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Galectin-1 confers immune privilege to human trophoblast: implications in recurrent fetal loss

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Mechanisms accounting for the protection of the fetal semi-allograft from maternal immune cells remain incompletely understood. In previous studies, we showed that galectin-1 (Gal1), an immunoregulatory glycan-binding protein, hierarchically triggers a cascade of tolerogenic events at the mouse fetomaternal interface. Here, we show that Gal1 confers immune privilege to human trophoblast cells through the modulation of a number of regulatory mechanisms. Gal1 was mainly expressed in invasive extravillous trophoblast cells of human first trimester and term placenta in direct contact with maternal tissue. Expression of Gal1 by the human trophoblast cell line JEG-3 was primarily controlled by progesterone and pro-inflammatory cytokines and impaired T-cell responses by limiting T cell viability, suppressing the secretion of Th1-type cytokines and favoring the expansion of $CD4^+CD25^+FoxP3^+$ regulatory T (T_{reg}) cells. Targeted inhibition of Gal1 expression through antibody (Ab)-mediated blockade, addition of the specific disaccharide lactose or retroviral-mediated siRNA strategies prevented these immunoregulatory effects. Consistent with a homeostatic role of endogenous Gal1, patients with recurrent pregnancy loss showed considerably lower levels of circulating Gal1 and had higher frequency of anti-Gal1 auto-Abs in their sera compared with fertile women. Thus, endogenous Gal1 confers immune privilege to human trophoblast cells by triggering a broad tolerogenic program with potential implications in threatened pregnancies.

Keywords: fetal loss / fetomaternal tolerance / galectin-1 / pregnancy / progesterone

Introduction

An essential feature of successful mammalian reproduction is maternal tolerance to the presence of semi-allogeneic fetus (Trowsdale and Betz 2006; Terness et al. 2007). Multiple tolerance mechanisms can operate at the fetomaternal interface including a shift toward a T helper (Th) type 2 cytokine profile (Blois et al. 2004; Chaouat 2007), differentiation and/or recruitment of $CD4^+CD25^+$ T regulatory (T_{reg}) cells (Aluvihare et al. 2004; Zenclussen et al. 2005; Kallikourdis et al. 2007; Fainboim and Arruvito 2011; Ramhorst et al. 2012) and expansion of decidual natural killer (NK) cells (Koopman et al. 2003; Kopcow et al. 2008). Moreover, other immunoevasive programs have been described including the promotion of apoptosis of uterine T cells via the Fas ligand (FasL) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand pathways (Phillips et al. 1999; Ohshima et al. 2001; Kayisli et al. 2003), activation of inhibitory signals such as programmed death ligand-1 (PD-L1; Guleria et al. 2005) and immunomodulation by neuropeptides and chemokines (Fraccaroli, Alfieri, Larocca, Calafat, Mor, et al. 2009; Fraccaroli, Alfieri, Larocca, Calafat, Roca, et al. 2009). In addition, a functional crosstalk between immune and endocrine systems has been reported to be crucial in the regulation of fetomaternal tolerance. Through direct or indirect mechanisms, progesterone, progesterone-induced blocking factor and human chorionic gonadotropin contribute to immune regulation during pregnancy (Arck et al. 2007; Arruvito et al. 2008).

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During the past few years, there has been an increasing appreciation for the impact of lectin–glycan interactions in the control of immune tolerance, homeostasis and inflammation (Kreisman and Cobb 2012; Rabinovich and Croci 2012). Gal1, a "proto-type" member of an evolutionarily conserved family of glycan-binding proteins (Rabinovich, Rubinstein, et al. 2002; Vasta 2012), elicits a broad spectrum of immunoregulatory activities in vivo including the resolution of autoimmune inflammation (Rabinovich et al. 1999; Toscano et al. 2006, 2007; Perone et al. 2009) and the promotion of tumor-immune escape (Rubinstein et al. 2004; Juszczynski et al. 2007; Banh et al. 2011; Soldati et al. 2011; Cedeno-Laurent, Watanabe, et al. 2012). Investigation of the mechanisms underlying these immunoregulatory effects revealed the ability of Gal1 to differentially regulate the survival of Th cell subsets (Toscano et al. 2007; Motran et al. 2008), to control T cell trafficking (He and Baum 2006; Norling et al. 2008) and to promote the differentiation of tolerogenic dendritic cells (DCs; Ilarregui et al. 2009; Kuo et al. 2011). Blockade of Gal1 expression in tumor tissue led to heightened T cell-mediated tumor rejection, increased secretion of Th1-type cytokines and decreased angiogenesis (Rubinstein et al. 2004; Thijssen et al. 2006; Juszczynski et al. 2007). In addition, Gal1-deficient (*Lgals1*$^{-/-}$) mice show greater Th1 and Th17 responses and were considerably more susceptible to autoimmune inflammation than their wild-type (WT) counterpart (Toscano et al. 2007), suggesting an essential role for this endogenous lectin in controlling immune tolerance and homeostasis.

Research over the past few years has identified essential roles of galectins, particularly Gal1, during the establishment and maintenance of normal pregnancy (Than et al. 2012). Gal1 is abundant in the placenta and female reproductive tract of various species (Phillips et al. 1996; Maquoi et al. 1997; Iglesias et al. 1998; Bozic et al. 2004; von Wolff et al. 2005; Dong et al. 2008) and is substantially increased in the late secretory-phase endometrium (von Wolff et al. 2005) and uterine decidual NK cells (Koopman et al. 2003). Using an established mouse model of stress-induced pregnancy failure, we found that *Lgals1*$^{-/-}$ mice had higher rates of fetal loss compared with WT mice in allogeneic, but not in syngeneic matings (Blois et al. 2007). Administration of recombinant Gal1 prevents fetal loss and restores tolerance in vivo (Blois et al. 2007). Interestingly, subsequent studies indicated that decidual NK cells induce the apoptosis of effector T cells via the secretion of Gal1 (Kopcow et al. 2008).

We undertook this study to further understand the pathophysiologic role of Gal1 in normal and pathologic human pregnancies. We found that this lectin is mainly expressed in invasive extravillous trophoblast cells of human first trimester and term placenta, is regulated by progesterone and pro-inflammatory cytokines and confers immune privilege to human trophoblast cells by limiting T cell viability, dampening the secretion of Th1-type cytokines and favoring the expansion of $CD4^+CD25^+FoxP3^+$ T_{reg} cells. Consistent with these findings, patients with recurrent pregnancy loss had considerably lower levels of circulating Gal1 and higher frequency of anti-Gal1 auto-antibodies (Abs) in their sera compared with fertile women. Our results suggest that Gal1 is part of the immunoevasive program displayed by human trophoblast cells with critical implications in threatened pregnancies.

Results

Expression and localization of Gal1 in human first trimester and term placenta

To investigate the potential role of Gal1 in human pregnancy, we first examined the expression and localization of this protein in trophoblast and non-trophoblast tissue of human placenta. In representative tissue samples, the expression of Gal1 was high in first trimester and term placenta (Figure 1A and B). In the first trimester and term placenta, Gal1 staining was prominent in extravillous cytotrophoblast cells located within decidua basalis, distal parts of cell islands and cell columns and in the basal plate, but was low or undetectable in trophoblast giant cells, villous cytotrophoblast and the syncytiotrophoblast (Figure 1A). In addition, Gal1 staining was prominent in decidua cells, villous endothelial and stroma cells (Figure 1A). However, the expression of this lectin was variable (up- or down-regulated) in the primary tissue of hydatidiform moles, suggesting its altered expression in gestational trophoblast diseases (Figure 1B). Moreover, Gal1 was highly expressed in the human choriocarcinoma cell line JEG-3, which recapitulates the phenotypical and functional properties of human trophoblast (Figure 1B). Thus, Gal1 is present, although at different extents, in normal and transformed invasive extravillous trophoblast in direct contact with maternal tissues.

