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Fetal Expression of Fas Ligand Is Necessary and Sufficient for Induction of CD8 T Cell Tolerance to the Fetal Antigen H-Y during Pregnancy

Melanie S. Vacchio1* and Richard J. Hodes*†

Interaction of Fas with Fas ligand (FasL) is known to play a role in peripheral tolerance mediated by clonal deletion of Ag-specific T cells. We have assessed the requirement for Fas/FasL interactions during induction of tolerance to the fetus. Using H-Y-specific TCR transgenic mice, we have previously demonstrated that exposure of maternal T cells to H-Y expressed by male fetuses results in deletion of 50% of H-Y-specific maternal T cells. The remaining H-Y-specific T cells were hyporesponsive to H-Y as assayed by decreased proliferative ability and CTL activity. To determine whether Fas/FasL interactions contribute to induction of maternal T cell tolerance, responsiveness to fetal H-Y was assessed in H-Y-specific TCR transgenic pregnant females that were deficient in functional Fas or FasL. Surprisingly, both deletion and nondeletion components of tolerance were abrogated in TCR transgenic H-Y-specific lpr (Fas-deficient) or gld (FasL-deficient) pregnant females. Experiments further revealed that expression of FasL by the fetus, but not by the mother, is necessary and sufficient for both components of maternal T cell tolerance to fetal Ags. Fas interaction with fetal FasL is thus critical for both deletion and hyporesponsiveness of H-Y-reactive CD8⁺ T cells during pregnancy. The Journal of Immunology, 2005, 174: 4657–4661.

Developing T cells undergo selective processes in the thymus so that self-reactive T cells are eliminated before entering the periphery where they potentially could do harm (reviewed in Ref. 1). However, a difficulty with this approach to self tolerance is that the entire realm of self Ags is not necessarily expressed in the thymus. Some Ags encountered in the periphery could be tissue-specific Ags that are sequestered and never circulate through the thymus. Others may be developmentally expressed Ags from genes turned on during later stages of life such as puberty, pregnancy, or lactation. Developmentally expressed proteins could be particularly problematic since much of T cell development occurs around the time of birth. Thus, circulating mature T cells could find themselves in contact with self Ags to which they are highly reactive. Multiple mechanisms of peripheral tolerance exist that prevent these cells from causing harm, including clonal deletion, immune deviation, suppression by regulatory cells, and the induction of unresponsiveness by mechanisms such as anergy and TCR or coreceptor down-regulation (reviewed in Ref. 2).

We have utilized pregnancy as a model to study peripheral T cell tolerance (3, 4). This model presents an ideal system to study exposure of naive T cells to Ags that have not been previously encountered. In addition, exposure to Ag occurs at physiologically relevant levels during pregnancy, avoiding concerns inherent in experimental systems in which supraphysiological levels of Ag are introduced by injection of Ag or overexpression of transgenic neo-

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*Abbreviations used in this paper: FasL, Fas ligand; WT, wild type.
FasL expression in the placenta was found midgestation and onward in placental trophoblasts that border maternal blood spaces and in the fetal placental endothelial cells. We have generated H-Y-specific TCR transgenic mice that were deficient in functional Fas or FasL, and addressed whether maternal T cell tolerance occurs to fetal H-Y. We report here that Fas/FasL interactions must be intact for maternal T cell tolerance to occur. Moreover, we have taken advantage of this Fas dependence to address the origin of the tolerizing cell. Surprisingly, we have found that fetal expression of FasL is both necessary and sufficient for clonal deletion and induction of hyporesponsiveness to fetal H-Y.

Materials and Methods

Mice

H-Y-specific TCR transgenic RAG-2 knockout (H-Yrag) mice were generated as previously described (3) and maintained in our breeding facility at Bioqual (Rockville, MD). H-Yrag mice were bred with B6.gld/gld mice deficient in functional Fasl (The Jackson Laboratory) to establish TCR transgenic RAG-2-deficient Fasl-deficient (H-Ytag, gld) lines. H-Y mice were also bred to mice deficient for Fas (B6/lpr/lpr; The Jackson Laboratory) to establish TCR transgenic RAG-2-deficient Fas-deficient (H-Yrag, lpr) lines. C57BL/6 mice were obtained from National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD).

