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TIM-3 Regulates Innate Immune Cells To Induce Fetomaternal Tolerance

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TIM-3 is constitutively expressed on subsets of macrophages and dendritic cells. Its expression on other cells of the innate immune system and its role in fetomaternal tolerance has not yet been explored. In this study, we investigate the role of TIM-3–expressing innate immune cells in the regulation of tolerance at the fetomaternal interface (FMI) using an allogeneic mouse model of pregnancy. Blockade of TIM-3 results in accumulation of inflammatory granulocytes and macrophages at the uteroplacental interface and upregulation of proinflammatory cytokines. Furthermore, TIM-3 blockade inhibits the phagocytic potential of uterine macrophages resulting in a build up of apoptotic bodies at the uteroplacental interface that elicits a local immune response. In response to inflammatory cytokines, Ly-6ChiGneg monocytic myeloid–derived suppressor cells expressing inducible NO synthase and arginase 1 are induced. However, these suppressive cells fail to downregulate the inflammatory cascade induced by IFN-γ and TNF-α by inflammatory granulocytes leads to abrogation of tolerance at the FMI and fetal rejection. These data highlight the interplay between cells of the innate immune system at the FMI and their influence on successful pregnancy in mice. The Journal of Immunology, 2013, 190:88–96.

Since Medawar’s first hypothesis on the mechanism of avoidance of immune attack by the semiallogeneic fetus, substantial research in reproductive and transplant immunology has addressed this paradigm. Successful pregnancy requires that the maternal immune system does not attack the fetus that has fetal histocompatibility Ags inherited from the father. A deleterious immune attack is avoided by orchestration of cellular, hormonal, and enzymatic factors. In recent years, it has become apparent that a Th2 cytokine profile is crucial to maintain successful pregnancy (1–3).

Pregnancy-induced regulatory T cells (4) and the negative co-stimulatory molecule programmed death ligand-1 (PD-L1) have been shown to be important in fetal acceptance in murine pregnancy (5, 6). Further, innate immune cells are also critical for initiating and coordinating an immune response against paternal Ags (7). Decidual macrophages and dendritic cells (DC) have also been shown to have a pivotal role in establishing a tolerogenic microenvironment at the fetomaternal interface (FMI) (8, 9).

In this study, we explore whether the molecule TIM-3 plays a role in inducing fetomaternal tolerance. TIM-3 was first described as a molecule specifically expressed on the surface of IFN-γ–producing Th1 and Tc1 cells (10). TIM-3 is a pattern recognition receptor specialized for recognition of phosphatidylserine exposed on apoptotic cells (11). Another ligand for TIM-3 is galectin-9 (12). Galectin-9 is an S-type lectin ubiquitously expressed in cells and on certain epitheliums (13). It binds to TIM-3 expressed on activated (IFN-γ–producing) Th1 and Tc1 but not to Th2 cells (10) to terminate T cell response by induction of apoptotic signals (12, 14). A role for TIM-3 has also been described in Th1 cell exhaustion of virus-infected CD8 cells (15–21).

Besides being expressed on activated T cells, TIM-3 is constitutively expressed on cells of the innate immune system in both mice and humans (10, 22, 23). TIM-3 expressed on DC and subsets of macrophages mediates phagocytosis of apoptotic cells and cross-presentation of Ags (23) and synergizes with TLRs to enhance inflammatory responses (22). Transgenic overexpression of TIM-3 on T cells results in an increase in the population of CD11b+Ly6Ghi granulocytic myeloid–derived suppressor cells (G-MDSC) in mice (24). The role of TIM-3 in innate immune cells is likely complex, as many cell types are involved in the regulation of the innate immune response by various mechanisms.

The role of TIM-3 has also been studied in allograft tolerance (14, 25). For example, TIM-3–deficient mice are reported to be refractory to tolerance induction by donor-specific transfusions or treatment by CTLA4-Ig or anti-CD40L (CD 154) Ab (14). However, little is known about whether TIM-3 plays a role in regulating the immune system at the FMI.