Gal1 contributes to the immunosuppressive activity of human trophoblast cells

To investigate whether Gal1 contributes to the immunosuppressive activity displayed by human trophoblast cells, we conducted a series of functional studies using different in vitro approaches. The first approach involved the study of the contribution of Gal1 secreted by JEG-3 cells to the modulation of T-cell immunity (Figure 2A–E). JEG-3 cells not only synthesized but also secreted substantial amounts of Gal1 to the extracellular medium (Figure 2A). Peripheral blood mononuclear cells (PBMCs) were polyclonally activated with the T cell mitogen phytohemagglutinin (PHA) and cultured in the absence or the presence of serum-free conditioned medium (CM) obtained from the JEG-3 cell line. Addition of JEG-3 CM to PBMC cultures induced a dose-dependent inhibition of T-cell proliferation, as shown by thymidine uptake ($P < 0.01$, Student's *t*-test; Figure 2B). To investigate the contribution of Gal1 to the suppressive activity of JEG-3 CM, we measured the T-cell proliferative response in the absence or the presence of an anti-Gal1 polyclonal immunoglobulin G (IgG) previously shown to have neutralizing activity (Rubinstein et al. 2004). Notably, Ab-mediated Gal1 blockade prevented the inhibition of T-cell proliferation induced by JEG-3 CM (Figure 2B). Notably, the T-cell suppressive activity of JEG-3 CM was even more pronounced when polyclonally activated PBMCs were further cultured in the presence of an optimal concentration of progesterone (10^{-5} M; Van Voorhis et al. 1989; Figure 2C). This suppressive effect was

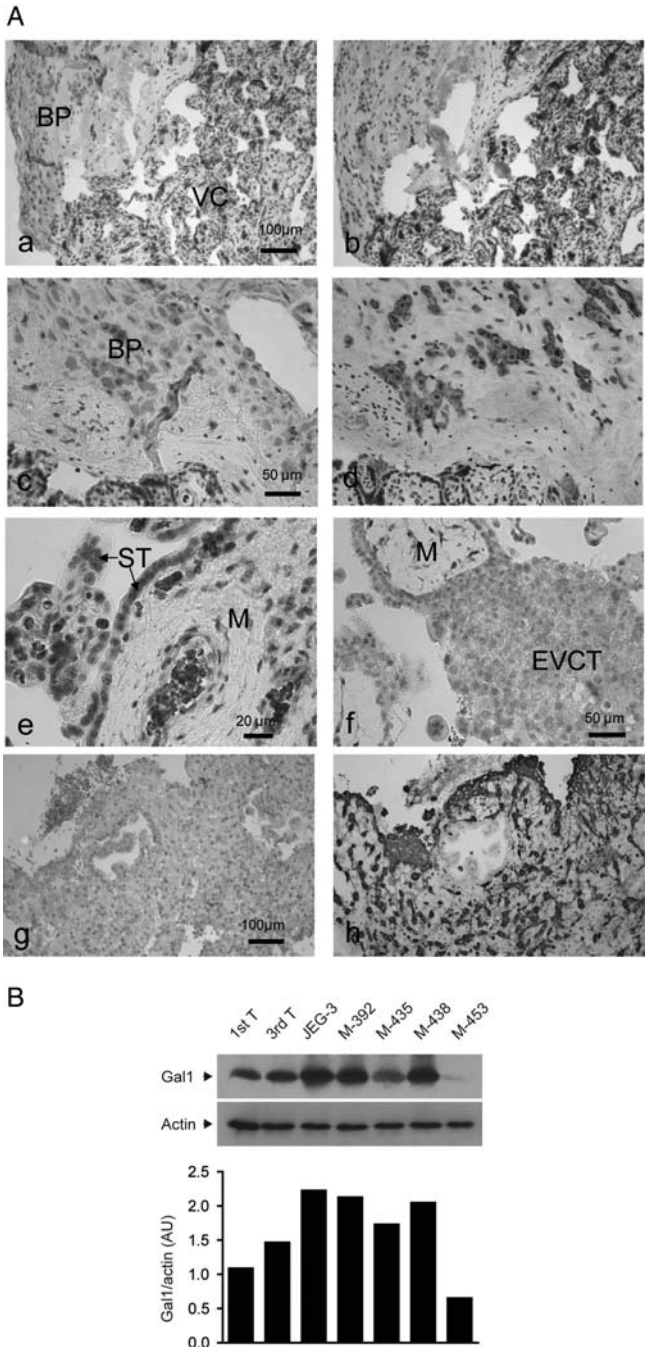

Fig. 1. Expression and localization of Gal1 in normal and pathological human placental tissue. (**A**) Immunohistochemistry of term (a–e) and first trimester (f–h) placenta. (a) shows the expression of Gal1 in the basal plate (BP) and the villous chorion (VC) when compared with the respective serial section negative control (b), where the primary anti-Gal1 Ab was replaced by non-immune rabbit IgG. Staining of serial sections with Ab recognizing Gal1 (c) or HLA-G (an extravillous trophoblast marker) (d) reveals that both decidua cells and the extravillous cytotrophoblast in the BP are positive for Gal1. (e) shows Gal1 in the villous mesenchyme (M), whereas the syncytiotrophoblast (ST) is negative. (f) shows the expression of Gal1 in the distal part of the extravillous cytotrophoblast (EVCT) cell column of an anchoring villus and in the mesenchyme (M). In trophoblast-invaded decidua basalis (g and h), both decidua cells and the extravillous trophoblast are positive for Gal1 (g), as revealed by a serial section where only the extravillous cytotrophoblast is stained for HLA-G (h). (**B**) Western blot analysis of Gal1 expression in whole cell lysates obtained from normal human placental tissue from first (1^{st}T) or third (3^{rd}T) trimester, JEG-3 choriocarcinoma cell line and primary tissue from several hydatidiform moles (M-329, M-435, M-438 and M-453). Equal amounts of total protein were subjected to SDS–PAGE on a 15% polyacrylamide gel, transferred onto nitrocellulose membranes and immunoblotted with a rabbit anti-Ga1 or a rabbit anti-actin polyclonal Ab. Immunoreactive protein bands were semi-quantified by densitometry and expressed as relative arbitrary units (AU) relative to β-actin. Data are representative of three independent experiments.

partially prevented when the anti-Gal1 Ab was incorporated into PBMC cultures (Figure 2C; $P < 0.01$, Student's *t*-test).

To study the mechanisms underlying the T-cell inhibitory effects of Gal1 and given the ability of this lectin to control the viability of activated and alloreactive T cells (Perillo et al. 1995; Pace et al. 1999; Rabinovich, Ramhorst, et al. 2002), we evaluated the contribution of T cell apoptosis to Gal1-mediated JEG-3-induced immunosuppression. Polyclonally activated PBMCs were cultured with JEG-3 CM in the absence or the presence of the specific anti-Gal1 polyclonal IgG, and T cell apoptosis was analyzed by annexin-V staining in the CD3-gated cell population. We found an increase of up to 22% in the frequency of apoptotic $CD3^+$ T cells when PBMCs were exposed to JEG-3 CM (Figure 2D). This effect was partially prevented when PBMC cultures were exposed to JEG-3 CM in the presence of the anti-Gal1 blocking IgG (Figure 2D; $P < 0.01$, Student's *t*-test). To confirm these findings, we developed an in vitro system by co-culturing JEG-3 cells with polyclonally activated PBMCs. In this second approach, JEG-3 subconfluent cells were co-cultured with PBMCs in the absence or the presence of the anti-Gal1 neutralizing IgG. We found a considerably lower frequency of annexin-V^+ T cells when the anti-Gal1 Ab was incorporated to co-cultures (Figure 2E; $P < 0.01$, Student's *t*-test). Collectively, these findings highlight the contribution of endogenous Gal1 to the pro-apoptotic activity of JEG-3 cells.

