INVESTIGATION OF TIM-3 AND GALECTIN-9 MOLECULES IN HEALTHY AND PATHOLOGIC PREGNANCIES AND IN INFERTILE WOMEN

Ph.D. Thesis

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INTRODUCTION

Foeto-maternal immune tolerance

At the time of implantation, the maternal immune system has to be altered to enable survival of the semi-allogeneic fetus. Pregnancy is an ideal condition to study active immunotolerance. During pregnancy the fetus will not be attacked or rejected by the maternal immune system but rather successfully accepted by the mother. Precise immunoregulation of the maternal immune system is critical for normal pregnancy and fetal development. Locally, recruited immune cells of the decidua come into direct contact with fetal antigens presented by trophoblast cells leading to immunoactivation and inducing tolerance via type 2 immunity particularly in the innate immune system. Uterine natural killer (NK) cells, the dominant lymphocyte subpopulation found in the decidua, play a central role in efficient placentation. Recognition of fetal non-polymorphic human leukocyte antigen (HLA) G and HLA-E by uterine NK cells usually induces the secretion of Th2-type cytokines and contributes to the success of pregnancy. On the other hand, recognition of paternal HLA-C molecules expressed on the trophoblast results in a local inflammatory response, which, by loosening the tissue, facilitates extravillous cytotrophoblast invasion. Additionally, interferon-g, produced during inflammation, promotes uterine vascular remodeling. Disturbed maternal recognition of fetal antigens may lead to pregnancy pathologies. Identified a special genetic combination of maternal killer immunoglobulin- like receptors and fetal HLA-C genes that results in inhibition of trophoblast invasion and leads to preeclampsia.

The participation of NK and NKT cells in the Th1/Th2 shifts of pregnancy suggests a dominant role of the innate rather than the adaptive immune system. The Th1/Th2 paradigm has recently been reconstituted to include a third population of T helper cells that produce IL-17, therefore these cells are designated as Th17 cells. This Th2 cytokine polarization occurs both at systemic level and at the fetal-maternal interface and the cause behind this cytokine shift are not clearly defined. Pregnancy as a physiological condition includes the altered ratio and function of different lymphocytes subpopulations compared to non-pregnant status. Therefore it is important to investigate and understand the immune regulatory mechanism behind these immunological changes.

TIM-3

The immunoglobulin superfamily member T-cell immunoglobulin mucin 3 (TIM-3) was first discovered in 2002 on interferon IFN-γ producing CD4+ (Th1) and on CD8+ T cytotoxic cells (Tc). TIM-3 expression was verified in a variety of immune cells, including Th1, Th17, NK cells, NKT cells, Tregs, and also on antigen presenting immune cells such as dendritic cells and monocytes. TIM-3 molecule has been implicated in both activation and inhibition of immune responses, but its *in vivo* function have remained unknown.

Consistent with its role as an inhibitory molecule, blockade of the TIM-3-TIM-3L pathway *in vivo* by blocking antibody or soluble TIM-3 Ig fusion protein, which serves to block TIM-3–Gal-9 interactions, exacerbates experimental allergic encephalomyelitis (EAE) and type 1 diabetes. Expression of TIM-3 on Th1 cells provides a key checkpoint that serves to dampen proinflammatory Th1-dependent T-cell responses and may contribute to the maintenance of pregnancy. In line with this, Chabtini et al. examined the TIM-3-expression on innate immune cells by using an allogeneic mouse model of pregnancy and indicated their possible role in the regulation of tolerance at the foeto-maternal interface. The only human study presented that TIM-

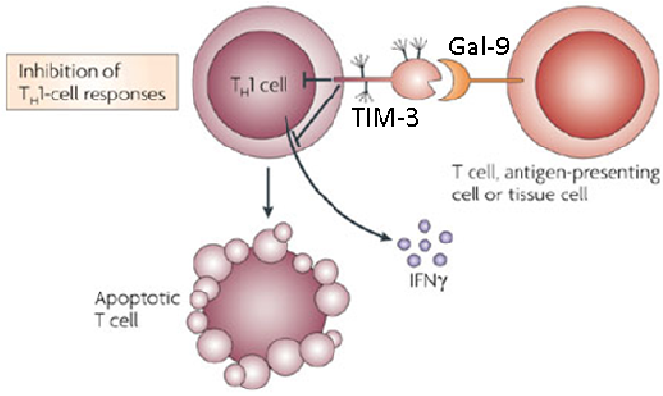
3 is upregulated by monocytes in peripheral blood of pregnant women indicate that abnormal TIM-3 expression might be related to the loss of pregnancy.