In this study, we use an MHC-mismatched pregnancy to explore the role of TIM-3 on uterine myeloid cells in inducing or maintaining fetomaternal tolerance. We find that TIM-3 is expressed on monocytes and granulocytes infiltrating the uterus as well as on...
different subsets of uterine macrophages and DC. Treatment of pregnant mice with a TIM-3–blocking Ab resulted in the failure of uterine macrophages to clear apoptotic and dying cells. The resulting inflammation activates two subsets of myeloid cells. The first subset, the suppressor monocytic myeloid–derived suppressor CD11b+Ly6ChiGneg cells (M-MDSC), upregulates the expression of inducible NO synthase (iNOS) and arginase 1; the second subset, the inflammatory CD11b+Ly6CintGhi cells, increases their production of proinflammatory cytokines such as IFN-γ and TNF-α. M-MDSC are unable to counterbalance the overpowering inflammation created by CD11b+Ly6ChiGneg cells, and the resultant effect is increased fetal resorption and death.

**Materials and Methods**

**Mice**

CBA/CaJ and C57BL/6 mice were purchased from The Jackson Laboratory and maintained in our animal facility according to institutional and National Institutes of Health guidelines. Seven- to eight-week-old females were mated to either C57BL/6 or CBA/CaJ males to induce pregnancy and inspected every morning for vaginal plugs. The day of visualization of a plug was designated as day 0.5 of pregnancy. In some experiments, pregnant mice were monitored until parturition and the number of live pups recorded. Mice were sacrificed before the termination of pregnancy, and the percentage of resorbed embryos was calculated (resorbed embryos/total embryos × 100).

**Treatment protocol**

Pregnant females received four injections of RMT3-23 (anti–TIM-3) Ab i.p. at doses of 500, 500, 250, and 250 µg at days 6.5, 8.5, 10.5, and 12.5, respectively.

**Cell preparation**

Uteri from pregnant mice were dissected free from the mesometrium and removed by cuts at the ovaries and cervix. These uteri were carefully dissected with scissors to remove fetal and placental tissue, washed twice in ice-cold PBS or HBSS, and shredded carefully. Minced uteri were enzymatically digested for 20 min at 37°C in HBSS/Ca/Mg containing 200 U/ml hyaluronidase, 1 mg/ml collagenase type IV, 0.2 mg/ml DNase, and 1 mg/ml BSA/fraction V as previously described with some modifications (26).

**In situ identification of nuclear DNA fragmentation**

CBA/CaJ females were mated with C57BL/6 males, and uteri were harvested on gestational days 7.5 and 10.5. Uteri were immersion-fixed in 10% buffered formalin and embedded in paraffin. After deparaffinization, uterine sections were stained using ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) according to the manufacturer’s instructions. Nuclei were counterstained with DAPI (ProLong Gold Antifade Reagent; Invitrogen Life Technologies, Grand Island, NY). Images were captured on Zeiss LSM 510 Meta confocal microscope (Carl Zeiss), and the number of TUNEL-positive cells was quantified as TUNEL-positive cells per mm² of tissue.

**RNA isolation and real-time PCR (TaqMan)**

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies). cDNA was synthesized using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen Life Technologies). mRNA synthesis system for RT-PCR (Invitrogen Life Technologies). cDNA was synthesized using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen Life Technologies). mRNA was used to quantify expression of several genes using TaqMan real-time PCR. The primer sequences and conditions for each gene are as follows:  

**Figure 1**

**Analysis of CD45^+ subpopulations in the pregnant uterus of CBA/CaJ × C57BL/6 mice.** Lymphocytes were isolated from uteri of pregnant mice at gestational days 7.5, 10.5, and 12.5. Uterine lymphocytes were gated based on CD45 staining, and the different cell populations were characterized by multicolor flow cytometry based on the unique phenotype of each cell. Statistical analysis was obtained using Kruskal–Wallis test. (A) There was no significant increase in any of the three subpopulations of DC CD11c^+11bhi, CD11c^+11blo, and CD11c^+11bneg in the uterus between days 7.5 and 10.5, but CD11c^+11bhi cells decrease significantly at day 12.5 (p = 0.1967, 0.0411, and 0.4236, respectively; n = 3–6 mice/group). (B) Uterine macrophages are described as F4/80^+ cells with variable expression of MHC II. From gestational day 7.5 to 10.5, there is a trend toward increase in MHC II^hi subpopulation in the uterus. A similar trend is observed in the F4/80^+MHC II^hi subpopulation, and the increase becomes significant by day 12.5 (p = 0.0093, n = 3–6 mice/group). (C) All three subpopulations of IMC/MDSC exist in the pregnant uterus. CD11b+Ly6ChiGneg M-MDSC increase in the uterus from days 7.5 to 12.5 (p = 0.0182, n = 3–6 mice/group). CD11b+Ly6ChiGneg granulocytic MDSC and CD11b+Ly6CintGhi cells, in contrast, peak at day 10.5 (p = 0.0093 and 0.0656, respectively).
expression of iNOS, arginase 1, IFN-γ, TNF-α, and GAPDH were examined using TaqMan gene expression assays. Triplicate samples were obtained in each condition. A comparative threshold cycle (CT) value was normalized for each sample using the formula: \( \Delta CT = CT_{\text{gene of interest}} - CT_{\text{GAPDH}} \), and the relative expression was then calculated using the equation \( 2^{-\Delta \Delta CT} \).