Since the induction of a local anti-inflammatory microenvironment is crucial for the control of a pro-inflammatory response occurring at early stages of human embryo implantation (Mor 2008) and Gal1 influences cytokine secretion through mechanisms that are independent of apoptosis (Rabinovich, Ramhorst, et al. 2002; Stowell et al. 2008), we then examined whether Gal1 secreted by JEG-3 cells controls cytokine secretion. Polyclonally activated PBMCs were cultured with JEG-3 CM in the absence or the presence of the anti-Gal1 blocking Ab. In addition, the direct effects of rhGal1 were also analyzed as a control. After 72 h, we collected PBMC supernatants and measured cytokine production by enzyme-linked immunosorbent assay (ELISA). Addition of JEG-3 CM to PBMCs led to a considerable reduction in interferon (IFN)-γ production, while substantially increased the amounts of interleukin (IL)-10 and IL-5 secretion; this effect was prevented when the anti-Gal1 neutralizing Ab was incorporated into PBMC cultures (Figure 3A–C; $P < 0.01$, Student's *t*-test). Of note, a similar effect was observed when we examined cytokine secretion following the co-culture of JEG-3 cells with activated PBMCs. This cytokine shift was prevented when co-cultures were performed in the presence of the anti-Gal1 neutralizing Ab or the specific disaccharide

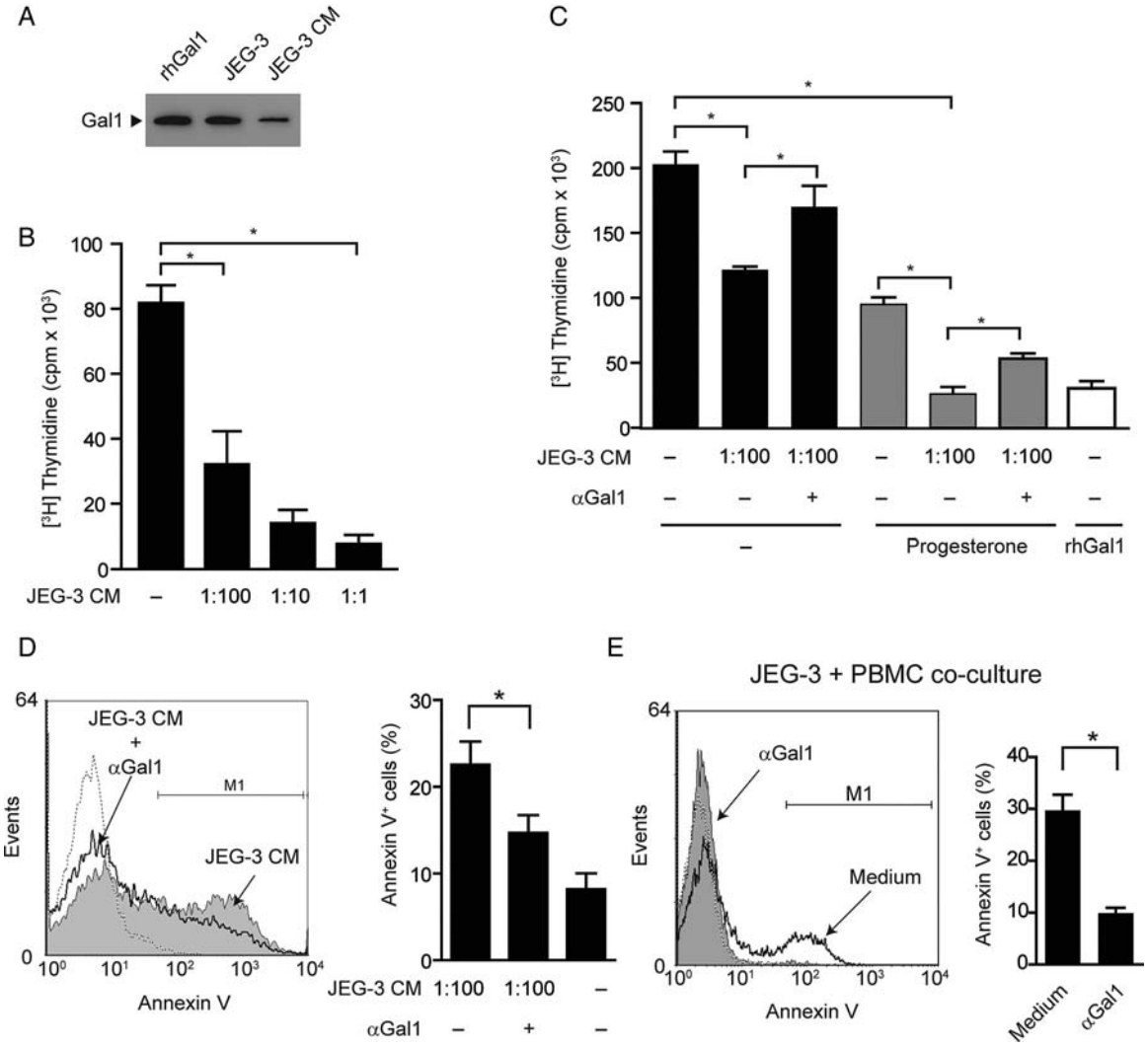

Fig. 2. Gal1 contributes to JEG-3-induced immunosuppression by controlling the viability of activated T cells. (**A**) Immunoblot analysis of secreted Gal1 in serum-free CM collected from 16 h semi-confluent JEG-3 cell cultures. Soluble proteins from JEG-3 CM were concentrated by precipitation with acetone and further subjected to SDS–PAGE (15 µg) and western blot analysis using a rabbit anti-Gal1 polyclonal Ab. rhGal1 and JEG-3 total cell lysates were used as positive controls. Data are representative of three independent experiments. (**B** and **C**) Human PBMCs were activated with PHA (1 µg/mL) and cultured during 72 h in the absence or the presence of serial dilutions of JEG-3 CM. Human PBMCs were also cultured with JEG-3 CM (dilution 1:100) in the absence or the presence of the anti-Gal1 polyclonal IgG (1:100) or progesterone (10^{-5} M). Exposure to rhGal1 (40 µg/mL) was used as a positive control. The proliferative response was monitored by [^{3}H]-thymidine incorporation. Results are expressed as cpm (mean ± SEM) of triplicate determinations of three independent experiments with a total of six donors individually analyzed. (**D**) Human PBMCs were activated with PHA for 72 h and further incubated for an additional 18 h with JEG-3 CM in the absence or the presence of the anti-Gal1 polyclonal IgG (1:100). T cell apoptosis was determined by staining the cells with fluorescein isothiocyanate (FITC)-annexin-V and phycoerythrin-labeled anti-CD3 mAb. Data are expressed as the percentage of annexin-V$^+$ cells within the CD3$^+$ population. Results are representative (left) or are the mean ± SEM (right) of three independent experiments of a total of three different donors individually analyzed. (**E**) JEG-3 cells (60% confluent) were co-cultured with human PBMCs in the absence or the presence of anti-Gal1 neutralizing IgG (1:100) for 48 h. PBMCs were recovered and cell death was analyzed by FITC-annexin-V and PE-anti-CD3 mAb. Data are expressed as the percentage of annexin-V$^+$ cells within the CD3$^+$ population. Results are representative (left) or are the mean ± SEM (right) of three independent experiments of three different donors analyzed. *$P < 0.01$, Student's *t*-test.

lactose (Figure 3D–F; $P < 0.01$, Student's *t*-test). However, addition of sucrose, a non-specific disaccharide, had no preventive effect (data not shown). Thus, endogenous Gal1 could also confer immune privilege to human trophoblast by altering the balance of pro- and anti-inflammatory cytokines. Remarkably, endogenous Gal1 promoted a robust increase in IL-10 (Figure 3B and E), an immunoregulatory cytokine that is essential for the promotion of fetomaternal tolerance (Trowsdale and Betz 2006).

Since T_{reg} cells are key components of tolerogenic programs during pregnancy (Leber et al. 2010) and Gal1 promotes T_{reg} cell expansion in murine pregnancy (Blois et al. 2007), we then evaluated whether Gal1 synthesized by human JEG-3 cells drives the expansion of T_{reg} cells. For this

