T cell apoptosis at the maternal–fetal interface in early human pregnancy, involvement of galectin-1

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The human fetus is not rejected by the maternal immune system despite expressing paternal antigens. Natural killer cells, the major lymphocyte population of the human decidua (dNKs), express genes with immunomodulatory potential. These include galectin-1 (gal1), a lectin with apoptotic activity on activated CD8$^+$ T cells, Th1 and Th17 CD4$^+$ cells. Although many cell types at the maternal–fetal interface also produce gal1, its production by dNKs has been used here to study its function in pregnancy. Media conditioned by dNKs containing gal1 induced apoptosis of activated T cells. This effect was blocked by anti-gal1 antibodies. Decidual T (dT) cells but not peripheral T (pT) cells bound gal1 and presented a distinct glycophenotype compatible with sensitivity to gal1. Annexin V staining, TUNEL, and hypodiploidy showed a substantial proportion of apoptotic dT cells. Immunohistochemistry revealed widespread expression of gal1 as well as periglandular apoptotic dT foci that colocalized with dNKs. Thus, secretion of gal1 by dNKs and other decidual cells contributes to the generation of an immune-privileged environment at the maternal–fetal interface.

maternal–fetal tolerance | NK cells | lectin | decidua

Human pregnancy is an immunological paradox. Despite expressing paternal antigens, the fetus is not rejected by the maternal immune system during successful pregnancies (1). During the first trimester of gestation, maternal immune cells account for 30% of human decidual cells (2). This infiltrate is abundant in natural killer cells (≈80%) and relatively scarce in CD3$^+$ T cells (≈10%). In contrast, NK cells and CD3$^+$ T cells represent about 10% and 80% of lymphocytes, respectively, in peripheral blood (2–5). Various mechanisms to suppress potentially alloreactive placental T cells and tolerize the maternal immune system toward the fetus have been proposed in mice and humans. These include placental expression of Fas and Fas ligand (6, 7), T cell starvation through tryptophan depletion by indolamine 2,3-dioxygenase (8), ligation of the inhibitory ligand PD-L1 expressed on T cells in the gravid uterus (9), Th2 cytokine polarization (10), suppression by CD4$^+$CD25$^+$ regulatory T cells (11, 12), and progesterone-induced Th2-type responses (13). A number of redundant immunosuppressive systems exist at the maternal–fetal interface, not very surprisingly in a system so essential for species survival.

Human decidual NK cells (dNKs) are phenotypically distinct from peripheral blood NK cells (pNKs) (4). They play a role in placental vasculature remodeling (14, 15). These CD56^{bright} dNKs overexpress many genes compared with CD56^{bright} and CD56^{dim} pNK subsets (4), and display reduced cytotoxic activity (16). One of the genes most up-regulated in dNKs encodes galectin-1 (gal1) (4). Gal1 is a 14-kDa secreted protein in the galectin family, whose members have 1 or 2 carbohydrate recognition domains with affinity for poly-*N*-acetyllactosamine (β-1,4-galactosyl-*N*-acetylglucosamine) moieties in cell surface glycoproteins (17). The immunosuppressive properties of gal1 have been studied extensively, with particular attention to its effects on T cells. Gal1 induces apoptosis of activated CD8 T cells (18) and induces Th2 cytokine shifts. Expression of C2GnT (core 2 β-1,6-*N*-acetylglucosaminyltransferase) by T cells, which initiates a second branch on O-glycans, later elongated with poly-*N*-acetyllactosamine moieties, renders activated T cells susceptible to gal1-induced apoptosis (19, 20). Differential glycosylation of Th1, Th2, and Th17 CD4 T cells results in differential sensitivity to gal1-induced apoptosis. Th1 and Th17 cells present complex N-glycans with poly-*N*-acetyllactosamine moieties that make them susceptible to gal1 (21). The α-2,6-sialylation of poly-*N*-acetyllactosamine moieties by ST6Gal1 interferes with gal1 binding (22), protecting Th2 cells from gal1-induced cell death (21). Many other effects of gal1 on cell adhesion, T cell proliferation, monocytes, and neutrophils have been reported (17, 23–26).

Fifteen galectins have been described in mammals, of which gal1, gal3, gal9, and gal13 occur in the human placenta (4, 27–31). Cycling endometrium produces gal1 during the secretory phase, and its expression is augmented during early pregnancy. It is expressed by various cells in the human placenta, including placental stromal cells, CD45$^+$ cells (29), cytotrophoblasts in cell columns, and syncytiotrophoblasts (28). Expression levels are reduced in patients with early pregnancy loss (27), suggesting that gal1 may play a role in maintaining pregnancy.

Given the abundance of NK cells compared with T cells in the human decidua (2–5), the overexpression of gal1 by dNKs at the transcriptional level (4), and the abundance of gal1 produced by several cell types in the human placenta (28, 29), we explored the immunosuppressive role of gal1 in protecting the fetus from potentially alloreactive T cells in the human decidua. The data presented here suggest a critical role for gal1 in human maternal–fetal tolerance.