Flow cytometry

For FACS analysis, cells were isolated from uterus tissue at different points of gestation, pretreated with Fc-blocking mAb clone 24G.2 for 10 min at 4°C, and stained with fluorochrome-labeled Abs against CD45 (30-F11), CD11c (N418), CD11b (M1/70), F4/80 (BM8), MHC class II (MHC II; Ia/ Ie), TIM-3 (B8.2C12), Ly-6C (HK1.4), Ly-6G (1A8), CD3 (17A2), CD4 (GK1.5), CD8 (53-6.7) purchased from BioLegend (San Diego, CA), Invitrogen, eBioscience (San Diego, CA), or BD Biosciences (San Jose, CA). The fluorochromes used were FITC, PE, PerCP-Cy5.5, allophycocyanin, PE-Cy7, allophycocyanin-Cy7, Alexa Fluor 700, PE-Texas red, and brilliant violet. Samples were washed with FACS buffer, acquired on a five-laser 12-Color BD LSR II FACS (BD Biosciences), and analyzed by FlowJo software (Tree Star, Ashland, OR). For sorting experiments, cells were isolated from uterine tissue as described earlier, stained with Abs against CD45 allophycocyanin (30-F11), CD11b PE-Cy7 (M1/70), TIM-3 PE (B8.2C12), Ly-6C FITC (HK1.4), and Ly-6G PerCP-Cy5.5 (1A8). Cells were sorted on a five-laser FACS aria (BD Biosciences) or MoFlo cell sorter (Beckman Coulter, Brea, CA) into TRizol reagent (Invitrogen Life Technologies).

For apoptosis experiments, cells were stained using the Annexin V apoptosis detection kit from BD Pharmingen (San Jose, CA), acquired on a two-color liquid FACSCalibur (BD Biosciences), and analyzed with FlowJo software (Tree Star).

Immunostaining

Immunofluorescence Ag labeling for TIM-3 was performed on the paraffin-embedded mouse uterus samples. Paraffin-embedded sections were prepared and treated with Trilogy (Cell Marque) for Ag retrieval. Sections were then incubated with 1 mg/ml sodium borohydride (ICN Chemicals) for 5 min at room temperature. After three washes with TBS, the sections were incubated with 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. The sections were then incubated with goat anti-TIM-3 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-Mac2 (1:300; Cedarlane Laboratories, Burlington, NC) primary Ab overnight at 4°C.

Slides were washed three times with TBS and incubated with Dylight 649–conjugated donkey anti-goat secondary Ab or Dylight 549–conjugated donkey anti-rabbit secondary Ab for 1 h at room temperature (1:200; Jackson ImmunoResearch Laboratories).

Slides were then washed three times with TBS and mounted with Prolong Gold anti-fade mounting media containing DAPI (Invitrogen). Confocal images were taken using a Zeiss LSM510 Meta confocal system and Zeiss LSM510 image acquisition software (20×0.8 Plan-Apochromat objective and a 40×/1.3 oil Plan-Apochromat objective; Carl Zeiss).

Cell lines: Tsras2 and RAW264.7

Tsras2 cell line was a kind gift from Adrian Erlebacher (NYU Langhorne Medical Center, New York, NY). Cells were maintained in DMEM: F12 supplemented with 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 1 μM sodium pyruvate, and 100 μM 2-ME.

RAW264.7 cell line (American Type Culture Collection) was maintained in complete DMEM with 1-glutamine supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES.