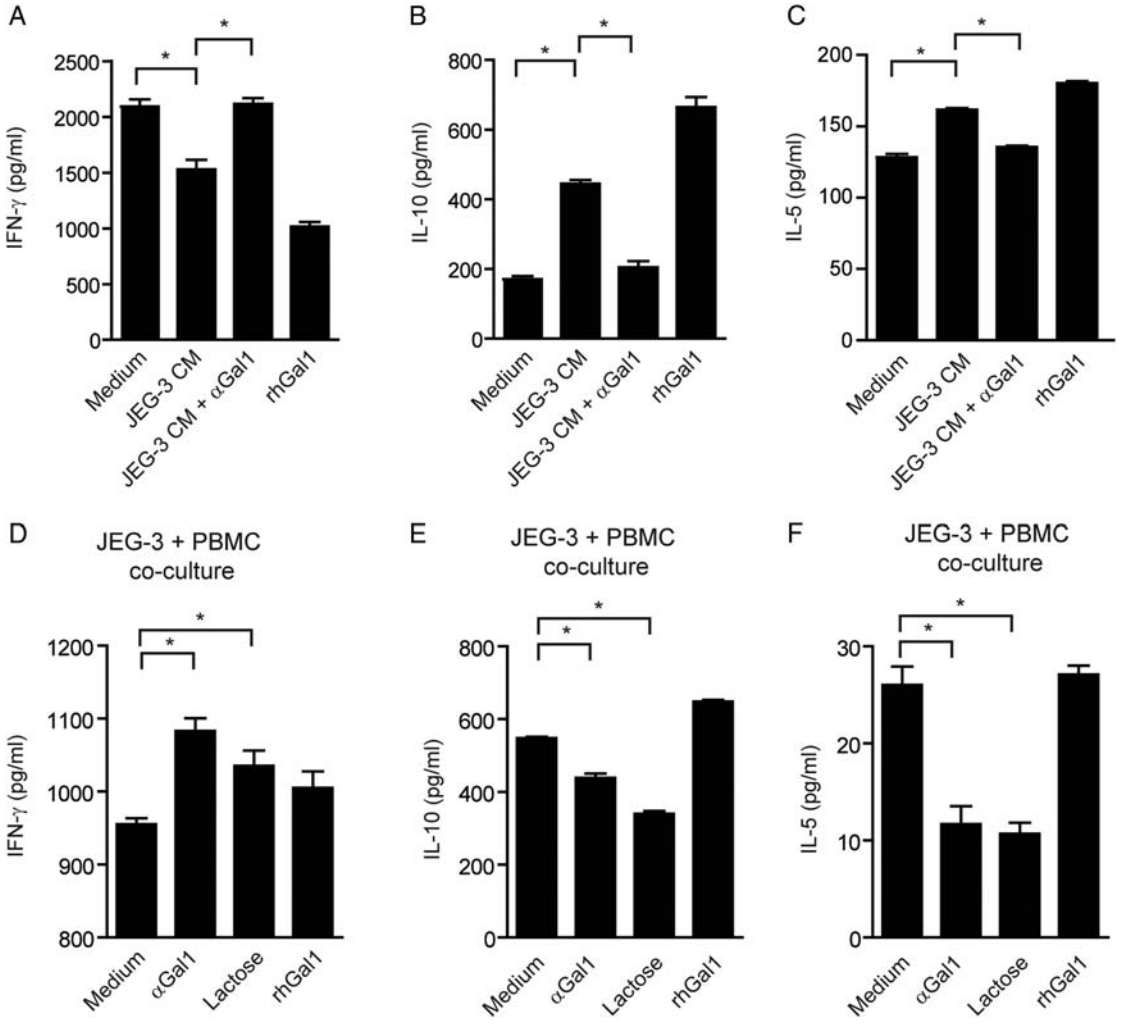

Fig. 3. Gal1 secreted by JEG-3 cells controls the balance of pro-inflammatory and anti-inflammatory cytokines. (**A**–**C**) Human PBMCs were activated with PHA (1 μg/mL) and cultured with CM from JEG-3 cells (dilution 1:100) in the absence or the presence of anti-Gal1 neutralizing IgG (1:100) during 72 h. PBMCs were also activated in the presence of rhGal1 (40 μg/mL). Results are the mean ± SEM of three independent experiments of three different donors individually analyzed. (**D**–**F**) JEG-3 cells (60% confluent) were co-cultured with PBMCs in the absence or the presence of anti-Gal1 neutralizing IgG (1:100) or lactose (30 mM) for 48 h. PBMCs were also cultured in the presence of rhGal1 (40 μg/mL). In both experimental settings, culture supernatants were collected to analyze the amounts of secreted cytokines (IFN-γ, IL-10 and IL-5) by capture ELISA. Results are the mean ± SEM of three independent experiments of three different donors individually analyzed. *$P < 0.01$, Student's *t*-test.

purpose, we assessed the frequency of $CD4^+CD25^+FoxP3^+$ T_{reg} cells within polyclonally activated PBMCs cultured for 72 h with JEG-3 CM in the absence or the presence of the anti-Gal1 neutralizing Ab or lactose. Incubation of PHA-activated PBMCs with CM from JEG-3 cells led to a considerable increase in the percentage of $CD25^+FoxP3^+$ cells within the CD4-gated T-cell population (Figure 4A). Incorporation of the anti-Gal1 monoclonal Ab (mAb) or the galectin-specific disaccharide lactose not only prevented the induction of T_{reg} cells by JEG-3 CM, but also induced a reduction in the frequency of T_{reg} cells, when compared with cells cultured with medium alone (Figure 4A; $P < 0.01$, Student's *t*-test), suggesting the essential contribution of endogenous Gal1 to trophoblast-induced T_{reg} cell expansion. As expected, CM from JEG-3 cells induced a higher frequency of T_{reg} cells in comparison with T cells directly exposed to rhGal1, suggesting the contribution of other soluble mediators to JEG-3-induced immunosuppression. These findings were verified by the western blot analysis of FoxP3 expression in PBMCs exposed to different stimuli (Figure 4B).

To confirm the contribution of endogenous Gal1 to the induction of T_{reg} cells, we knocked down Gal1 in JEG-3 cells using siRNA-mediated silencing strategies. Transduction of JEG-3 cells with retroviral vectors expressing a scrambled (Scr) oligonucleotide was used as a negative control. The efficiency of Gal1 silencing was verified by western blot analysis (Figure 4C). Polyclonally activated PBMCs were cultured with CM obtained from Gal1 knockdown JEG-3 cells (CM KD) or from JEG-3 cells transduced with a Scr control oligonucleotide (CM Scr). Targeted inhibition of