Timed pregnant females were generated by mating females with males for 18 h. The day the pair was separated was counted as day 0 (d0) of gestation. Pregnant females were sacrificed at day 18 of gestation (d18). Sex of the fetuses from each pregnancy was determined microscopically, and the number of males per litter was noted. All studies were performed under animal study protocols approved by the Animal Care and Use Committees of the National Cancer Institute and Bioqual, Inc.

Flow cytometric analysis

Cells were resuspended in FACS buffer (0.2% BSA, 0.01% sodium azide in HBSS without phenol red), then 1 × 10⁶ cells stained with the desired directly conjugated Abs for 30 min at 4°C, washed three times in FACS buffer, then incubated with avidin-CyChrome for 10 min, if required, followed by extensive washing in FACS buffer. The Ab that recognizes the clonotypic H-Y-specific TCR expressed by these transgenic mice, T3.70, was a generous gift from Dr. Elizabeth Shores (U.S. Food and Drug Administration, Bethesda, MD). Other fluorochrome-labeled Abs were purchased from BD Pharmingen. Cells were analyzed on a FACScan cytometer (BD Biosciences). Although all ungated events were saved for later analysis, 10,000 events in a live gate were acquired. The number of T3.70-expressing T cells was calculated from the total spleen cell number multiplied by the percentage of T3.70-expressing cells obtained from the flow cytometric data as previously described (4).

Cell culture

Single cell suspensions were prepared and maintained in complete tissue culture medium at 37°C in 5% CO₂ as previously described (4).

Cellular proliferation assay

Spleen cells were labeled with 1 μM CFSE (Molecular Probes) as previously described (10) and cultured at 4 × 10⁵ cells/well in 96-well U-bottom plates for 72 h in the absence or presence of titrated H-Y peptide (KCS RNRQYL) (11) or with 4 × 10⁵ irradiated C57BL/6 male spleen cells as a source of H-Y-expressing APCs. Cultures were harvested at 72 h and analyzed for binding of T3.70 (for the detection of the clonotypic H-Y-specific T cells) (12) and annexin-V-PE as previously described. Data are expressed as the percentage of T3.70-expressing cells that have greater than three divisions (4). Cells were analyzed on a FACScan cytometer (BD Biosciences).

Embryo transfers

Briefly, blastocyst stage embryos were flushed from the fallopian tubes of B6 or B6.gld females mated 24 h previously to B6 or B6.gld males. Fertilized eggs were selected and oviductal transfers performed into H-Yrag or H-Yrag gld pseudopregnant females. Pregnant females were sacrificed at 18 days gestation, embryos were sexed, and spleens collected for further analysis.

Results

Functional Fas/FasL interactions are required for clonal deletion of H-Y-reactive T cells during pregnancy

To determine the role that Fas and FasL may play in the induction of peripheral tolerance, we compared the ability of pregnancy to induce clonal deletion and alter T cell responsiveness in TCR transgenic H-Y-specific T cells from females that expressed either wild-type (WT) (H-Y WT) or nonfunctional Fas (H-Y lpr) or Fasl (H-Y gld). In all cases, we have used TCR transgensics that are RAG-2 knockout to prevent complications from expression of endogenous TCR chains. As previously reported (3), the number of clonotypic T cells in the spleen and lymph nodes of female TCR transgenic mice decreased in an Ag-specific manner during pregnancy. When compared with nonpregnant controls, the number of clonotypic T cells decreased by approximately 50% (4.4 vs 2.0 × 10⁶, p = 0.05) in the spleens of H-Y WT females at day 18 of pregnancy (d18) when male fetuses were present (Fig. 1). We have