Results

Human dNKs and Decidual Macrophages Express gal1. Gene expression profiles showed that gal1 mRNA is overexpressed by dNKs compared with CD56^{bright} (20 times) and CD56^{dim} (5–10 times) pNKs (4). Differences in gal1 expression also were found at the protein level by Western blot analysis (Fig. 1*A*). Western blot analysis and RT-PCR also showed gal1 expression by decidual macrophages, the second most populous leukocyte in the decidua (data not shown). Although many cells in addition to dNKs in the human decidua produce gal1, its secretion by dNKs is used here to study further its effects in human pregnancy.

Conditioned media (CM) from dNKs or CD3$^-$CD56$^+$ pNKs

Author contributions: H.D.K., J.L.K., and J.L.S. designed research; H.D.K., F.R., Y.L., and J.L.K. performed research; H.D.K., F.R., Y.L., D.S.J.A., J.L.K., and J.L.S. analyzed data; and H.D.K., F.R., and J.L.S. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0809233105/DCSupplemental.

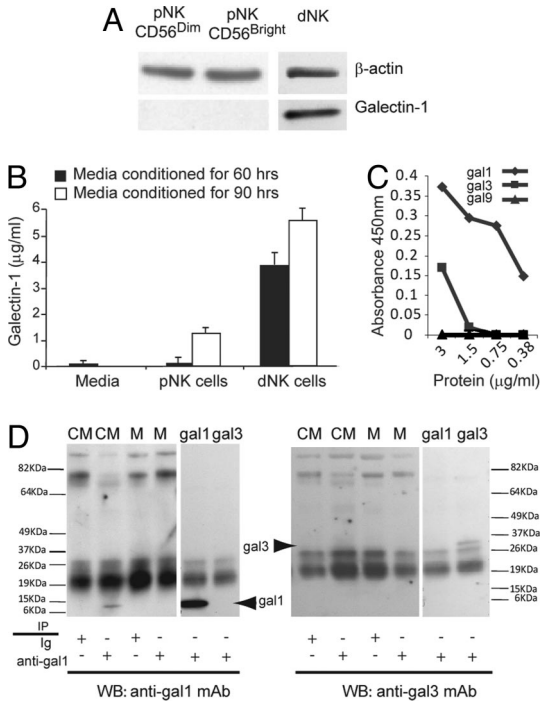

Fig. 1. Gal1 is expressed and secreted by dNKs but not by pNKs. (*A*) Western blot of fresh CD56^{dim} pNK, CD56^{bright} pNK, and dNK lysates developed with anti-gal1 mAb (*Lower*) and anti-β-actin mAb (*Upper*). Gal1 was visualized as a 14-kDa band. Five dNK and and 5 pNK cell samples from different donors were analyzed. Similar results were obtained with decidual macrophages (data not shown). (*B*) Concentration of gal1 in media conditioned by CD56^{+}CD16^{-}CD3^{-} dNKs and CD56^{+}CD3^{-} pNKs for 60 h (filled bars) and 90 h (open bars). Incubations were done in the presence of 12 ng/mL IL-15. The average of 3 independent experiments is shown. (*C*) Crossreactivity of the antibody used in *B* with recombinant human gal1 (diamonds), gal3 (squares), and gal9 (triangles) in ELISA assays. (*D*) Immunoprecipitation of gal1 from media conditioned by dNKs (CM), control media not exposed to dNKs (M), recombinant human gal1 (gal1), and recombinant human gal3 (gal3) with the same antibody as used in *B* (anti-gal1) or with an isotype control (Ig). Immunoprecipitates were resolved by Western blotting with anti-gal1 (*Left*) or anti-gal3 (*Right*) mAbs. The additional bands are derived from immunoglobulins and protein G. One experiment of 3 is shown.

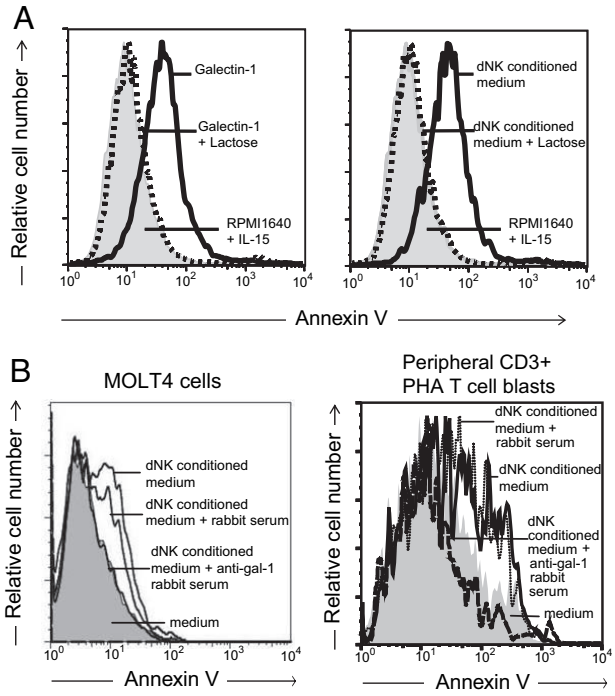

Fig. 2. Medium conditioned by dNKs and containing gal1 has apoptotic activity on T cells and MOLT4 cells. (*A*) Annexin V staining of MOLT4 cells treated (open histogram) with gal1 (*Left*) or medium conditioned by dNKs (*Right*) in the presence (dotted line histogram) or absence (solid line histogram) of 50 mM lactose. Shaded histograms correspond to cells incubated with control medium. Incubations (30 min at 37°C) were done in the presence of 0.5 mM DTT to prevent gal1 oxidative inactivation. (*B*) Blocking of apoptosis induction of MOLT4 cells (*Left*) and peripheral T cell blasts (*Right*) by dNK conditioned medium with anti-gal1 polyclonal antibodies. Control medium (shaded histogram), dNK conditioned medium (solid line histogram), conditioned medium plus nonimmune rabbit serum (dotted line histogram), and conditioned medium plus anti-gal1 rabbit serum (dashed line histogram) are shown.