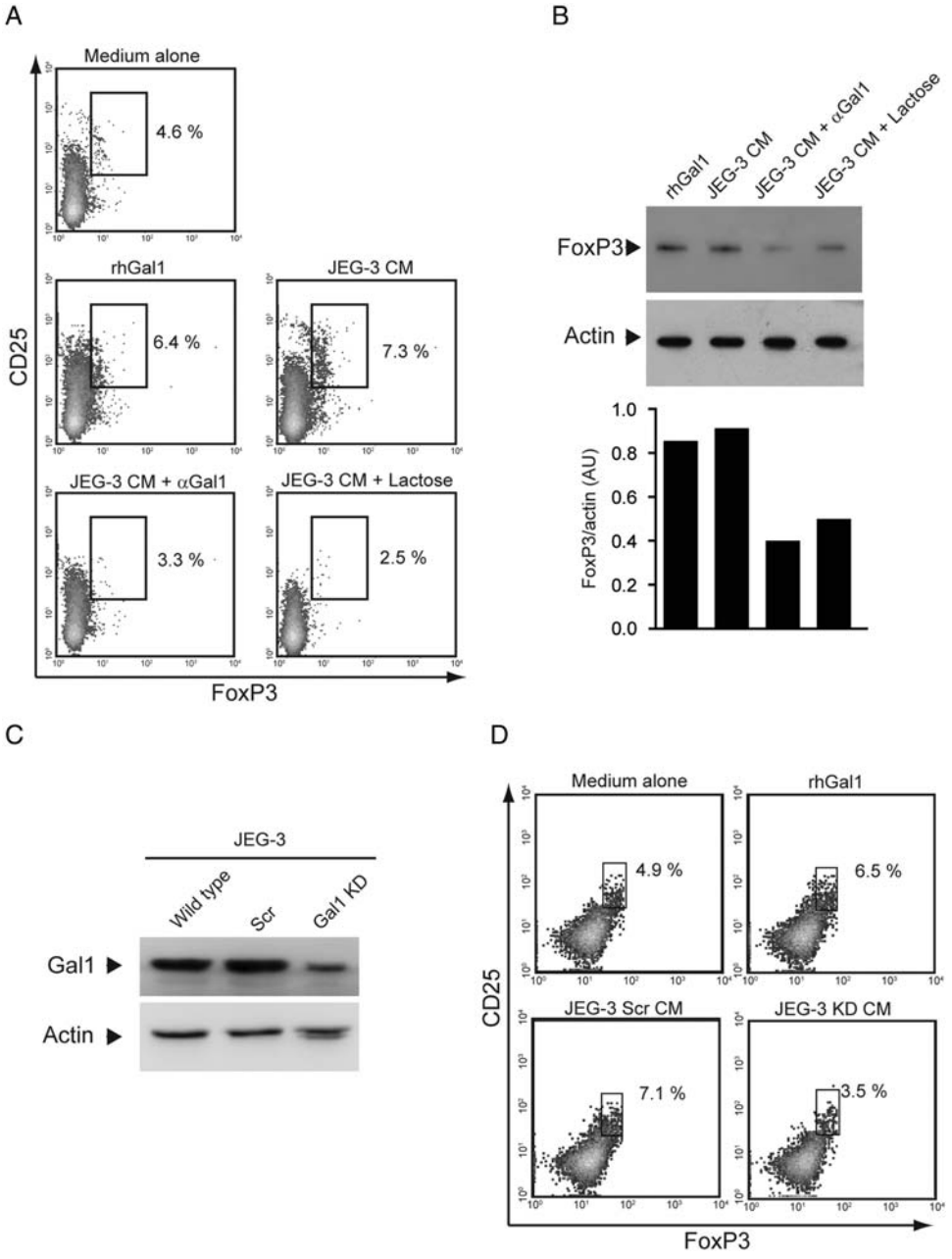

Fig. 4. Gal1 produced by JEG-3 cells increases the frequency of T_{reg} cells. (**A** and **B**) PBMCs were activated with PHA (1 μg/mL) and cultured with JEG-3 CM (dilution 1:100) in the absence or the presence of the anti-Gal1 neutralizing IgG (1:100) or lactose (30 mM) for 72 h. PBMCs were also activated in the presence of rhGal-1 (40 μg/mL). (A) The percentage of $CD25^+FoxP3^+$ T_{reg} cells was analyzed in the CD4-gated T cell population by flow cytometry. Results are representative of three independent experiments of three donors individually analyzed. (B) Western blot analysis of FoxP3 expression. Equal amounts of protein lysates (30 μg) were subjected to SDS–PAGE on a 10% polyacrylamide gel, transferred onto nitrocellulose membranes and immunoblotted with goat-anti-FoxP3 Ab and rabbit anti-actin polyclonal Ab. Immunoreactive protein bands were semi-quantified relative to actin and expressed as arbitrary units (AU). Results are representative of three independent experiments of three donors individually analyzed. (**C**) Western blot analysis of Gal1 and actin expression in JEG-3 cells transduced or not with retroviral vector expressing Gal1 siRNA or an Scr oligonucleotide sequence. Results are representative of four independent experiments. (**D**) PBMCs were activated with PHA (1 μg) and cultured in the absence or the presence of CM from Gal1 knockdown JEG-3 cells (JEG-3 KD CM; 1:100) or Scr-transfected JEG-3 cells (JEG-3 Scr CM) for 72 h. PBMCs were recovered and the frequency of $CD4^+CD25^+FoxP3^+$ T cells was analyzed by flow cytometry. Results are expressed as the percentage of $CD25^+Foxp3^+$ cells within the $CD4^+$ T cell population. Results are representative of three independent experiments of three donors individually analyzed.

Gal1 gene (*Lgals1*) expression considerably prevented induction of T_{reg} cells by JEG-3 CM (Figure 4D; $P < 0.01$, Student's *t*-test).

Collectively, our results indicate that endogenous Gal1 may confer immune privilege to human trophoblast cells through mechanisms involving induction of T cell apoptosis, modulation

of the pro-inflammatory/anti-inflammatory cytokine balance and control of T_{reg} cell frequency.

Progesterone and pro-inflammatory cytokines up-regulate Gal1 expression in human JEG-3 cells

Given the immunoregulatory role of progesterone during pregnancy (Arck et al. 2007), we examined whether this hormone regulates Gal1 expression in human trophoblast. Similar to the effects of progesterone in mouse pregnancy (Blois et al. 2007), progesterone induced a dose-dependent up-regulation of Gal1 in human JEG-3 cells (Figure 5A). Furthermore, the exposure of JEG-3 cells to recombinant human (rh) IL-2 or rhTNF-α led to a substantial up-regulation of Gal1 (Figure 5B). In contrast, a modest decrease in Gal1 expression was observed when JEG-3 cells were exposed to rhIL-12 or rhIL-4 (Figure 5B). Thus, Gal1 appears to be a transcriptional target of both progesterone and pro-inflammatory cytokines in human trophoblast cells, suggesting its potential role at early stages of the implantation period where these mediators play a pivotal role.

Levels of circulating Gal1 and frequency of anti-Gal1 auto-Abs in sera from patients with recurrent spontaneous abortions

Given the higher rates of fetal loss observed in *Lgals1*$^{-/-}$ compared with WT mice in allogeneic matings (Blois et al. 2007) and the important contribution of Gal1 to the immunosuppressive activity of human JEG-3 cells, we next determined the levels of circulating Gal1 and the prevalence of anti-Gal1 auto-Abs in sera from patients with recurrent spontaneous abortion (RSA; $n = 76$) and sera collected from fertile women (defined as women with at least two successful pregnancies; $n = 48$). Using a capture ELISA, we detected substantially lower amounts of circulating Gal1 in sera from RSA patients compared with fertile women (Figure 6A; $P < 0.01$, Mann–Whitney U-test). On the other hand, using a direct ELISA designed to detect serum anti-Gal1 auto-Abs (Romero et al. 2006), we found higher prevalence of Gal1-immunoreactive sera in RSA patients ($n = 66$ immunoreactive sera) compared with fertile women in which only 10 sera immunoreacted with rhGal1 (Table I). Immunoreactivity of representative sera from RSA patients and fertile women was confirmed by western blot analysis (Figure 6B). Thus, lower levels of circulating Gal1 correlate with higher prevalence of anti-Gal1 auto-Ab in sera from RSA patients compared with fertile women.

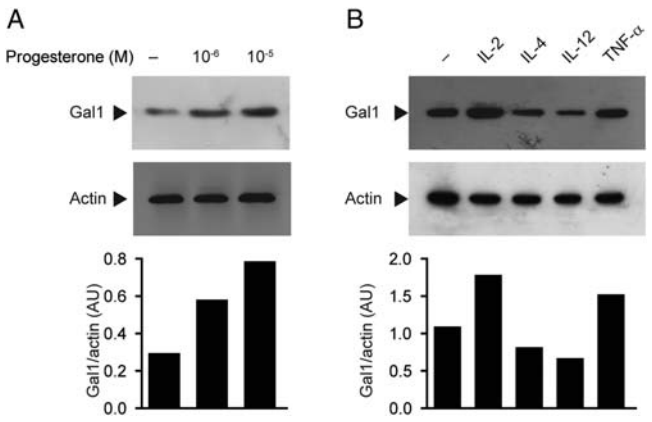

Fig. 5. Progesterone and pro-inflammatory cytokines up-regulate Gal1 expression in human JEG-3 cells. (**A** and **B**) Subconfluent JEG-3 cells were incubated in the absence or the presence of progesterone (10^{-5} or 10^{-6} M), rhIL-2 (8 ng/mL), rhTNF-α (10 ng/mL), rhIL-12 (2 ng/mL) and rhIL-4 (5 ng/mL) for 24 h. Equal amounts of protein (10 μg) from whole cell lysates were subjected to SDS–PAGE on a 15% polyacrylamide gel, transferred onto nitrocellulose membranes and immunoblotted with rabbit anti-Gal1 or anti-actin polyclonal Ab. Immunoreactive protein bands were semi-quantified by densitometry and expressed as arbitrary units (AU) relative to β-actin. Results are representative out of four independent experiments.

Discussion

Trophoblast cells play a key role in preventing allorecognition by displaying multiple immunoevasive programs (Mor and Cardenas 2010). These include the expression of HLA-G which prevents killing by maternal NK cells, release of pro-apoptotic molecules such as FasL, expression of immune inhibitory molecules such as PD-L1, expansion of T_{reg} cells and synthesis of immunosuppressive cytokines (Terness et al. 2007; Saito et al. 2010). In addition, placental hormones,

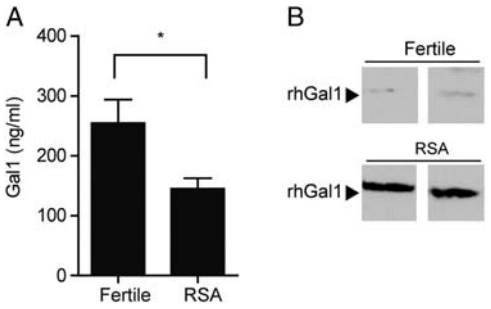

Fig. 6. Circulating levels of soluble Gal1 and anti-Gal1 auto-Ab in sera from RSA patients and fertile women. (**A**) Serum samples from women with RSA ($n = 76$) and women who had at least two successful pregnancies (fertile; $n = 48$) were analyzed by capture ELISA to determine the levels of circulating Gal1. Results are expressed as the media of Gal1 in ng/mL ± SEM of sera from RSA patient vs fertile women. *$P < 0.01$. (**B**) IgG immunoreactivity against rhGal1 (western blot analysis) of two representative sera from RSA patients and fertile women. Analysis of the prevalence of anti-Gal1 mAb in patients with RSA and fertile women by ELISA is shown in Table I.