![FIGURE 1. Fas/FasL interactions are required for tolerance induction of H-Y-reactive T cells during pregnancy. a, Decreased recovery of H-Y-specific T cells from d18 WT H-Y, but not Fas- or FasL-deficient pregnant females. Spleen cells from nonpregnant or d18 pregnant females were analyzed for expression of T3.70. Results represent pooled data from multiple experiments. b, H-Y-specific T cells recovered from Fas- or FasL-deficient pregnant females do not exhibit hyporesponsiveness to male APCs. 4 × 10⁵ CFSE-labeled spleen cells from either nonpregnant or d18 pregnant WT H-Y, H-Y/lpr, or H-Y/gld mice were cultured in the presence of 4 × 10⁶ male spleen cells for 72 h. Proliferation was assessed by dye dilution after gating on T3.70 annexin V- cells, and the percentage of live clonotype-expressing cells that had divided more than three times was determined. Results represent pooled data from multiple experiments. All pregnancies were assessed and determined to have male fetuses in the litters. For a and b, WT nonpregnant, n = 10; WT pregnant, n = 19; gld nonpregnant, n = 16; gld pregnant, n = 18; lpr nonpregnant, n = 3; lpr pregnant, n = 4; *, p < 0.05; **, p < 0.01).](http://www.jimmunol.org/Downloadedfrom)
previously shown that there is no deletion of H-Y-specific T cells in pregnant H-Yrag females when there are no males in the litter and when H-Y Ag is therefore absent (4). In contrast, there was no significant decrease in clonotype-positive T cells in the spleens of pregnant H-Yrag/lpr mice (Fig. 1), indicating that clonal elimination is Fas-dependent. Furthermore, no significant decrease was observed in the numbers of H-Y-specific T cells in H-Yrag gld pregnant females, in which the FasL molecule is rendered nonfunctional by a naturally occurring point mutation. These results demonstrate that Fas/FasL interactions are required for clonal deletion of H-Y-specific T cells during pregnancy.

In vivo encounter with H-Y during pregnancy results in hyporesponsiveness in remaining H-Y-specific T cells only when Fas/FasL interactions are intact

We next addressed the ability of exposure to H-Y during pregnancy to induce Ag-specific hyporesponsiveness in the remaining (nondeleted) T cells in the absence of Fas/FasL interactions. CFSE-labeled spleen cells from H-Yrag lpr, gld, or WT mice were cultured in the presence of male APCs, then T3.70+ T cells were assessed for proliferation (Fig. 1b). There was a significant decrease in the ability of the remaining clonotype-expressing T cells from d18 pregnant WT H-Yrag females to proliferate to H-Y in vitro compared with control nonpregnant WT H-Yrag. However, when either Fas or FasL was nonfunctional in H-Yrag lpr or gld pregnant females, respectively, there was no decrease in the proliferative ability of T3.70-expressing cells from these mice. These data indicate that the Fas/FasL pathway must be functional not only for clonal deletion to occur, but also for induction of proliferative hyporesponsiveness in response to fetal Ags during pregnancy.

Fetally expressed FasL is necessary for induction of tolerance in H-Y-specific T cells during pregnancy

FasL expression is limited in its distribution and is particularly prominent on activated T cells and CTLs (13) but has been reported in other tissues, such as the placenta (9). To address whether placental expression of FasL is important for tolerance induction or whether FasL expression by maternal cells is sufficient, we designed experiments to selectively manipulate FasL expression on fetal cells. Embryos from either B6 pregnant females (expressing FasL) or from B6.gld pregnant females (lacking expression of functional FasL) were transferred into pseudopregnant WT FasL-expressing H-Yrag females. T cells from females that were recipients of WT embryos had decreased numbers of T3.70-expressing cells in the spleen, comparable to that seen in conventionally pregnant WT H-Yrag pregnant females (Fig. 2a). In contrast, T cell numbers were not decreased in female recipients of gld embryos. The proliferative capacity of CFSE-labeled spleen cells from H-Yrag embryo recipients was assessed upon in vitro culture with male APCs (Fig. 2b). Only T3.70-expressing cells from those females that had received WT embryos had decreased proliferative capacity in response to H-Y presented by male APCs. T cells from females that had received gld embryos were not reduced in their proliferative response to male APCs, responding equivalently to nonpregnant WT H-Y controls. These results demonstrate that FasL expressed on fetal placental tissues is of critical importance for both clonal deletion and induction of hyporesponsiveness in H-Y-specific T cells during pregnancy.

