

21 measurement prior to the replacement of embryos in both cohorts, with which

22 any changes following ET could be compared and quantified; moreover, the

23 observation that the relevant baseline immunological parameters are the same

24 between those with success or failure of implantation suggest that these

25 parameters are unlikely to be confounding factors.

26 **Limitations of current study**

1   Whilst we have found that successful embryo implantation is associated with a,

2   transient and significant response in the maternal peripheral blood immune

3   system, it is not possible to conclude in this study if the lack of a normal

4   maternal immune response is the cause or consequence of the implantation

5   failure; further well-designed prospective studies are required. A further

6   limitation of the study is that the measurements in peripheral blood may not

7   reflect what is happening at the site of implantation. Other limitations of this

8   study include the relatively small sample size, which did not enable as to

9   analyze the possible impact of various confounding variables such as patient

10   population (different underlying causes of infertility), the type of embryo

11   transfer cycle (fresh or frozen), the methods of endometrium preparation

12   (natural or artificial) and embryo quality.

13   **Clinical implication**

14   The significant and transient reduction of Tim-3 and PD-1 was detected on day

15   3 after ET, but the changes reverted back to normal or above baseline level by

16   the 9th day after ET. It implies that in order to ascertain if there is an underlying

17   immune cause of implantation failure, serum markers should be measured as

18   soon as an embryo is replaced; doing the measurement after the pregnancy

19   test result is known (9 days after ET) is already too late. The monitoring of any

20   proposed immune therapy to improve embryo implantation should also

21   commence prior to confirmation of a pregnancy test result.

22   **CONCLUSION**

23   To conclude, we have found that successful embryo implantation is

24   characterized by a biphasic immunological response in peripheral blood

25   lymphocytes, with a transient and significant reduction in Tim-3 and PD-1

26   expression, signifying a pro-inflammatory response 3-6 days after ET, followed

1 by a rebound increase in Tim-3 expression consistent with the beginning of a

2 second phase of anti-inflammatory response.

3 **Conflict of interest**:

4 The authors declare no financial or commercial conflict of interest.

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4

5   **Figure Legends**

6   Figure 1. Differential Tim-3 and PD-1 expression in pNK, T and NK-like T cells.

7   (A-C) Gating strategy of peripheral NK cells (CD3$^-$CD56$^+$), T cells (CD3$^+$CD56$^-$),

8   NK-like T cells (CD3$^+$CD56$^+$), CD8$^+$T cells (CD3$^+$CD56$^-$CD8$^+$ CD4$^-$) and CD4$^+$T

9   cells (CD3$^+$CD56$^-$CD4$^+$CD8$^-$). (D-G) Gating strategy and representative plots

10 of flow cytometric analysis of Tim-3$^+$NK cells, Tim-3$^+$NK-like T cells,

11 Tim-3$^+$CD4$^+$T cells and Tim-3$^+$CD8$^+$T cells. (H-K) Gating strategy and

12 representative plots of flow cytometric analysis of PD-1$^+$NK cells, PD-1$^+$NK-like

13 T cells, PD-1$^+$CD4$^+$T cells and PD-1$^+$CD8$^+$T cells.

14 Figure 2. Comparison of Tim-3 and PD-1 expression on peripheral NK subsets,

15 NK-like T and T cell subtypes between women who did and did not conceive

16 over a nine-day period after ET. (A) Tim-3$^+$CD56^{dim}NK, (B) Tim-3$^+$CD56^{bright}NK

17 cells, (C) Tim-3$^+$CD8$^+$T cells, (D) Tim-3$^+$CD4$^+$T cells, (E) Tim-3$^+$NK-like T cells,

18 (F) PD-1$^+$CD8$^+$T cells, (G) PD-1$^+$CD4$^+$T cells, (H) PD-1$^+$NK-like T cells.

19 Significant difference was found in (A), (D) and (E) at three and six days after

20 ET between two groups. The percentage of (C) was significantly lower in

21 pregnant women compared with non-pregnant women at three days after ET.

22 No significant difference was found in (B, F-H). Asterixis indicate comparison

23 which showed a significantly different result, whereas if the asterixis is not

24 shown, it means the results were not significantly different. The concentrations

1   of circulating Gal-9 (I) and PD-L1 (J) were compared between pregnant and

2   non-pregnant women at serial time points. No significant difference in

3   concentration of serum Gal-9 and PD-L1 was observed within groups and

4   between two groups.

5