Table I. Prevalence of serum anti-Gal1 auto-Abs in populations of RSA patients and fertile women

	Reactive sera	Non-reactive sera	Total
RSA patients	66	10	76
Fertile women	10	38	48
Total	76	48	124

Serum samples from women with RSA ($n = 76$) and women who had at least two successful pregnancies (fertile; $n = 48$) were analyzed by direct ELISA to determine the prevalence of anti-Gal1 auto-Abs (Gal1-immunoreactive sera). Reactivity was considered positive if optical density was 2 or more standard deviations above the mean of normal values (cut-off). Chi-square test: df, 54.03; $P < 0.0001$.

mainly progesterone, favor immune tolerance by controlling regulatory cell networks during pregnancy (Arck et al. 2007). In contrast, syncytiotrophoblast cells at the surface of chorionic villi maintain a state of mild systemic inflammation via the activation of innate immunity (Mor 2008).

Using a model of stress-induced pregnancy failure, we previously demonstrated a pivotal role for Gal1 in fetomaternal tolerance through the amplification of a cascade of immunosuppressive events (Blois et al. 2007). Mice lacking Gal1 (*Lgals1*$^{-/-}$) showed higher rates of fetal loss compared with their WT counterpart in allogeneic, but not syngeneic matings (Blois et al. 2007). Although original studies showed that *Lgals1*$^{-/-}$ mice developed normally and did not show any obvious phenotypic abnormality (Poirier and Robertson 1993), recent findings demonstrated significant immunological alterations in mice lacking Gal1 (Blois et al. 2007; Toscano et al. 2007; Rajasagi et al. 2012). Here, we identified a role for Gal1 in normal and pathologic human pregnancy. Our results show that Gal1 is mainly expressed in invasive extravillous trophoblast cells, in maternal decidua cells and to some extent also in the villous mesenchyme of human first trimester and term placenta. In addition we demonstrate that Gal1 is regulated by progesterone and pro-inflammatory cytokines and confers immune privilege to human trophoblast cells via the activation of multiple tolerogenic programs. Accordingly, patients with recurrent pregnancy loss had lower levels of circulating Gal1 and higher prevalence of anti-Gal1 auto-Abs compared with fertile women. Given the emerging role of lectin–glycan interactions in the control of immune cell homeostasis (Rabinovich et al. 2012), our findings and those of other groups, add a new piece to the puzzle of fetomaternal tolerance during pregnancy.

We found a progesterone-Gal1 synergism that control immune tolerance in both mouse (Blois et al. 2007) and human settings. This immune-endocrine crosstalk was confirmed by phylogenetic footprinting studies highlighting steroid-responsive elements in *Lgals1* gene that were gained after the emergence of mammalian placentation (Than Romero et al. 2008). Consistent with these findings, recent studies revealed that Gal1 markedly reduces the incidence of resorptions in mice missing the immunophilin FK506-binding protein (FKBP)52, a co-chaperone that optimizes progesterone receptor (PR) signaling in the uterus (Hirota et al. 2012). Notably, Gal1 was significantly down-regulated in both *Fkbp52*$^{-/-}$ and *Pgr*$^{-/-}$ uteri compared with WT uteri, suggesting that uterine Gal1 is an important downstream target of progesterone-FKBP52-PR signaling in the uterus (Hirota et al. 2012). In addition, Gal1 has been shown to act as a regulator of progesterone synthesis by the chorionic carcinoma cell line BeWo (Jeschke et al. 2004).

Expression of Gal1 was found to be dynamically regulated in pro- or anti-inflammatory microenvironments. We previously showed that tolerogenic stimuli such as IL-10, vitamin D3 and vasoactive intestinal peptide up-regulate Gal1 in immature and mature DCs (Ilarregui et al. 2009). In contrast to those findings, here we show that pro-inflammatory and T-cell activating cytokines such as TNF-α and IL-2 considerably up-regulate Gal1 in the human JEG-3 choriocarcinoma cell line, often used as a model of human trophoblast. As an initial pro-inflammatory cytokine response is critical for the implantation process (Mor 2008), it is possible to speculate that Gal1 might affect critical biological events associated with implantation. However, no changes in implantation were found between *Lgals1*$^{-/-}$ and WT mice exposed to stress (Blois et al. 2007). Alternatively, given the broad immunosuppressive activity of Gal1, it is possible that pro-inflammatory cytokines may up-regulate Gal1 as a homeostatic mechanism to favor the resolution of exacerbated T cell responses. Supporting this assumption, Gal1 augments at the peak of the inflammatory disease during autoimmune neuroinflammation (Ilarregui et al. 2009). However, IL-2 and TNF-α may also act, under certain circumstances, as potent immunosuppressive mediators. For example, IL-2 acts in conjunction with transforming growth factor-β to promote T_{reg} cell differentiation (Hoyer et al. 2008). Furthermore, engagement of TNF receptor p55 can also transduce inhibitory signals triggered by TNF-α (Eliçabe et al. 2010). The ability of these cytokines to up-regulate Gal1 expression suggests the possibility that this endogenous lectin could mediate these inhibitory effects.

Endowed with the capacity to suppress T cell responses, T_{reg} cells hold the promise of limiting autoimmune pathology and sustaining tolerance at the fetomaternal interface (Leber et al. 2010). Trophoblast cells play an active role in promoting recruitment and differentiation of maternal T_{reg} cells (Ramhorst et al. 2012). Reproductive failure could result from the inability of T_{reg} cells to function during the preimplantatory phase (Arruvito et al. 2007; Kwak-Kim et al. 2009). Notably, Gal1 has emerged as a key component of T_{reg} cell biology. While this endogenous lectin contributes to the immunosuppressive activity of T_{reg} cells (Garín et al. 2007) through binding to the ganglioside GM1 on effector T cells (Ledeen et al. 2012), it also favors the expansion of inducible $CD25^{+}FoxP3^{+}$ T_{reg} cells and IL-10-producing T_{reg} type 1 cells (Toscano et al. 2006; Blois et al. 2007; Juszczynski et al. 2007; Cedeno-Laurent, Opperman, et al. 2012). Accordingly, we found here that human trophoblast cells directly augment the frequency of $CD4^{+}CD25^{+}FoxP3^{+}$ T_{reg} cells and promote the synthesis of IL-10 through mechanisms involving Gal1 secretion. Noteworthy, IL-10 has been shown to be a key mediator of the immunoregulatory activity of Gal1 both in vitro (van der Leij et al. 2007) and in vivo (Blois et al. 2007; Ilarregui et al. 2009).

Undoubtedly, one of the best studied functions of Gal1 is the induction of T cell apoptosis (Perillo et al. 1995; Pace et al. 1999). However, other studies have questioned these observations, suggesting that Gal1 does not alter T cell viability in the absence of the reducing agent dithiothreitol (Stowell et al. 2008). Here, we show, using co-culture experiments without exogenous addition of rhGal1, that trophoblast cells negatively regulate T cell survival via Gal1-mediated mechanisms. However, as previously demonstrated, trophoblast-derived Gal1 may also participate in non-immunological processes, including trophoblast cell invasion (Kolundžić et al. 2011), syncytium formation (Fischer et al. 2011) and angiogenesis (Thijssen et al. 2006).