Fetally expressed FasL is sufficient for tolerance induction of H-Y-specific T cells during pregnancy

Whereas the previous experiments demonstrate that fetally derived FasL is necessary for tolerance induction of peripheral T cells during pregnancy, these results do not preclude a role for maternally expressed FasL in tolerance induction. We therefore performed the reverse experiments in which maternal cells were deficient in functional FasL, and the fetal tissues varied in their
expression of FasL. This was accomplished by breeding H-Yrag gld females to B6 males (that express FasL) or B6.gld males (that lack functional FasL) as controls. When both fetuses and mothers lacked FasL, there was no deletion of peripheral T cells, whereas deletion was observed when only the fetus, and not the mother, expressed FasL (Fig. 3a). Furthermore, T3.70-expressing cells from pregnant H-Yrag gld females that carried gld embryos did not exhibit hyporesponsiveness to in vitro challenge with H-Y using male APCs (Fig. 3b), whereas T3.70-expressing cells from pregnant H-Yrag gld females carrying WT embryos exhibited significantly reduced proliferation to in vitro challenge with H-Y. Although to date, Fas expression has not been identified in the fetal placenta, experiments were performed in which the number of H-Y-specific T cells was assessed in Fas-deficient H-Yrag lpr females that carried WT fetuses. There was no significant difference in recovery of H-Y-specific T cells whether the fetal placenta was of WT or lpr origin (5.2 ± 1.8 vs 7.5 ± 2.2 × 10⁶). Overall, these results indicate that fetal expression of FasL is both necessary and sufficient for the induction of both clonal deletion and hyporesponsiveness to H-Y during pregnancy.

FIGURE 3. FasL expression on maternal cells is not required for the induction of tolerance in H-Y-reactive T cells during pregnancy. a, Recovery of H-Y-specific T cells from d18 pregnant H-Y gld mice is decreased only when the fetus expresses FasL. Spleen cells from nonpregnant or pregnant H-Y gld females were analyzed for expression of T3.70. Results represent pooled data from multiple experiments. b, Proliferative ability of H-Y-specific T cells from d18 pregnant H-Y gld mice is decreased when the fetus expresses FasL. 4 × 10⁶ CFSE-labeled spleen cells from either nonpregnant or d18 pregnant WT H-Y mice were cultured in the presence of 4 × 10⁵ male spleen cells for 72 h. Proliferation was assessed by dye dilution after gating on T3.70⁻ annexin V⁻ cells, and the percentage of live clonotype-expressing cells that had divided more than three times was determined. Results represent pooled data from multiple experiments. For a and b, gld nonpregnant, n = 11; gld pregnant with WT fetus, n = 11; gld pregnant with gld fetuses, n = 18. All pregnancies were assessed and determined to have male fetuses in the litters; *, p < 0.05; **, p < 0.01.

Discussion

We have found that Fas/FasL interactions are required for the induction of both clonal deletion and hyporesponsiveness in maternal T cells to the fetal Ag, H-Y. Although FasL is expressed on activated T cells and CTL and has been shown to be involved in fratricidal/suicidal cell death by interacting with Fas expressed on T cells, FasL expression on maternal T cells was dispensable for the induction of clonal deletion. Using combinations of embryo transfers or specific breeding strategies, we demonstrate that interaction of Fas-expressing maternal T cells with FasL-expressing fetal cells is not only both necessary and sufficient for the induction of maternal T cell deletion during pregnancy but, surprisingly, is required for the induction of hyporesponsiveness as well.

Our work, as well as that of others, has demonstrated that the maternal immune system is aware of the fetus and becomes tolerant to the fetus either transiently (14, 15) or long-term as we have observed (3). Little is understood about how fetal Ags are recognized by the mother or how this recognition leads to tolerance. Fetal Ags could be picked up and processed by maternal APCs, then presented to maternal T cells. Studies have recently revealed that cross-presentation of soluble Ag via MHC class I can and does occur, resulting in tolerance induction (reviewed in Ref. 16). For example, Ags from the pancreas are captured by dendritic cells that then migrate to the draining lymph node where the Ag is then presented to CD8 T cells (17). After initial activation, reactive T cells become unresponsive or are deleted. Mechanisms such as this may be one way in which maternal T cells encounter fetal Ags such as H-Y and become tolerant. Alternatively, Ag contact could occur directly between the fetal cells and maternal T cells, leading to tolerance. Our findings demonstrate that fetal cells must express FasL for tolerance mechanisms to occur and therefore implicate the fetal cell in a functional role beyond simply acting as a donor of cross-presented tolerizing Ag.