Apoptotic cells preparation and coculture with RAW264.7 cell line

Apoptotic cells were prepared by incubating the Tsras2 trophoblast cell line from FVB mice with 1% H2O2 for 10 min at 37°C. The percent of apoptosis was assessed using the Annexin V apoptosis detection kit (BD Biosciences).

For measuring in vitro activation of macrophages by apoptotic cells, 1 million RAW264.7 cells were pretreated with Fc block followed by RMT3-23 Ab or control and cocultured with 0.1 × 10^6 apoptotic trophoblast cells for 24 h at 37°C. After 24 h, cells were washed twice with PBS, and RNA was isolated according to the protocol described earlier.

In vitro phagocytosis assay

RAW264.7 macrophage cell line or uterine F4/80+ macrophages flow sorted from gestational day 10.5 uterus of CBA/Ca females (mated with C57BL/6 males) were plated on a 48- or 96-well plate at a density of 5 × 10^5 cells/well for 4 h at 37°C and then washed twice to remove nonadherent cells. The adherent cells were then incubated with RMT3-23 Ab (30 μg/ml) for 30 min prior to culture with FITC-labeled beads (Cayman Chemicals, Ann Arbor, MI) for 2 h at 37°C. The cells were washed three times with PBS to remove unbound beads, and fluorescence intensity was assessed using an inverted microscope or by flow cytometry on an FACSCalibur (BD Biosciences) according to the manufacturer’s instructions.

Statistics

Unpaired two-tailed t test was used to analyze the statistical significance between two groups. Kruskal–Wallis followed by Dunn’s post hoc test was used to analyze the statistical significance among multiple groups. A p value <0.05 was considered statistically significant.

Results

Myeloid cells in allogeneic murine pregnancy

The mouse model of allogeneic pregnancy involving mating of CBA/Ca females with C57BL/6 males (27, 28) was used to study the changes taking place in the composition of cells infiltrating the uterus during gestation day 10.5 using Ab against TIM-3 (red) counterstained with DAPI (blue). Arrows show positive TIM-3 staining. Original magnification ×200/0.8 (A) and ×40/1.3 (B) oil Plan-Apochromat–objective.

![FIGURE 2. Immunofluorescence staining. Immunofluorescence staining of uterine tissue from MHC-mismatched pregnancy (H2k) on gestational day 10.5 using Ab against TIM-3 (red) counterstained with DAPI (blue). Arrows show positive TIM-3 staining. Original magnification ×200/0.8 (A) and ×40/1.3 (B) oil Plan-Apochromat–objective.](image-url)
FMI on gestational days 7.5, 10.5, and 12.5. (See Supplemental Figs. 1 and 2 for gating strategy.) Analysis of DC subtypes showed that CD11b^hi and CD11b^neg subtypes do not vary between days 7.5 and 12.5. In contrast, CD11b^lo cells decreased significantly in number in late pregnancy (day 12.5) (Fig. 1A). Analysis of macrophages in the uterus and draining para-aortic and iliac LNs of pregnant mice showed two subtypes: mature F4/80^+MHC II^hi cells and immature F4/80^+MHC II^lo cells (Fig. 1B). Immature macrophages reside predominantly in the uterine mucosa. The percentage of these uterine F4/80^+MHC II^lo cells increases significantly from 0.8% in the peri-implantation period (day 7.5) to 7.8% at late gestation (day 12.5).

Inflammatory monocytes (IMC)/MDSC are phenotypically characterized as CD11b^+Gr1^+ cells. Our results show that these cells are further divided into three subsets at the uteroplacental interface based on staining by specific Gr1 mAbs (Ly6C and Ly6G) (Fig. 1C). G-MDSC have a CD11b^+Ly6C^loG^hi phenotype and constitute 0.5–3% of the CD11b^+Gr1^+ population. M-MDSC have a CD11b^+ly6C^hiG^NEG phenotype and constitute 10–30% of the population. Granulocytes make up 2–4% of CD11b^+Gr1^+ cells and have a CD11b^+Ly6C^intG^hi phenotype. Resident monocytes, which constitute the rest of the population, are Ly-6C and G negative (data not shown). CD11b^+Ly6C^NEGG^hi cells remain unchanged throughout pregnancy compared with G-MDSC and M-MDSC, which peak at mid- (day 10.5) and late gestation (day 12.5), respectively. Table I summarizes the mean percentage of myeloid cells on gestational days 7.5, 10.5, and 12.5 in the uterus.