The leading causes of early pregnancy loss include chromosomal defects, endocrine disorders, anatomical abnormalities of the female genital tract and immunological or psychological factors (Kwak-Kim et al. 2009). In human pregnancy, the

implantation period is characterized by an initial local inflammatory response that later shifts toward a tolerogenic state (Mor and Cardenas 2010). An inappropriate dysregulation of the pro-inflammatory/anti-inflammatory balance, caused by increased Th1 cytokine responses, reduced FoxP3 expression or a limited repertoire of NK cell inhibitory receptors, may represent a major cause of fetal loss (Ledee-Bataille et al. 2004; Jasper et al. 2006; Baek et al. 2007; Kwak-Kim et al. 2009; Teklenburg et al. 2010; Toth et al. 2010). Here, we found a significant reduction in soluble Gal1 and a higher prevalence of anti-Gal1 auto-Abs in sera from patients with RSA compared with fertile women, suggesting an alternative mechanism to explain pregnancy loss in RSA patients. Whether fluctuations in the levels of soluble Gal1 or anti-Gal1 auto-Abs may have a direct pathogenic role in RSA by interfering with tolerogenic circuits or whether these changes could arise as a consequence of the inflammatory response generated during pregnancy failure remains to be investigated. Supporting these findings, proteomic analysis of placental villous showed the lower expression of Gal1 in placental tissue from patients with early pregnancy loss compared with placental tissue from normal pregnant women (Liu et al. 2006). However, in recent studies, the levels of serum Gal1 and anti-Gal1 Ab did not differ significantly between healthy pregnant women and patients with pre-eclampsia (Molvarec et al. 2011); yet, Gal1 expression was markedly up-regulated in placental tissue from patients with pre-eclampsia (Than, Erez, et al. 2008). These results underscore possible differences in the expression and activity of local vs systemic Gal1 in different pathophysiologic settings. In this regard, anti-Gal1 IgG auto-Abs have been identified in sera from patients with autoimmune uveitis (Romero et al. 2006), systemic lupus erythematosus (Montiel et al. 2010) and Chagas' disease cardiomyopathy (Giordanengo et al. 2001), suggesting an increased prevalence of these auto-Abs in immunologically related disorders associated with chronic inflammation. In addition, given the lack of reliable biomarkers of early pregnancy failure, soluble Gal1 or anti-Gal1 Ab may serve as possible biomarkers of fetal loss. As an ideal biomarker should reflect an important feature of a given disease, future studies are warranted to investigate whether these biochemical variables correlate with other clinical parameters of RSA patients.

In summary, our findings demonstrate an essential role of endogenous Gal1 in conferring immune privilege to human trophoblast cells via induction of multiple tolerogenic mechanisms. In addition, our study identifies significant alterations of circulating Gal1 and anti-Gal1 auto-Ab in human failing pregnancies. The current wealth of preclinical information allows the visualization of strategies through which the manipulation of lectin–glycan interactions may contribute to restore tolerance to fetal or trophoblast antigens with the ultimate goal of re-establishing immune cell homeostasis in failing pregnancies.

Materials and methods

Reagents

L-PHA, lactose, sucrose, protease inhibitor cocktail, progesterone, Tween-20, NP-40, horseradish peroxidase (HRP)-labeled goat anti-human IgG, rhTNF-α, rhIL-2 and rhIL-4 were purchased from Sigma Chemical Co. (St Louis, MO). Dubelcoo's modified Eagle's medium (DMEM), RPMI 1640 medium and L-glutamine were purchased from Invitrogen Life Technologies (Carlsbad, CA). HRP-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG were from Vector Lab. (Burlingame, CA) and rhIL-12 was from BD Biosciences (San José, CA). Goat anti-human FoxP3 Ab was purchased from Abcam (Cambridge, MA). Production and purification of rhGal-1 was accomplished essentially as described (Rabinovich et al. 1999). The rabbit anti-Gal1 polyclonal IgG was generated and used essentially as described (Rubinstein et al. 2004). All other chemical reagents were commercially available analytical grade.

Patients

RSA patients were defined as women with a history of two or more consecutive pregnancy losses before week 12 of gestation after excluding any infectious, endocrine or anatomic disorders. Criteria for exclusion of patients were: (i) occurrence of anti-phospholipid Ab, (ii) hepatitis B or C virus infection, (iii) human immunodeficiency virus infection and (iv) balanced translocation. Control fertile women were defined as women who had two or more normal pregnancies without any miscarriage. The Investigation and Ethics Committee at the Hospital de Clínicas "José de San Martín" has approved this study and all patients provided their written consent to participate.

Placental tissues and immunohistochemistry

Term placenta samples were obtained from clinically normal pregnancies. First trimester placenta and decidual tissues (weeks 6–8 of pregnancy) were obtained following elective termination of pregnancies. Tissue collection was approved by the Ethics Committee of the Medical University of Graz. Samples were fixed in 4% buffered formalin, embedded in paraffin following the standard protocols and cut. The 5-µm sections were dried on superfrost slides (Menzel-Gläser, Braunschweig, Germany), deparaffinized in xylen and rehydrated using a graded series of ethanol. Rehydrated sections were tested for the suitable antigen retrieval procedure, which involved boiling of the slides in a pressure cooker at 120°C for 7 min in an antigen retrieval buffer at pH 9.0 (Eubio, Vienna, Austria). The staining method was standardized using the Lab Vision autostainer and the Ultravision LP HRP detection system (Lab Vision, Fremont, CA) specific for both mouse and rabbit Abs. Endogenous peroxidase was blocked with 3% hydrogen peroxide, the reaction was developed using AEC (Dako, Denmark) and the washing steps were carried out in Tris-buffered saline supplemented with 0.5% Tween-20. All Abs used were diluted in UltraAb diluent (Lab Vision). The polyclonal rabbit anti-Gal1 Ab and the negative control (rabbit Ig, NeoMarkers, Fremont, CA) were diluted to a working concentration of 2 µg/mL. As a positive control for the identification of extravillous trophoblasts, we used 0.5 µg/mL of the mouse mAb 4H84 specific for HLA-G (BD Pharmingen). Slides were counterstained with Mayer's hemalum (Merck, Germany) and mounted in Kaiser's glycerol gelatine (Merck).

Culture of the human JEG-3 choriocarcinoma cell line and preparation of CM

The human JEG-3 choriocarcinoma cell line was regularly maintained in DMEM supplemented with 10% fetal calf serum (FCS), penicillin–streptomycin and 2 mM L-glutamine (DMEM/FCS). To obtain JEG-3 CM, subconfluent (70%) JEG-3 cell cultures were incubated for 18 h with DMEM supplemented with penicillin–streptomycin, sodium pyruvate and L-glutamine. Subsequently, CM was concentrated by precipitation with acetone (24 h at −20°C) and centrifugation (20 min at 300 × *g*). Protein pellets were reconstituted in phosphate-buffered saline (PBS).

Generation of Gal1 knockdown JEG-3 transfectants

To obtain Gal1 knockdown stable transfectants, JEG-3 cells were transduced with pSIREN-RetroQ retroviral vector (BD Clontech, Mountain View, CA) containing Gal1-specific siRNA or Scr oligonucleotide as described (Juszczynski et al. 2007). JEG-3 cells were transduced for 48 h in Opti-MEM I Reduced Serum Medium (Invitrogen Life Technologies) without antibiotics. The Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies) was used according to the manufacturer's instructions. Cells were then maintained under continuous puromycin selection (1.8 µg/mL) for 15 days. Stable transfectants and controls were screened for Gal1 expression by western blot analysis.

Mononuclear cell isolation and stimulation

PBMCs were isolated from healthy fertile women volunteers (total $n = 27$) by Ficoll-Paque™ Plus gradient centrifugation. Cells were cultured (10^6/mL) in RPMI 1640 supplemented with 10% heat-inactivated, pooled normal human AB serum, sodium pyruvate, glutamine and penicillin–streptomycin (RPMI/PHS). In different sets of experiments, PBMCs were stimulated with PHA (1 µg/mL) and cultured in the absence or the presence of increasing dilutions of the JEG-3 CM (1:1 to 1:100), progesterone (10^{-5} M) or rhGal1 (40 µg/mL). To evaluate the contribution of endogenous Gal1 to the immunosuppressive activity of JEG-3 CM, PBMCs were stimulated under similar conditions in the presence of lactose (30 mM) or anti-Gal1 polyclonal IgG (10 µg/mL).

Co-cultures of JEG-3 and PBMCs

JEG-3 cells were cultured in 24 flat bottom polystyrene plates (Becton Dickinson, Franklin Lakes, NJ) in complete DMEM 10% FCS (Gibco, Invitrogen Life Technologies). At 60% of confluence, PBMCs (5×10^5 cells/well) were added in the absence or the presence of rhGal1 (40 µg/mL), lactose (30 mM) or anti-Gal1 polyclonal Ab (10 µg/mL). After 48 h of culture, PBMCs were recovered and used for analysis of apoptosis or cytokine determination.