were analyzed by sandwich ELISA assays. After 60 h in culture (with IL-15), 4 μg/mL gal1 was detected in CM from dNKs but not from pNKs (Fig. 1*B*). After 90 h, gal1 also was detected in CM from pNKs, although at much lower levels. Ex vivo dNKs but not ex vivo pNKs also secreted gal1 in the absence of IL-15 in 18-h cultures (data not shown). The cross-reactivity of the antibody used in the ELISA assays was tested against 2 other galectins present in the human placenta that were available to us in recombinant form. The antibody cross-reacted slightly with recombinant human galectin-3 (gal3) at high protein concentrations, but not with recombinant human galectin-9 (gal9) by ELISA (Fig. 1*C*). However, only gal1 was immunoprecipitated from CM from dNKs. Anti-gal1 mAb but not anti-gal3 mAb produced bands in Western blots of the immunoprecipitate (Fig. 1*D*), confirming that dNKs secrete gal1.

Media Conditioned by Human dNKs Have Apoptotic Activity on Activated T Cells. Gal1 has apoptotic activity on activated T cells. Both gal1 and CM from dNKs induced apoptosis of MOLT4 T cells, a gal1-susceptible T cell tumor line. Apoptosis induction was blocked in the presence of 50 mM lactose, which competes with gal1 for binding to poly-*N*- acetyllactosamines (18) (Fig. 2*A*), and anti-gal1 antibodies (Fig. 2*B*), indicating that gal1 is the major contributor to the apoptotic activity of CM from dNKs. Similar results were obtained using CD3^{+} T cells activated for 5 days with phytohemagglutinin (PHA) and IL-2.

Decidual and Peripheral Human T Cells Have Different Glycophenotypes and gal1-Binding Capacity. Secretion of gal1 in the decidua by dNKs and other cells (28, 29) might contribute to an immunosuppressive microenvironment at the maternal–fetal interface, thereby protecting fetal cells from activated alloreactive maternal T cells. The gal1-binding capacity of decidual T cells (dTs) and peripheral blood T cells (pTs) was therefore explored using biotinylated gal1. CD3^{+} dTs, but not pTs from pregnant and nonpregnant female donors, bound biotinylated gal1, and binding was glycan-dependent because it could be competed with 50 mM lactose (Fig. 3*B* and statistics in Fig. 3*D*).

Gal1 binds poly-*N*-acetyllactosamine moieties on N-glycans or core 2 O-glycans. On N-glycans, it binds to unsialylated or α-2,3-sialylated poly-*N*-acetyllactosamine moieties. The latter can be detected with the lectin *Maackia amurensis* agglutinin (MALII) (21). The α-2,6-sialylation of *N*-acetyllactosamines on N-glycans reduces gal1 binding and can be identified with the lectin *Sambucus nigra* agglutinin (SNA) (Fig. 3*A*). Both dTs and pTs bound biotinylated SNA equally and did not bind MALII (Fig. 3*C*), suggesting that these cells do not bind gal1 through N-glycans.

On O-glycans, the lectin peanut agglutinin (PNA) recognizes asialo core 1 O-glycans, the substrate on which the enzyme