Since it has been previously reported that Fas signaling is a major mechanism for peripheral clonal deletion in response to self Ags, it is not surprising that absence of Fas or its ligand abrogates clonal deletion. However, it is surprising that signaling through Fas would result in hyporesponsiveness in the remaining T cells. Although it has not previously been observed that direct signaling through Fas on a Fas-expressing cell can lead to hyporesponsiveness, we cannot rule out that Fas/FasL interactions may, under certain conditions, trigger an uncharacterized pathway inducing tolerance by means other than apoptosis. Alternatively, induction of an apoptotic pathway in a population of T cells via Fas/FasL interactions could somehow influence the fate of the surviving T cells, rendering them hyporesponsive. Our observation in pregnancy has similarities to a previously reported system characterized by Griffith et al. (18) studying another immunologically privileged site. TNP-coupled Fas-expressing T cell interactions with FasL-expressing cells in the anterior chamber of the eye were required to induce systemic tolerance. Induction of apoptosis via Fas/FasL was critical for the induction of immune deviation resulting in decreased delayed-type hypersensitivity and IFN-γ production, whereas cell death by other mechanisms such as necrosis did not result in tolerance. It was proposed that induction of apoptosis in these T cells by FasL-expressing cells in the anterior chamber of the eye resulted in IL-10 expression by the dying cells. IL-10 expression, in turn, modulated local APC function resulting in immune deviation in the periphery (18, 19). We have previously demonstrated that although numbers of clonotype-expressing T cells decrease during pregnancy, those remaining T3.70-expressing T cells do not have altered cytokine expression (4). Although we do not observe altered IFN-γ production in the surviving
T3.70-expressing cells (data not shown) as noted in the system of Griffith et al. (18), we do observe hyporesponsive proliferative and CTL responses in these T cells.

We have previously shown that CD28/B7 interactions are required for induction of tolerance to H-Y during pregnancy. There is a striking similarity between the requirement for the Fas/FasL pathway and for the CD28/B7 pathway in the induction of CD8 T cell tolerance in this system. Activation of both pathways is required for the induction of clonal deletion and for the induction of hyporesponsiveness in H-Y-specific T cells upon encounter with Ag. Interestingly, these pathways appear to be non-redundant. One explanation of this non-redundancy is that the two pathways are linked (e.g., expression of FasL is dependent on CD28-mediated signals). Recently a functional CD28 response element in the FasL promoter has been found, linking CD28 activation with CD95L up-regulation (20). It is thus possible that recognition of H-Y and subsequent activation via both TCR and CD28 would result in up-regulation of FasL on the activated T cell such that tolerance could be mediated through maternal T–T interactions. However, experiments in this study argue against this possibility. Embryo transfer experiments in which functional FasL is expressed only in the mother revealed a lack of tolerance induction. Furthermore when reverse experiments were performed in which the mother lacked FasL, and only the fetus expressed FasL, deletion and hyporesponsiveness did occur. This points to critical Fas/FasL interactions occurring between maternal and fetal cells in the process of tolerance induction and not simply interactions between maternal T cells.

Another possibility is that both the CD28/B7 pathway and Fas/FasL pathways are important for two independent but essential steps in the tolerance induction pathway. One scenario is that both B7 and FasL, as well as the H-Y peptide/MHC complex, are encountered by a maternal T cell on the same fetal cell. Others have demonstrated placental B7.1 expression present but highly restricted in the placenta, primarily on a population of fetal macrophages (21). Maternal T cells could interact with this small population of fetal macrophages between the mother and the conceptus.

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Disclosures

The authors have no financial conflict of interest.

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