Proliferation assays

PBMCs (10^5/well) from healthy fertile women volunteers ($n = 6$) were stimulated with PHA and cultured in 96-well U-bottomed polystyrene plates in the absence or the presence of JEG-3 CM (1:1 to 1:100), progesterone (10^{-5} M), rhGal1 (40 µg/mL), lactose (30 mM) or anti-Gal1 IgG (10 µg/mL) for 72 h. Cells were then pulsed with [^{3}H]-thymidine (1 µCi/well; Perkin Elmer, Boston, MA) during the last 18 h of culture. Cells were harvested on glass fibber filters using a Packard Filtermate cell harvester (Packard Instruments, LaGrange, IL). Incorporated radioactivity was measured in a liquid scintillation β-counter (Packard Instruments). All experiments were conducted in triplicate. Results were expressed as mean cpm ± SEM of triplicate wells.

Apoptosis assays

PBMC healthy fertile women volunteers ($n = 3$) were stimulated with PHA for 48 h and further incubated for 18 h in the absence or the presence of JEG-3 CM (1:100) or JEG-3 CM (1:100) plus anti-Gal1 IgG (10 µg/mL). In other set of experiments, PBMCs (2×10^6 cells/mL) were co-cultured with subconfluent (60%) JEG-3 cell cultures during 48 h in DMEM complete medium. Thereafter, non-adherent cells were recovered, washed and incubated with a PE-anti-CD3 mAb for 30 min at 4°C. Apoptotic cells were identified by staining with fluorescein isothiocyanate-labeled annexin-V. Ten thousand events were acquired in a FACSAria II flow cytometer® (BD Biosciences). T cell apoptosis was determined as the percentage of annexin-V$^+$ cells within the CD3-gated T cell population using the WinMDI software®.

Western blot analysis

Gal1 expression was analyzed by western blot in whole cell or tissue protein lysates of first (1stT) and third (3rdT) trimester normal placental tissue, JEG-3 choriocarcinoma cells and several hydatidiform moles (M-329, M-435, M-438 and M-453). Gal1 expression was also analyzed in JEG-3 cells incubated with progesterone (10^{-5} and 10^{-6} M), rhIL-4 (5 ng/mL), rhIL-12 (2 ng/mL), rhTNF-α (10 ng/mL) and rhIL-2 (50 U/mL) for 72 h. FoxP3 expression was analyzed in PBMCs isolated from healthy donors stimulated with PHA and rhGal-1 (40 µg/mL) or JEG-3 CM (1:100) in the absence or the presence of lactose (30 mM) or anti-Gal1 IgG (10 µg/mL) for 72 h.

Cells or minced tissue samples were incubated with lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid, 1% NP-40) and protease inhibitor cocktail for 20 min on ice. Samples were centrifuged at 15,000 × *g* for 20 min at 4°C and whole protein cell lysates were stored at −70°C until use. Protein concentration was estimated by the Micro-BCA Protein Assay kit (Pierce Biotechnology, RO). Western blot analysis was performed essentially as described (Fraccaroli, Alfieri, Larocca, Calafat, Mor, et al. 2009). Briefly, equal amounts of proteins were resolved on a 10% (FoxP3) or a 15% (Gal1) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto nitrocellulose membranes and probed with a goat anti-FoxP3 Ab or a rabbit anti-Gal1 Ab. Bound Abs were detected with HRP-labeled anti-goat IgG or anti-rabbit IgG followed by development with enhanced chemiluminiscence kit (Amersham). Equal loading was checked by Ponceau S staining (Sigma Chemical Co, St Louis, MO) and by probing striped membranes with a rabbit anti-actin polyclonal Ab (Santa Cruz Biotech, CA). RhGal1 (30 ng) was used as a positive control for western blot detection.

To assess the presence of anti-Gal1 auto-Abs in serum samples, 1 μg of rhGal1 was resolved on a 15% SDS–PAGE, transferred onto nitrocellulose membranes and probed with different dilutions of sera from patients presenting RSA and women who had at least two successful pregnancies (fertile). Bound Ab was detected using HRP-conjugated anti-human IgG. Protein bands were analyzed with the ImageJ 1.440 analysis software (NIH).

Cytokine determination

ELISA for human IL-5, IL-10 and IFN-γ was performed according to the manufacturer's instructions (Endogen; Pierce Biotechnology).

Flow cytometry analysis of T_{reg} cells

Flow cytometry analysis of T_{reg} cells was performed according to the manufacturer's instructions (Human Regulatory T Cell Staining Kit; eBioscience, San Diego, CA). Briefly, 1×10^6 cells were stained with CD4/CD25 cocktail. After 30 min, cells were washed with staining buffer and then incubated with the fixation/permeabilization buffer for 1 h. After washing, non-specific binding sites were blocked by adding 2 μL (2% final) normal rat serum for 15 min. Then, cells were incubated with the anti-human FoxP3 (PCH101) Ab or rat IgG2a isotype control for 30 min at 4°C. Finally, cells were washed with permeabilization buffer and analyzed. Ten thousand events were acquired in a FACS Aria II cytometer® (BD Biosciences). The percentage of $CD25^+FoxP3^+$ cells was analyzed within the CD4-gated cell population using the WinMDI software®.

ELISA for Gal1 detection

Soluble Gal1 was detected using an in-house ELISA. Briefly, high binding 96-well microplates (Costar®, Corning Inc., Corning, NY) were coated with capture Ab (2 μg/mL purified rabbit anti-Gal1 polyclonal IgG) in 0.1 M sodium carbonate pH 9.5. After incubation for 18 h at 4°C, wells were rinsed three times with wash buffer (0.05% Tween-20 in PBS) and incubated for 1 h at room temperature with blocking solution (2% BSA in PBS). Samples and standards (100 μL) were diluted in 1% BSA and incubated for 18 h at 4°C. Plates were then washed and incubated with 100 ng/mL biotinylated detection Ab (purified rabbit anti-Gal1 polyclonal IgG) for 1 h. Plates were rinsed three times before adding HRP-labeled streptavidin (0.33 μg/mL; Sigma Chemical Co.) for 30 min. After washing, 100 μL of TMB solution (0.1 mg/mL tetramethylbenzidine and 0.06% H_2O_2 in citrate-phosphate buffer pH 5.0) was added to the plates. The reaction was stopped by adding 4N H_2SO_4. Optical densities were determined at 450 nm in a Multiskan MS microplate reader (Thermo Electron Corporation, Waltham, MA). A standard curve ranging from 2.5 to 160 ng/mL rhGal1 was run in parallel.

ELISA for anti-Gal1 autoAb

Serum levels of anti-Gal1 Ab were determined by ELISA in sera from RSA patients and women who had at least two successful pregnancies (fertile). Briefly, microtiter plates (Nunc, Rochester, NY) were coated with 5 μg/mL rhGal1 in PBS, blocked and incubated with a 1:100 dilution of human serum. Bound IgG was detected by incubation with HRP-conjugated goat anti-human IgG (1:1000). Optical densities were measured at 490 nm in an ELISA reader (Bio-Rad Richmond, CA). A result was considered positive if optical density was two or more standard deviations above the mean of normal control sera.

Statistical analysis

Significance was analyzed by Student's *t*-test. Mann-Whitney *U* test and χ^2 (chi-square) test were used for comparison of human samples and Newman Keuls was used for multiple comparisons using the GraphPad Prism4 software (GraphPad, San Diego, CA).

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Conflict of interest

None declared.

Abbreviations

Ab, antibody; CM, conditioned medium; DC, dendritic cell; DMEM, Dubelcoo's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FasL, Fas ligand; FCS, fetal calf serum; FKB52, FK506-binding protein 52; FoxP3, Forkhead box P3; Gal1, galectin-1; HRP, horseradish peroxidase; IFN, interferon; IgG, immunoglobulin G; IL, interleukin; NK, natural killer; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PD-L1, programmed death ligand-1; PHA, phytohemagglutinin; PR, progesterone receptor; rh, recombinant human; RSA, recurrent spontaneous abortion; Scr, scrambled; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Th, T helper; TNF, tumor necrosis factor; T_{reg}, T regulatory; WT, wild type.

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