Galectin-9 (Gal-9)

Gal-9 was initially characterized as an eosinophil specific chemoattractant and subsequently, Gal-9 was found to possess a variety of biological functions, including roles in cell differentiation, aggregation, adhesion, the induction of cell death, and the inhibition of cancer metastasis. Gal-9 has anti-metastatic effect in cancer cells, like breast cancer and oral squamous cell carcinoma. Furthermore Gal-9 induces immunotolerance in certain autoimmune diseases. Recently proved that Gal-9 causes apoptosis in activated CD4+ Th cells, which is not occur in resting cells. In tumor induced immunsuppressive status Gal-9 has antitumor activity via TIM-3/Gal-9 pathway while in hyperimmun conditions causes apoptosis in TIM-3+ Th1, Th17 and CD8+ Tc cells. Recently, human Gal-9+ Th cells were identified expressing Gal-9 on their surface and secreting Gal-9 upon TCR stimulation resulting in the regulation of Th17/Treg development. Many studies examined the role of Gal-9 in immunological contexts, which can influence the immune system in different ways, either by exacerbating the inflammatory process or by acting as an anti-inflammatory agent.

TIM-3/Gal-9 interaction

As a member of the immunglobulin superfamily TIM-3 is a transmembran protein, it was firstly described on differentiated Th1 cells surface. Gal-9 was identifed as a ligand of TIM-3 receptor and is widely distributed throughout various tissues, being particularly abundant in the liver, lung, thymus and some muscles. Some proinflammatory cytokines as IFN-γ or IL-1β induce Gal-9 expression by endothelial and fibroblast cells. TIM-3 has a major role in regulation of Th1 immunity and some autoimmune diseases. Isolation of innate and adaptive immune cells from patients with MS exhibit intense TIM-3 expression by dendritic cells which promotes TNF-α secretion. In Th1 immune responses TIM-3 expression by differentiated Th1 cells increased compared to dendritic cells and it's associated with the up-regulation of Gal-9 mediated signal pathway which lead to elevated IFN-γ secretion. Further study revealed that Gal-9 induce TIM-3 expression in Th1 cells and contribute to terminate Th1 immune responses.



Effect of the TIM-3 and Gal-9 connection

Engagement of TIM-3 by its ligand Gal-9 negatively regulates IFN-γ secretion, influences the ability to induce T cell tolerance and triggers a significant signal cascade to induce apoptosis

of Th1 type immune cells. Thus, engagement of TIM-3 by Gal-9 may function as a negative regulator, abrogating Th1- and Th17 driven immune responses and may modulate the Th1/Th2 balance. In this regard, it is possible that TIM-3/Gal-9 interaction could play an important role in the regulation of maternal immune tolerance toward the fetus and may be a potent regulator of the adaptive and innate immune responses. Although data about the TIM-3/Gal-9 pathway in the pathogenesis of human diseases is emerging their possible role during human pregnancy is not precisely known.

AIMS OF THE STUDY

Here we investigated the possible role of TIM-3 and Gal-9 molecules in healthy pregnancy, in pathological pregnancy and in infertile women.

1. Possible role of TIM-3/Gal-9 pathway in healthy pregnancy

How TIM-3 receptor and Gal-9 ligand expression change in the peripheral blood throughout healthy pregnancy analyzing different lymphocyte subpopulations? What are the functional characteristics of the TIM-3+ lymphocyte subpopulations: Does cytotoxic activity or cytokine production differ from TIM-3 negative subsets? Is there any correlation between different stages of pregnancy and serum soluble Gal-9 levels?

2. Possible role of TIM-3/Gal-9 pathway in early-onset preeclampsia

Are there any differences in TIM-3 and Gal-9 molecule distribution or expression pattern in peripheral blood between women with early-onset preeclampsia and in healthy pregnant women? What are the functional consequences of these changes?

3. Possible prognostic role of TIM-3/Gal-9 pathway after IVF treatment

How frequency, TIM-3 expression pattern and functional activity of NK cells change after IVF treatment? Are these differences influence the embryo transfer outcome?

MATHERIALS AND METHODS

In our experiments the following techniques were used:

- PBMC (peripheral blood mononuclear cells) isolation on Ficoll gradient
- PBMC cryopreservation and thawing
- Labeling lymphocytes for flow cytometric analyses
- Intracellular staining with anti-perforin
- CD107a cytotoxicity assay
- FoxP3 intracellular staining
- Flow cytometry
- Magnetic activated cell sorting (MACS)
- Fluorescent cell sorting
- PMA/Ionomycin treatment
- Cytokine detection with Cytometric Bead Array (CBA)
- Soluble Gal-9 measuring with ELISA method
- Statistical analyses with SPSS V.20.