**TIM-3 characterization at the uteroplacental interface**

To address whether TIM-3 is involved in promoting immune cell homeostasis at the FMI, TIM-3 expression was analyzed on gestational day 10.5 in uterine sections obtained from allogeneically mated mice. TIM-3 expression was observed in the uterus as determined by immunofluorescence staining (Fig. 2).

In addition to the above-described TIM-3 expression at the uteroplacental interface by histological methods, we assessed TIM-3 expression on myeloid cells present in the uterus and draining LNs by flow cytometry on gestational days 7.5, 10.5, and 12.5. It was observed that in the draining LN, TIM-3 expression is significantly upregulated at midgestation (day 10.5) in all three DC subsets, namely CD11c^+11b^, CD11c^+11b^lo, and CD11c^+11b^neg (data not shown). TIM-3 expression was significantly upregulated at midgestation (day 10.5) in uterine CD11c^+11b^hi and CD11c^+11b^neg DCs as well. Conversely, uterine CD11b^lo DCs expressed relatively constant levels of TIM-3 at day 10.5 gestation (Fig. 3A).

Similar to DCs, macrophages are known to constitutively express TIM-3 (23, 29, 30). We also evaluated whether TIM-3 expression on mature and immature uterine macrophage populations and on cells residing in the draining para-aortic and iliac draining LNs play a role in modulating alloimmune pregnancy. The draining LNs contained very few macrophages (data not shown). The gestational uterus contained a higher number of macrophages compared with the regional draining LNs, and the majority of cells were immature, expressing low levels of MHC class II. TIM-3 expression remained constant in both subsets of uterine macrophages during early stages of pregnancy, but immature cells were observed to downregulate TIM-3 expression at day 12.5 (Fig. 3B).

Different subsets of myeloid progenitor and suppressor cells were observed to express TIM-3 in a unique manner in pregnancy. TIM-3 expression in the uterus was negative in the three subsets of myeloid-derived cells at gestational day 7.5 and they all upregulate TIM-3 expression at midgestation (Fig. 3C). Thus,
pregnancy in mice results in variable expression of TIM-3 on the surface of the different cells residing locally in the uterus and regional LNs.

In vivo role of TIM-3 in pregnancy
To investigate the role of TIM-3 in fetomaternal tolerance, we studied the effect of TIM-3 blockade on syngeneic and allogeneic pregnancy. Syngeneic pregnancy did not show any difference in litter size after treatment (Fig. 4A). Pregnant CBA/CaJ females (mated with C57BL/6 males) challenged with TIM-3–blocking Ab (RMT3-23) according to the protocol described earlier were observed to be more susceptible to fetal loss as manifested by a reduction in litter size (Fig. 4B) and increased rate of resorption at midgestation compared with untreated pregnant controls (Fig. 4C). These data show that TIM-3 plays a protective role during successful pregnancy in vivo.

Homing of macrophages and granulocytes and accumulation at the uteroplacental interface
TIM-3 blockade was observed to result in infiltration of macrophages and inflammatory Ly-6CintGhi cells at the uteroplacental interface at midgestation in allogeneic (Fig. 5A–C) but not in syngeneic pregnancy (Fig. 5D, 5E). Granulocytes are phenotypically characterized by coexpression of Ly-6C and Ly-6G (Ly-6CintGhi cells). Uterine Ly-6CintGhi cells were also observed to express TIM-3. We isolated uterine TIM-3–positive and TIM-3–negative Ly-6CintGhi cells from both treated and untreated allogeneically impregnated mice. We observed that TIM-3+ and TIM-3– Ly-6CintGhi cells upregulate TNF-α mRNA following TIM-3 blockade. TIM-3+ Ly-6CintGhi cells also upregulated

FIGURE 4. Effect of TIM-3 blockade on fetal loss and litter size. CBA/CaJ females were mated to either CBA/CaJ males (syngeneic) or C57BL/6 males (allogeneic) and challenged with four doses of RMT3-23 or left untreated (control). Number of live pups at birth (mean litter size ± SEM) in syngeneic (n = 5 to 6/group; p = 0.3845) (A) and allogeneic (n = 8–11/group; p < 0.0001) mating (B) is shown as a scatter dot plot. (C) The mean percentage of fetal loss ± SEM at gestational days 10.5 (n = 12 to 13/group; p = 0.0006) in allogeneic mating is shown as scatter dot plot.