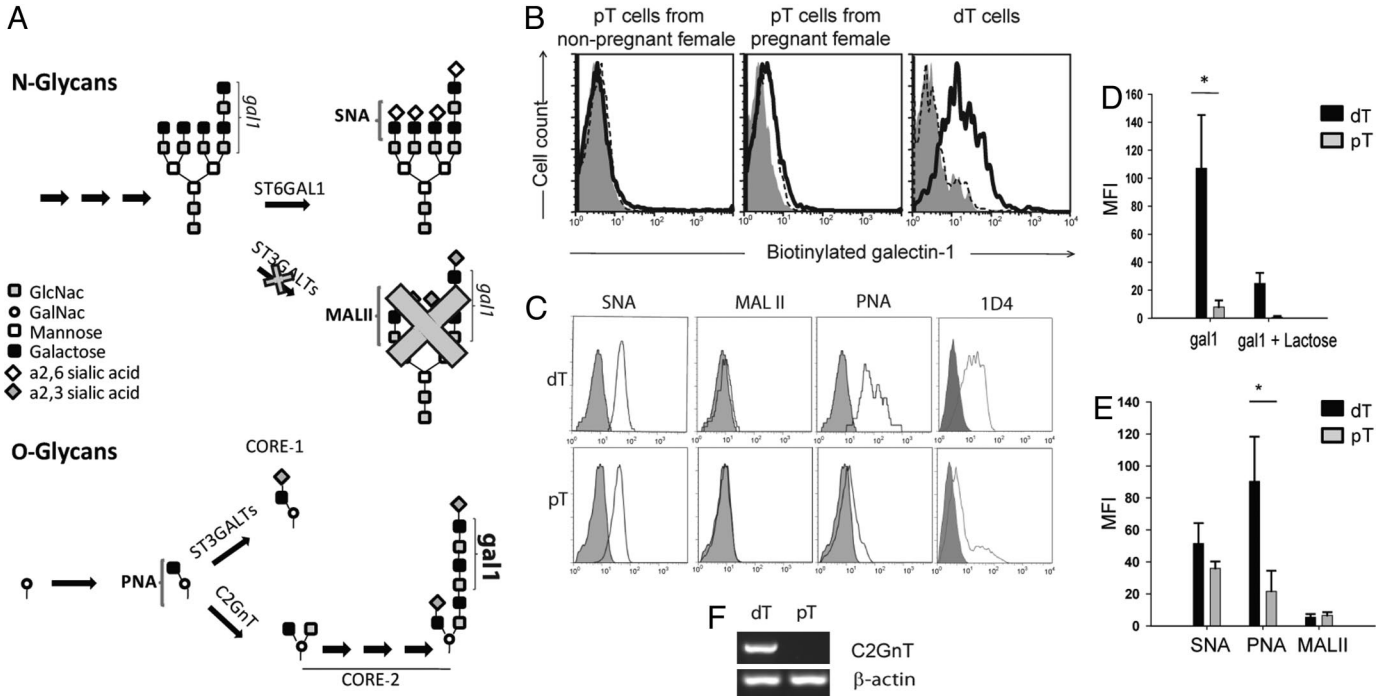

Fig. 3. Differential gal1 binding and glycophenotype of decidual and peripheral blood CD3$^+$ T cells. (*A*) Biosynthetic pathways of tetraantenary N-glycans and core 2 O-glycans indicating the structures recognized by lectins SNA, PNA, MALII, and gal1. Crossed structures are absent from dTs and pTs based on data shown in *C*. The potential gal1-binding sites in N-glycans (italicized) are masked by α-2,6-sialylation and by the failure to form α-2,3-sialylated *N*-acetyllactosamine. Core 2 O-glycans are recognized by gal1 on dT cells (in bold). (*B*) Binding of biotinylated gal1 to dTs (*Right*) and pTs from pregnant (*Center*) and nonpregnant (*Left*) female donors in the presence (dashed line histograms) or absence (solid line histograms) of 50 mM lactose. (*C*) Binding of biotinylated SNA, MALII, and PNA or anti-core 2 O-glycosylated CD43 mAb 1D4 to dTs (*Upper*) and pTs (*Lower*). In *B* and *C*, histograms correspond to fresh lymphocyte preparations gated on CD3$^+$ cells. Bound biotinylated lectins were detected by staining with fluorescently labeled streptavidin. Gray shaded histograms correspond to CD3$^+$ gated cell preparations incubated only with fluorescently labeled streptavidin. Statistics comparing the staining with biotinylated gal1 (*D*) and biotinylated lectins SNA, PNA, and MALII (*E*) in dTs and pTs as described in *B* and *C* are shown. Asterisks indicate statistical significance in *t* test comparisons involving the groups denoted by the overlying lines (*, $P \leq 0.05$). Results correspond to 8 decidual and 4 peripheral blood samples. Error bars represent standard error. (*F*) Expression of C2GnT by dTs and pTs evaluated by RT-PCR.

C2GnT adds the second branch of core 2 O-glycans (19, 20), later elongated with poly-*N*-acetyllactosamine moieties, to which gal1 binds. The dTs bound significantly more PNA than the pTs (Fig. 3*C* and statistics in Fig. 3*E*). Furthermore, only dTs expressed C2GnT mRNA (Fig. 3*F*) and had core 2 O-glycosylated CD43, stained with antibody 1D4 (Fig. 3*C*). These differences in O-glycosylation patterns are likely to explain the differential gal1 binding capacity of the 2 cell types.

A Population of Apoptotic T Cells Is Present in the Human Decidua. Given the capacity of dTs to bind gal1, their apoptotic profile was studied. Staining freshly isolated decidual lymphocytes with anti-CD56 or anti-CD3 mAbs and Annexin V revealed that CD3$^+$ dTs (Fig. 4*A Center*) but not the majority of CD56$^+$ dNKs (Fig. 4*A Left*) were apoptotic. In contrast, no Annexin V staining was detected on CD3$^+$ T cells from peripheral blood lymphocyte preparations exposed to an isolation procedure similar to that used to obtain decidual lymphocytes (Fig. 4*A Right*). Statistical comparison showed that differences in the percentage of Annexin V-positive CD3$^+$ T cells were highly significant ($P < 6.7 \times 10^{-6}$) (Fig. 4*A′*). DNA fragmentation and hypodiploidy, 2 late apoptosis events, were evaluated by TUNEL and propidium iodide (PI) staining, respectively. TUNEL revealed that up to 60% of CD3$^+$ dTs were apoptotic (Fig. 4 *B* and *B′*). However, total decidual lymphocytes, most of which are NK cells, were negative for TUNEL labeling (Fig. 4*B Lower Right*). Analysis of CD3$^+$ pTs, isolated in the same fashion as dTs by anti-CD3 FACS sorting, showed that on average, only 10% of CD3$^+$ pTs were positive for TUNEL staining, in contrast to 45% of CD3$^+$ dTs ($P < 0.0016$) (Fig. 4*B′*). Statistically significant differences also were observed in TUNEL analyses of dTs and pTs isolated as CD14$^-$, CD56$^-$, and CD4$^+$/CD8$^+$ lymphocytes to avoid possible anti-CD3-induced activation and apoptosis (Fig. 4 *B* and *B′*). PI staining showed similar results (Fig. 4 *C* and *C′*).