RESULTS

1. Possible role of TIM-3/Gal-9 pathway in healthy pregnancy

We investigated the percentage of CD3+ T cells, CD4+ Th, CD8+ Tc cell subpopulations, NK cells, NKT cells and Gal-9+ Th cells in the peripheral blood of normal pregnant women during each trimester of pregnancy and in non-pregnant women. The frequency of NK cells and NK^{dim} cells throughout pregnancy was lower and the frequency of NK^{bright} cells was higher than in non-pregnant women but these results did not reach the level of significance. The frequency of Gal-9+ Th cells were approximately 1% in non-pregnant women and we detected an increased frequency throughout pregnancy reaching 2,39 % in the third trimester. The frequency of Gal-9 Th cells in the third trimester was significantly higher than in non-pregnant women, as well as women in the first and second trimester.

Investigating TIM-3 expression by CD8+ T cells we found a decrease in the 2nd trimester compared to other trimesters and to the samples from the non-pregnant group, however this change did not reach the level of statistical significance. Furthermore TIM-3 expression was significantly increased by NK cells in samples from the 3rd trimester compared to the samples in the 2nd trimester. Analyzing the NK cell subsets, the TIM-3 expression by NK^{dim} cells was significantly increased in samples from the 3rd trimester compared to the samples from the 2nd trimester and from non-pregnant women.

Th1, Th2 and Th17 cytokines were analyzed by CBA system, where IL-4, IL-6 and IL-1 cytokines were under the detectable level. CD8+ Tc cells expressing TIM-3 produced significantly lower level of proinflammatory (IL-2, TNF-α and IFN-γ) and Th17 cytokines compared to TIM-3 negative counterparts in the 1st and 3rd trimester of pregnancy and in healthy non-pregnant controls. IL-2 cytokine production by TIM-3+ NK^{dim} cells was significantly lower compared to TIM-3 negative NK^{dim} cells in the 1st and 2nd trimester of pregnancy. In the 2nd trimester of pregnancy IFN-γ cytokine production by TIM-3+ NK^{bright} cells was significantly higher compared to TIM-3 negative NK^{bright} cells.

Investigating the cytotoxic activity of TIM-3+ CD8+ Tc cells during pregnancy, we found that CD107a expression was significantly higher in samples from 3rd trimester compared to 1st and 2nd trimester and non-pregnant women. CD8+ Tc cells expressing TIM-3 in the 3rd trimester of pregnancy showed significantly increased CD107a expression compared to TIM-3 negative CD8+ Tc cells. Furthermore TIM-3 positive NK cells and NK^{dim} subset showed similar CD107a expression pattern, where the cytotoxic activity was significantly increased in samples from 3rd trimester compared to the non-pregnant group and 1st trimester group. Analyzing CD107a expression by the NK^{bright} subpopulation showed no significant differences. Interestingly cytotoxic activity of TIM-3+ NK cell and NK^{dim} cell were significantly lower in non-pregnant women and in all trimesters compared to TIM-3 negative counterparts.

Serum Gal-9 levels differ significantly between non-pregnant and healthy pregnant women in each trimester. Analyzing Gal-9 levels throughout pregnancy we found an increasing tendency with a significant elevation of serum Gal-9 concentration in the 2nd and 3rd trimester compared to the 1st trimester.

2. Possible role of TIM-3/Gal-9 pathway in early-onset preeclampsia

We compared the frequency of CD3+ T cells, helper and cytotoxic T cell subpopulations, regulatory T cells, NK cells, NK^{dim} cells, NK^{bright} cells, and NKT cells among peripheral blood mononuclear cells in women with early-onset preeclampsia and in healthy pregnant women. Compared to healthy pregnant controls, in the peripheral blood of early-onset preeclamptic

women there is a significant decrease in the frequency of regulatory T cells and in the frequency of NK^{bright} cells.

Investigating peripheral blood mononuclear cells of women with early-onset preeclampsia, our results showed a decreased TIM-3 expression by CD8+ Tc cells, NK cells and NK^{dim} cells compared to healthy pregnant women

Analyzing the Gal-9 expression of peripheral lymphocytes we found a notably increased frequency of Gal-9 positive CD8+ Tc, NK and NK^{bright} cells in the case of early-onset preeclamptic patients when compared to healthy pregnant controls while the frequency of Gal-9+ Treg cells did not changed.

Investigating the cytotoxic activity of CD8+ Tc and NK cells, we found that only TIM-3 positive CD8+ Tc and NK cells showed increased cytotoxicity in women with early-onset preeclampsia compared to healthy pregnant women. Interestingly, TIM-3 positive NK^{dim} cells from women with early-onset preeclampsia showed significantly increased CD107a expression compared to healthy pregnant women and this difference was not observed in the case of NK^{bright} cells.