FIGURE 5. Effect of TIM-3 blockade on immune cells at midgestation. CBA/CaJ females were mated to either CBA/CaJ or C57BL/6 males and challenged with RMT3-23 Ab. (A) Increase in the percentages of F4/80+MHC IIhi and F4/80+MHC IIlo cells in the uteri of allogeneically challenged mice at midgestation (n = 6–8 mice/group; p = 0.0027 and 0.0001 for MHC IIhi and MHC IIlo cells, respectively) is shown. (B) This bar graph shows increase in the absolute number of uterine macrophages in allogeneic pregnancy (n = 6–8 mice/group; p = 0.0036 and 0.0047 for MHC IIhi and MHC IIlo cells, respectively) at midgestation similar to (A). (C) This bar graph shows an increase in uterine CD11b+Ly6CintGhi cell population in the uteri of allogeneically mated anti–TIM-3–treated mice at midgestation (n = 3–4 mice/group; p = 0.0098). (D) This bar graph shows that challenge with RMT3-23 in syngeneically mated mice does not result in an increase in uterine CD11b+Ly6CintGhi cell population at midgestation (n = 5–6 mice/group; p = 0.9710). (E) Syngeneic pregnancy does not result in an increase in the percentages of F4/80+MHC IIhi and F4/80+MHC IIlo cells in the uteri of challenged mice (n = 5–8 mice/group; p = 0.1581 and 0.1609 for MHC IIhi and MHC IIlo cells, respectively).
IFN-γ mRNA (Fig. 6A). In addition, macrophages (RAW 264.7) cocultured in vitro with apoptotic trophoblast cells (Tsras2 cells) in the presence of TIM-3–blocking Ab were also observed to upregulate mRNA expression of TNF-α (Fig. 7C). In contrast, ex vivo–isolated suppressor cells such as CD11b+Ly6ChiGneg upregulated the expression of the immunoregulatory molecules iNOS and arginase 1 following treatment with RMT3-23 (Fig. 6B).

Our results suggest that the above-mentioned suppressor cell population fails to counterbalance the potent proinflammatory effect of CD11b+Ly6CintGhi cells in vivo, thereby leading to pregnancy failure.

**FIGURE 6.** Regulation of iNOS, arginase I, and cytokine production by IMC/MDSC and granulocytes. Cells were sorted from uteri of pregnant CBA/CaJ females at gestational day 12.5. mRNA was isolated from these cells after ex vivo purification, and real-time PCR was performed to examine the relative mRNA expression of iNOS, arginase I, TNF-α, and IFN-γ. (A) CD11b+Ly-6CintGhi cells show no difference in the expression of iNOS and arginase 1 (data not shown) but significantly upregulate TNF-α and IFN-γ expression (10–20-fold increase). (B) In contrast, INOS and arginase I (Arg1) expression was upregulated in CD11b+ Ly-6CnegGneg cells from Ab-treated mice compared with untreated control (6–8-fold increase in expression), whereas TNF-α and IFN-γ were undetectable.

**FIGURE 7.** Effect of TIM-3 blockade on phagocytosis of macrophages. (A) RAW264.7 cells expressing TIM-3 were pretreated with 30 μg RMT3-23 mAb and cultured with FITC-labeled phagocytosis beads (Cayman phagocytosis) according to the manufacturer’s protocol (Cayman Chemical) for 2 h at 37°C. Engulfment of beads was quantified by flow cytometry using an FACSCalibur instrument (BD Biosciences). RMT3-23 treatment results in decrease in phagocytosis of TIM-3+ RAW264.7 cells. (B) Uterine F4/80+ macrophages were sorted from gestational day 10.5 pregnant mice, pretreated with 30 μg RMT3-23 mAb, and cultured with FITC-labeled phagocytosis beads for 2 h at 37°C. Phagocytosis of beads was analyzed by inverted microscopy (original magnification ×100/0.35). We observed a decrease in phagocytosis of beads by F4/80+ cells following treatment. (C) RAW264.7 macrophages were treated with 30 μg RMT3-23 or control IgG mAb and cultured with apoptotic Tsras2 cells for 24 h at 37°C. RNA was isolated from these cells, and mRNA expression of TNF-α, iNOS, and Arg1 was quantified by RT-PCR.