Human dTs Form Periglandular Apoptotic Foci. Anti-CD3 and TUNEL staining of serial sections of decidual tissue revealed that CD3$^+$ dTs formed periglandular foci (Fig. 5 *A* and *B*) that colocalized with foci of apoptotic lymphocytes identified by TUNEL staining (Fig. 5 *A′* and *B′*). Away from these dense periglandular foci, interstitial CD3$^+$ cells were identified, and apoptosis identified by TUNEL was seen only in scattered single cells. Tissue samples analyzed were fixed immediately after sample collection, ruling out the possibility of apoptosis induction due to tissue manipulations. Periglandular apoptotic foci were also infiltrated by CD56$^+$ NK cells (Fig. 5*B′′*). Efforts to costain single tissue sections for TUNEL and CD3 were unsuccessful because of high nonspecific background generated by the combination of the staining techniques. Nevertheless, the pattern of TUNEL staining correlates with CD3 staining in serial sections, and the histochemical data combined with flow cytometric analysis of Annexin V staining, TUNEL, and hypodiploidy confirm the apoptotic nature of a major population of dTs but not of dNKs.

In some histological sections, T cell aggregates with relatively low levels of apoptosis were noted. This may reflect that not all dTs are apoptotic, in agreement with the finding that a major proportion of but not all dTs were apoptotic in Annexin V stainings, TUNEL, and hypodiploidy analyses (Fig. 4).