3. Possible prognostic role of TIM-3/Gal-9 pathway after IVF treatment

Comparing the data from the two dates, significant differences were only seen in the failed IVF group. In the peripheral blood of failed IVF patients there was a significant increase in the NK cell ratio within the lymphogate after 1 week of the procedure affecting both the NK^{bright} and the NK^{dim} subsets. After IVF, NK cells of failed IVF women expressed significantly more CD160 and NKG2D receptors compared with their expression before IVF. Changes in these NK cell receptor expressions applied to both NK cell subsets.

In the peripheral blood of women with failed IVF attempts, significantly more peripheral NK^{bright} cells contained cytotoxic perforin molecules. In the case of women with failed IVF, the ratio of activated and degranulated NK cells significantly increased after IVF, shown by the increased CD107a expression. These findings predominantly affect the NK^{dim} subpopulation.

In women with failed conception, the ratio of peripheral NKT-like cells significantly increased after the IVF procedure. After IVF, there was a significant decrease in perforin-containing NKT-like cells compared with their percentage before fertilization in the successful IVF group. NKT-like cells of failed IVF women expressed significantly more CD160 and NKG2D receptors compared with their expression before IVF performance. CD160 expression of NKT-like cells in women with successful IVF significantly decreased after the embryo transfer.

SUMMARY

1. Possible role of TIM-3/Gal-9 pathway in healthy pregnancy

1.1 TIM-3 receptor expression by peripheral blood lymphocytes show dynamic changes during healthy pregnancy both in the adaptive and innate immune system.

1.2 NK^{dim} subpopulation exhibit the highest TIM-3 expression level in healthy pregnancy.

1.3 However TIM-3 expression by CD8+ Tc cells do not change in the 1^{st} and 2^{nd} trimester of pregnancy, but the decreased proinflammatory cytokine production by TIM-3+ CD8+ Tc subset may contributes to the development of maternal tolerance.

1.4 The increased cytotoxic activity of TIM-3+ CD8+ Tc cells in 3^{rd} trimester of pregnancy could contribute to the proper immunological environment for delivery.

1.5 The cytotoxic activity of TIM-3+ NK cells is significantly lower compared to TIM-3 negative NK cells in all investigated groups.

1.6 In the 1^{st} and 2^{nd} trimester of pregnancy TIM-3 expression by NK cells does not change, but IL-2 production by TIM-3+ NK^{dim} cells was decreased, and IFN-γ production by TIM-3- NK^{bright} cells was increased.

1.7 Increased TIM-3 expression by NK and NK^{dim} cells is associated with elevated cytotoxic activity in the 3^{rd} trimester of pregnancy.

1.8 Serum Galectin-9 levels differ significantly between non-pregnant and healthy pregnant women in each trimester. Gal-9 level during pregnancy shows an increasing tendency with a significant elevation of serum Gal-9 concentration in the 2^{nd} and 3^{rd} trimester compared to the 1^{st} trimester. In line with this the proportion of Gal-9+ Th cells significantly increase in the 3^{rd} trimester. With the elevated soluble Gal-9 level could explain the reduced proinflammatory cytokine production by TIM-3+ CD8+ Tc cells and the reduced cytotoxic activity of the TIM-3+ NK and NK^{dim} cells.

2. Possible role of TIM-3/Gal-9 pathway in early-onset preeclampsia

2.1 Surface expression of TIM-3 receptor by the CD8+ Tc, NK and NK^{dim} cells is reduced in women with early-onset preeclampsia.

2.2 In women with early-onset preeclampsia the surface expression of Gal-9 molecule by CD8+ Tc, NK and NK^{bright} cells were increased.

2.3 Reduced TIM-3 expression by TIM-3+ CD8+ Tc and TIM-3+ NK^{dim} cells may contribute to the increased cytotoxic activity of these subsets which can not compensate by the elevated Gal-9 expression in women with early-onset preeclampsia.

3. Possible prognostic role of TIM-3/Gal-9 pathway after IVF treatment

3.1 In the peripheral blood of women with failed IVF attempts there was a significant increase in the NKT and NK cell ratio after 1 week of the procedure affecting both the NK^{bright} and the NK^{dim} subsets.

3.2 In the peripheral blood of failed IVF patients, significantly more peripheral NK^{bright} NK cells contained cytotoxic perforin molecules.

3.3 After the embryo transfer, NK and NKT cells of failed IVF women expressed significantly more CD160 and NKG2D receptors compared with their expression before IVF which is correlated with IVF failures.

3.4 TIM-3 expression by NK and NKT cells do not change in the investigated groups

3.5 Elevated cytotoxic activity of the NK and NK^{dim} subpopulations after 1 week of the procedure correlated with failed IVF outcomes.

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Cumulative impact factor of articles related to thesis: **9,35**
Oral presentations related to thesis : **7**
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