**TIM-3 expressed on uterine macrophages recognizes apoptotic cells**

To investigate whether TIM-3 plays a role in phagocytosis and clearance of apoptotic cells from the uteroplacental interface, TIM-3 expression on the surface of uterine F4/80+ macrophage population (Fig. 3B) as well as on RAW264.7 macrophages was first confirmed (Fig. 7A). Next, uterine F4/80+ macrophages were isolated from gestational day 10.5 uteri of CBA/CaJ mice, and their phagocytic properties were analyzed in the presence of RMT3-23 Ab. FcR of uterine macrophages were blocked to rule out the possibility that binding of RMT3-23 to FcR might affect phagocytosis. Macrophages were then pretreated with RMT3-23.
or PBS and incubated ex vivo with FITC-labeled beads. It was observed that both treated and untreated uterine macrophages are capable of internalizing the particles as shown in Fig. 7B. Interestingly, RMT3-23–treated cells were shown to engulf significantly fewer particles (Fig. 7B). In another experiment, RAW264.7 cells were pretreated with RMT3-23 Ab and incubated with FITC-labeled beads for 2 h before quantitative analysis by flow cytometry. Similarly, RMT3-23–treated RAW264.7 macrophages engulfed significantly fewer particles (Fig. 7A). Thus, TIM-3 blockade using RMT3-23 treatment significantly abrogates phagocytosis at the uteroplacental interface. As described in the previous section, macrophages cocultured with apoptotic trophoblast cells in vitro in the presence of anti–TIM-3 Ab upregulated mRNA expression of TNF-α (Fig. 7C).

**TIM-3 blockade results in accumulation of apoptotic cells at the uteroplacental interface**

To evaluate whether TIM-3 blockade in vivo has any effect on apoptosis at the uteroplacental interface, pregnant CBA/CaJ females were injected with RMT3-23 at days 6.5 and 8.5 of pregnancy. Uteri were harvested at day 10.5, and apoptotic cells were detected by TUNEL method on uterine sections; staining for Annexin V/7-aminoactinomycin D was performed on uterine cell preparation. Significantly increased numbers of TUNEL+ cells were observed in uterine sections from the RMT3-23–treated group as compared with that from the untreated control group (Fig. 8A, 8B). In addition, Annexin V staining showed increased apoptosis of uterine CD4+ population (Fig. 8C), but not CD8+ cells (data not shown).

This result is consistent with the possibility that more cells undergo apoptosis upon blockade of TIM-3. Alternatively, the observation of increased numbers of apoptotic cells could be a result of defective clearance of these cells by phagocytic cells.

Apoptotic and dying cells have been shown to send a danger signal that can regulate the immune system and enhance inflammation (31–33).

**Discussion**

The FMI is a complex environment because multiple mechanisms operate to protect the fetus against attack by the maternal immune system. These include complement system (34, 35), catabolism of tryptophan by IDO (27), regulation by galectin-1 (36), regulatory T cells (4, 37–39), and T cell apoptosis (40). Previously, we described a role for the inhibitory costimulatory molecule PD-L1 in maintaining tolerance at the FMI (5, 6, 41).

In this study, we have examined the role of TIM-3 molecule and contributions of macrophage and myeloid cells to fetomaternal tolerance, areas that have not been explored till date. TIM-3 is expressed on the surface of terminally differentiated T cells, DCs, and macrophages. On the surface of T cells, TIM-3 binds to galectin-9 and inhibits Th1 response by triggering T cell death (12, 42). On APCs, TIM-3 synergizes with TLR and increases the secretion of proinflammatory cytokines (22). TIM-3 also functions as a phosphatidylserine receptor and is involved in the clearance of apoptotic cells and Ag cross-presentation (23, 43). Our study demonstrates the involvement of TIM-3 in promoting tolerance at the FMI.

We observed that TIM-3 is expressed on the surface of innate immune cells, namely uterine macrophages and IMC/MDSC in the CBA/CaJ (× C57BL/6) model of allogeneic pregnancy. Our study shows an important role for TIM-3 in modulating the function of these two cell types. This is a significant finding given that innate immune cells constitute >80% of the cells found in the decidua/uterus during pregnancy (26, 44).