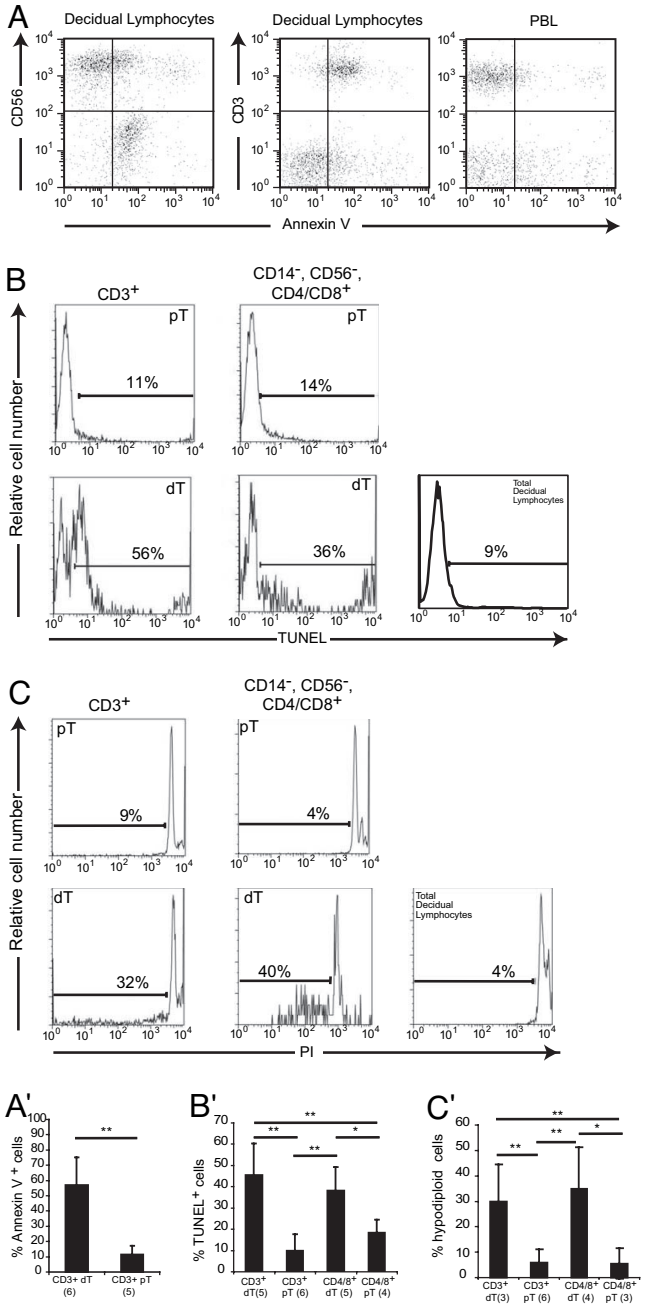

Fig. 4. Freshly isolated decidual lymphocytes contain a population of Annexin V-positive T cells. (*A*) Annexin V and anti-CD56 (NK cells; *Left*) or anti-CD3 (T cells; *Center*) staining of freshly isolated decidual lymphocytes, and peripheral blood lymphocytes subjected to an isolation procedure similar to the one used to obtain decidual lymphocytes (*Right*). Note the presence of $CD56^-$ and $CD3^+$ dT lymphocytes positive for Annexin V staining. (*B* and *C*) BrdU staining for TUNEL analysis (*B*) and PI staining for hypodiploid DNA content analysis (*C*) of FACS-sorted $CD3^+$ T cells (*Left*) and FACS-sorted T cells isolated as $CD14^-$, $CD56^-$ and $CD4^+/CD8^+$ cells (*Center*) obtained from total decidual lymphocyte (dt; *Lower*) or from peripheral lymphocyte preparations (pt; *Upper*). *Lower Right* panels display results for total decidual lymphocytes. Numbers indicate the percentage of cells with DNA fragmentation in *B* and of subdiploid cells in *C*. Panels correspond to independent dT cell preparations. Peripheral T cells shown were obtained from a single preparation. (*A′*, *B′*, and *C′*) Statistics comparing the abundance of Annexin V^+ (*A′*), $TUNEL^+$ (*B′*), and hypodiploid cells (*C′*) in $CD3^+$ or $CD4^+/8^+$ T cells preparations from decidual and peripheral blood lymphocytes analyzed as described in *A*, *B*, and *C*. Asterisks indicate statistical significance in *t* test comparisons involving the groups denoted by the overlying lines (**, $P \leq 0.01$; *, $P \leq 0.05$). Numbers in parentheses indicate the number of samples analyzed. Error bars represent standard deviation.

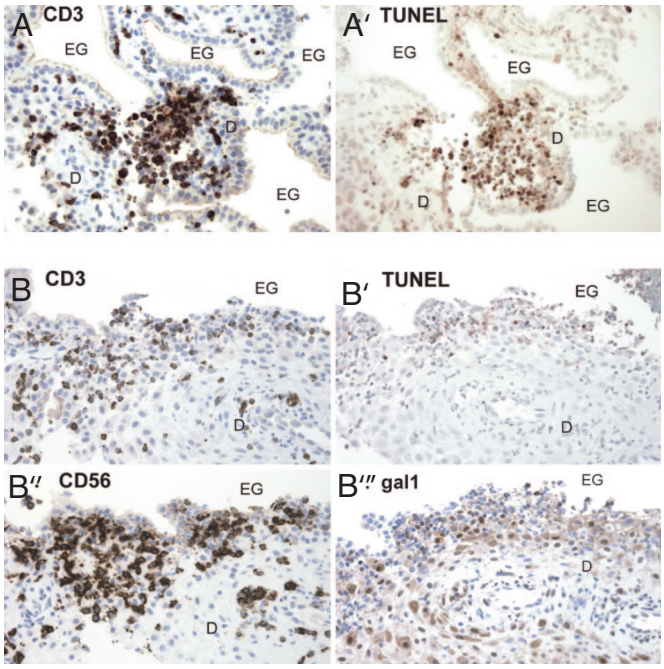

Fig. 5. Infiltrating T cells form periglandular apoptotic foci in the human decidua. CD3 (*A* and *B*), CD56 (*B″*), and gal1 (*B‴*) immunostaining and TUNEL staining (*A′* and *B′*) of 4-μm serial histological sections (*A* with *A′*; and *B* with *B′*, *B″*, and *B‴*) of first-trimester human decidua of 6 weeks' gestational age. Images are representative of 2 samples from 2 different donors. EG, endometrial gland; D, decidua.

Staining of decidual sections revealed widespread expression of gal1 in cells with different morphology (Fig. 5 *B‴*), indicating that many cell types in addition to dNKs may contribute to the generation of an immunosuppressive environment through gal1 expression.

Discussion

Various immunosuppressive mechanisms have been proposed to protect the fetus from potentially alloreactive T cells (6–13). Here, we described a novel mechanism likely involved in the induction of apoptosis of dTs in the human placenta mediated by gal1. The dTs express CD69, and ≈50% are $HLA\text{-}DR^+$, indicating an activated phenotype (2). Gal1 has the capacity to induce apoptosis of activated T cells (18), and dTs have the capacity to bind gal1 (Fig. 3*B*). Furthermore the glycophenotype of dTs, distinct from that of pTs, is compatible with their activated profile, differentially binding PNA, expressing C2GnT, and presenting core 2 O-glycans, and suggests that gal1 binds these cells through O-glycans (Fig. 3).

In serial sections from early human placentas, $CD3^+$ T cells formed periglandular foci (Fig. 5) that colocalized with the foci of TUNEL-positive apoptotic lymphocytes. The combined analysis of immunohistochemical sections and flow cytometric analyses of Annexin V, PI, and TUNEL staining support the presence of apoptotic dTs and nonapoptotic dNKs at this site. Scattered interstitial T cells also were present, some of which were nonapoptotic, and a few of which were apoptotic. Many other decidual cells also expressed gal1, contributing to the generation of a local immunosuppressive environment.

Media conditioned by dNKs contained gal1 at a lower concentration (1–4 μg/mL; Fig. 1*B*) than that of recombinant gal1 used to induce T cell apoptosis. It is possible that other proteins secreted by dNKs could synergize with the apoptotic effect of gal1 secreted by dNKs. PP14, a glycoprotein overexpressed by dNKs (4) that shares immunosuppressive properties with gal1, is

a candidate for such an interaction. Like gal1, PP14 also induces T cell apoptosis (32), colocalizes with CD45 on the cells to which it binds (33), and reacts with *N*-acetyllactosamines (34). Interestingly, PP14 is also expressed by glandular epithelium around which apoptotic T cells are seen, and has poly-*N*-acetyllactosamine moieties (35) to which gal1 could potentially bind. An interesting difference between the 2 proteins is that whereas α-2,6-sialylation of CD45 glycans on T cells negatively regulates gal1-induced apoptosis, it favors the activity of PP14 (22, 34). The presence of both proteins at the maternal–fetal interface may modulate a broad range of T cells.

Annexin V staining revealed the externalization of phosphatidylserine (PS) due to loss of membrane asymmetry during early apoptosis. DNA fragmentation, as evidenced by TUNEL, and hypodiploidy are late apoptosis events. It has been proposed that gal1-induced PS exposure can contribute to leukocyte homeostasis by phagocytic recognition (36). The lower percentage of TUNEL-positive or hypodiploid decidual T cells compared with Annexin V-positive decidual T cells (Fig. 4) could result from clearance of PS-positive cells before or at the point at which DNA fragmentation and hypodiploidy can be detected.

To establish whether gal1 is necessary for maintaining a successful pregnancy, animal models are required. Mice that are gal1$^{-/-}$ breed normally (37), but a syngeneic mating places little immunological stress on pregnancy. Experiments comparing syngeneic and allogeneic matings [CBA/Caj (H2^{k}), C3H/j (H2^{k}), and Balb/cJ (H2^{d}) males] failed to show differences in the litter size or embryo resorption rates of pregnant C57BL/6 (H2^{b}) gal1$^{-/-}$ females (data not shown). However, it was reported recently that gal1$^{-/-}$ 129P3/J females (H2^{b}) mated with allogeneic DBA/J2 males (H2^{d}) present greater fetal resorption rates than wild-type 129P3/J females, whereas no differences were observed in syngeneic matings (38), thus supporting an important role for gal1 in maternal–fetal immune tolerance. The fact that augmented embryo resorption rates in gal1$^{-/-}$ pregnant females are evident only in matings with certain strain combinations is indicative of the presence of redundant systems to prevent fetal rejection. This redundancy may be important only in genetic backgrounds that pose an increased immunological stress. As multiple galectins are present in the human decidua (28–31), multiple galectins present in the murine placenta (39) could, conceivably, compensate for each other.

Particular attention has been paid recently to the role of regulatory T cells in maternal–fetal tolerance (11, 12). Interestingly, CD4^{+} CD25^{+} regulatory T cells have been shown to mediate their suppressive activity through gal1 (40).

The periglandular localization of apoptotic dTs is intriguing, given that glandular epithelial cells in the decidua basalis are the frontier to the intervillous space. Furthermore, it has been reported that some uterine glandular epithelial cells have MHC class II expression (41) and antigen-presenting capacity (42). Speculatively, presentation of fetal antigens by these cells in a galectin-rich environment could promote the survival of fetal antigen-specific tolerogenic T cells and the death of activated antigen-specific Th1, Th17, and reactive CD8 T cells.

In conclusion, human dTs are shown here to have a particular glycophenotype that gives them the capacity to bind gal1. A significant proportion of those cells are apoptotic and colocalize with NK cells that, among other decidual cells, secrete apoptotically active gal1. These data, together with recent findings on the role of gal1 in murine pregnancies, point to an important role for gal1 in human maternal–fetal tolerance.

Methods

Isolation of Decidual and Peripheral Lymphocytes. Maternal decidual tissue was obtained from discarded material of elective first-trimester terminations and processed as described previously (4). The dNKs were FACS sorted as CD56^{bright}CD16^{-}CD3^{-} cells, and dTs as CD3^{+} or as CD4^{+}/CD8^{+} CD14^{-} CD56^{-} cells using a mixture of anti-CD4 and anti-CD8 mAbs conjugated to the same fluorophore.

Peripheral lymphocytes were isolated from leukopacks obtained from anonymous donors (Massachusetts General Hospital, Boston, MA) using density gradients (Ficoll-Hypaque; Amersham Biosciences) directly or, for NK cell preparations, after NK cell enrichment using Rosettesep (StemCell Technologies Inc.) following the manufacturer's instructions. For generation of pNK CM, cells were further isolated by FACS sorting as CD56^{+}CD3^{-} lymphocytes. For Western blotting, CD56^{bright} pNKs were isolated as CD56^{bright}CD16^{-}CD3^{-} cells, and CD56^{dim} pNKs as CD56^{dim}CD16^{+}CD3^{-} cells.

Mock isolation of peripheral lymphocytes as if they were decidual lymphocytes was done by exposing peripheral lymphocytes, after density gradient separation, to the same procedure used to obtain decidual cells (4), including the time elapsed before sample processing. For TUNEL assays and hypodiploid DNA content analyses, pTs were further isolated by FACS sorting by using the same staining scheme as described above for dTs.

Media Conditioned by NK Cells. FACS-sorted dNKs or CD56^{+} pNKs were incubated at 37°C at a density of 1×10^{6} cells/mL in RPMI 1640 containing 10% FCS for 60 or 90 h in the presence of 12 ng/mL IL-15, or for 18 h without IL-15. CM were screened for gal1 content by ELISA. Media generated over 60 h in the presence of IL-15 were used for induction of T cell apoptosis.