During pregnancy, apoptosis is particularly seen in trophoblast and uterine NK cells (45, 46). Apoptosis of these cells is increased in threatened pregnancy situations such as pre-eclampsia and intrauterine growth restriction (47, 48). Trophoblast cells express paternal Ags. Therefore, mechanisms must exist to clear apoptotic trophoblast cells so that the cells themselves or the paternal Ags released by these cells do not cause tissue damage and fetal rejection (49). Macrophages have been shown to play a key role in...
mediating the resolution of inflammatory conditions by phagocytosing and clearing apoptotic cells (50). Our data show that following TIM-3 blockade, apoptotic cells accumulate at the uteroplacental interface because of a deficiency in the phagocytic capacity of uterine macrophages, leading to inflammatory events and subsequent fetal rejection (Figs. 4, 6A, 7, 8).

Our data do not support the hypothesis that reduced phagocytosis of apoptotic cells by macrophages results in reduced presentation of Ags and hence reduced alloimmune responses. Rather, our alloimmune response data obtained by stimulating splenocytes from pregnant females with irradiated stimulants from allogeneic males showed no differences in Th1/Th2 or Th17 alloimmune responses following TIM-3 blockade (data not shown). Also, we did not see a difference in the size of DC population subtypes (CD11c+11bhi, CD11c+11bmo, and CD11c+11bneg) following anti-TIM-3 treatment (data not shown). Therefore, it is unlikely that phagocytosis and cross presentation of cell-associated Ags with MHC class I molecules is mediated by DCs (31, 51–53). Further studies are needed to determine if cross presentation of Ags by DCs is affected despite unchanged size of population subtypes.

MDSC, and CD11b+Ly6CintGhi granulocytes, can be distinguished. Cells with CD11b+Ly6CintGhi phenotype have no suppressive activity (54). G-MDSC and M-MDSC are capable of suppressing T cells (55) and are shown to modulate immune responses. TIM-3 blockade has been shown to increase disease severity in experimental autoimmune encephalitis model with a concurrent increase in infiltration of activated macrophages at the site of injury (10). TIM-3 expression was also observed in MDSC (Fig. 3). Based on the timed expression of either Ly6C or Ly6G, three populations, CD11b+Ly6C+Gm-MDSC, CD11b+Ly6C+Gneg M-MDSC, and CD11b+Ly6C+Ghi granulocytes, can be distinguished. Cells with CD11b+Ly6C+Gm-MDSC phenotype have no suppressive activity (54). G-MDSC and M-MDSC are capable of suppressing T cells (55) and are shown to modulate immune response in various disease models (54, 56–60). All three subtypes were observed to express TIM-3 in this study (Fig. 3). CD11b+Ly6C+Gneg M-MDSC and CD11b+Ly6C+Ghi inflammatory granulocytes (Fig. 1) predominated at the uteroplacental interface. Results obtained in this study suggest that both M-MDSC and CD11b+Ly6C+Ghi cells become activated as a consequence of inflammatory changes occurring in the uterus. Activated M-MDSC upregulate iNOS and arginase 1 (Fig. 6B), and CD11b+Ly6C+Ghi granulocytes upregulate the expression of TNF-α and IFN-γ in response to treatment or inflammation (Fig. 6A). Sorption observed in this study could be a result of the failure of M-MDSC to counterbalance the effect of inflammatory CD11b+Ly6C+Ghi granulocytes. TIM-3 blockade has been shown to result in proinflammatory cytokine release in a model of liver ischemia (61). Also, in a TIM-3–transgenic mouse model, overexpression of TIM-3 on T cells resulted in an expansion of CD11b+Gr1+F4/80G-MDSC and suppression of T cell responses, whereas other cellular compartments remained unchanged between transgenic mice and littermate control (24).

We conclude that TIM-3 blockade results in a decrease in phagocytic properties of uterine macrophages, leading to a failure to clear apoptotic and dead cells from the uterus. This leads to accumulation of apoptotic cells at the uteroplacental interface, causing activation of inflammatory granulocytes. The result is increased local inflammation and fetal rejection. Future studies will address the relative contributions of various innate immune cells in TIM-3–modulated fetomaternal tolerance and interactions between them.

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Disclosures

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References