ELISA Assays. Inmunolon 2 ELISA plates (DynatechLaboratories Inc.) were covered with polyclonal anti-gal1 antibodies, 10 μg/mL (Peprotech Inc.), and washed with washing buffer (0.05% Tween-20 in PBS). Plates were blocked with 1% BSA in PBS. Individual wells were incubated with serial dilutions of gal1 or media conditioned by dNKs or pNKs for 1 h at room temperature. Wells were washed and incubated with biotinylated polyclonal anti-gal1 antibodies (0.3 μg/mL in PBS 0.1% BSA; Peprotech Inc.). Plates were washed 6 times and incubated with HRP-conjugated streptavidin (Zymed). After 6 additional washes, a colorimetric reaction was developed with TMB substrate (Pierce). The reaction was stopped by adding one volume of 2 N H_2SO_4. Absorbance at 450 nm was recorded.

Immunoprecipitation. Protein G Sepharose beads (Sigma–Aldrich) were coated with polyclonal anti-human gal1 antibody (10 μg/mL; Peprotech) or control IgG. A total of 20 μL coated beads was added to 100 μL dNK CM. Beads were collected 12 h later by centrifugation. After 4 washes with PBS, SDS loading buffer was added, and samples were boiled. Beads were precipitated by centrifugation, and the bead-free supernatant was analyzed by Western blotting with anti-gal1 mAb (NCL Gal1; Novocastra) or anti-gal3 mAb (clone 9C4; Lab Vision).

Apoptosis Induction by Recombinant gal1 and Media Conditioned by dNKs. MOLT4 cells or peripheral blood lymphocytes activated for 5 days in RPMI media containing 10% FCS plus 2 μg/mL PHA and 200 U/mL IL-2 were incubated at 37°C with fresh dNK CM or recombinant gal1 (13 μM). Incubations were done in the presence of 0.5 mM DTT with or without the addition of 50 mM lactose, anti-gal1 rabbit serum, or nonimmune rabbit serum. Apoptosis induction was interrupted at 45 min of incubation by the addition of lactose to a final concentration of 100 mM. Cells were washed twice with cold PBS and stained on ice with allophycocyanin-conjugated anti-CD3 antibodies and then stained with FITC-conjugated Annexin V and 7AAD (BD PharMingen). Samples were analyzed with a flow cytometer. The percentage of CD3^{+} apoptotic cells over untreated CD3^{+} control cells was calculated by Overton histogram accumulative substraction (43) with the software Flow Jo (Treestar).

TUNEL and Hypodiploidy Assays. Total decidual lymphocytes, peripheral blood lymphocytes, or FACS-sorted dTs were processed with the APO-BRDU apoptosis assay kit (BD PharMingen) following the manufacturer's instructions. Some samples were also stained with propidium iodide (PI) to quantitate DNA ploidy. Fluorescein-Br-dUTP incorporation and PI staining were measured in a FACScalibur flow cytometer (Becton–Dickinson).

Biotinylation of gal1 and Lectin Staining. Recombinant gal1 was biotinylated by incubation with Sulfo-NHS-Biotin (Pierce) according to the manufacturer's instructions. Unconjugated biotin was removed by dialysis. Cells were incubated with biotinylated gal1 (15 μM), biotinylated SNA (20 μg/mL; E-Y Laboratories), biotinylated PNA (20 μg/mL; Sigma–Aldrich), or biotinylated MALII (20 μg/mL; Vector Laboratories) (21). Bound biotinylated lectins were detected with phycoerithrin-conjugated streptavidin (Sigma–Aldrich). Nonspecific binding was determined with phycoerithrin-conjugated streptavidin alone.

Antibodies, Lectins, and Recombinant Proteins. Recombinant human gal1 was kindly provided by Linda Baum (University of California, Los Angeles, CA) and Gabriel Rabinovich (Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina); recombinant human gal9 was provided by Vijay Kuchroo (Brigham and Women's Hospital, Harvard Medical School, Boston, MA). Recombinant human gal3 was from Peprotech Inc.

The following murine mAb and the proteins conjugated with FITC, PE, CyChrome, were used in FACS analysis or sorting: anti-CD3, anti-CD56, anti-CD16, anti-CD4, anti-CD8, anti-CD14, IgG isotype controls, and Annexin V were all from BD PharMingen. Anti-core 2 O-glycosylated CD43 antibody 1D4 was from MBL International. Rabbit anti-gal1 antibody used in immuohistochemistry was provided by Gabriel Rabinovich (44). Blocking anti-gal1 rabbit serum was kindly provided by Linda Baum (45). This serum has no cross-reactivity with gal3 (L. Baum, personal communication) and did not cross-react with gal9 in Western blot analysis (data not shown).

Immunohistochemistry, Western blotting, and RT-PCR are described in detail in supporting information (SI) *Methods*.

ACKNOWLEDGMENTS. We thank Linda Baum and Gabriel Rabinovich, who kindly provided recombinant gal1 and anti-gal1 immune serum. This research was funded by National Institutes of Health Grant AI053330 to J.L.S.

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