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*J Immunol* 2009; 182:8080-8093; doi: 10.4049/jimmunol.0804018

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Cross-Presentation of Male Seminal Fluid Antigens Elicits T Cell Activation to Initiate the Female Immune Response to Pregnancy

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The events that generate T cell-mediated immune tolerance in early pregnancy are ill-defined. To investigate the significance of seminal fluid Ags in activating maternal T cells, and define the underlying Ag presentation pathways, OVA-specific T cells were adoptively transferred to female mice inseminated by males ubiquitously expressing membrane-bound OVA. OVA-reactive CD8+ OT-I and CD4+ OT-II T cells transferred to mated recipients expressed activation markers CD25 and CD69 and proliferated vigorously in the para-aortic lymph nodes, but not in distal lymph nodes or spleen, and OT-I T cells expressed IFN-γ and IL-2. In contrast, OT-I T cells transferred later in pregnancy or up to 10 days postpartum expressed CD25 and CD69 and proliferated in all peripheral lymphoid tissues examined. OVA Ag was present predominantly in the plasma fraction of seminal fluid, and seminal plasma, but not sperm, was necessary for T cell proliferation. Female H-2Kb bone marrow-derived cells expressing TAP were essential for OT-I T cell proliferation, but responses were not elicited by OVA Ag presented by paternal MHC in seminal fluid or associated with placental cells. This study shows that at conception, seminal fluid drives activation and expansion of paternal Ag-reactive CD4+ and CD8+ T cell populations, and female APCs have an essential role in cross-presenting Ag to CD8+ T cells via a TAP-dependent pathway. Delivery of paternal Ags and immune-deviating cytokines by seminal fluid at conception may activate Ag-dependent CD4+ and CD8+ regulatory T cells mediating tolerance of pregnancy. The Journal of Immunology, 2009, 182: 8080–8093.

A repertoire of immune evasion strategies contributes to survival of the semiallogeneic fetus in pregnancy, acting to silence or modulate induction and effector mechanisms in both the innate and adaptive immune compartments (1, 2). TCR-transgenic models have been informative in identifying the underlying regulatory mechanisms, and show that T cell awareness of paternal MHC and other conceptus Ags results in a transient state of active immune tolerance in pregnant females (3–5), which is mediated largely by T regulatory (Treg)3 cells (6, 7). Inducible CD4+ and CD8+ Treg cells generated in peripheral tissues require Ag-driven activation and proliferation to achieve their full suppressive function (8, 9). The events driving activation of the Treg cell pool during early pregnancy are not well defined, and the nature and origin of the eliciting Ags are unclear (10). Experiments in TCR-transgenic systems specific for paternal H-Y Ag (4), paternal MHC class I (5), or using OVA as a model paternal Ag (11) suggest that Ags shed by the placenta and cross-presented by maternal dendritic cells (DCs) elicit T cell responses in midgestation (11), but this pathway does not explain observations that CD4+CD25+ Treg cell abundance increases within days after mating and before embryo implantation (6, 12), in an Ag-specific manner (13, 14). CD8+ cells with regulatory function are also implicated in suppression of fetal immune rejection from early pregnancy (15, 16). This is consistent with indications of Ag recognition in maternal MHC-reactive CD8+ T cells, accompanied by functional tolerance of paternal tumor challenge evident from the time of implantation (3, 12).

Female reproductive tissues are exposed to paternal Ags at two distinct stages of pregnancy, as follows: first, when seminal fluid is introduced at coitus (17), and second, when placental trophoblast cells invade maternal tissues after embryo implantation (18, 19). Given the evidence for adaptation in the maternal T cell compartment before embryo implantation, we hypothesized that female T cells can recognize and respond to paternal Ags in seminal fluid, and that exposure to seminal fluid Ags at the time of conception may constitute the initial priming event in the maternal immune response to pregnancy. The female reproductive tract is well equipped to initiate immune responses at insemination, because APCs are abundant in the uterine environment at this time. Indeed, seminal fluid has an active role in APC recruitment. Soluble factors in the seminal plasma induce expression of proinflammatory cytokines and chemokines in epithelial cells lining the cervix and uterus, causing recruitment of macrophages, DCs, and granulocytes that accumulate near the epithelial surface and persist there for several days (20).
Hostile immune responses to seminal Ags would be incompatible with maintenance of fertility, with adverse effects on sperm viability and oocyte fertilization (21) as well as pregnancy, because the conceptus shares MHC and other paternal Ags with those in semen (22, 23). However, seminal plasma is a rich source of immune-deviating agents PGE and TGF-β (24, 25) that are identified as potent inducers of Treg cells (26, 27). There is some existing evidence that female exposure to seminal fluid results in functional tolerance to male Ags. Female mice exposed to seminal plasma even in the absence of sperm or a conceptus show evidence of T cell activation (28) and hyporesponsiveness in type 1 immunity to male MHC Ags, and tolerate challenge with male tumor cells or skin grafts (12, 29–31). We have recently shown that the tolerance-inducing effect of seminal fluid transmission at coitus is associated with expanding CD4+CD25+ Treg cell populations in para-aortic lymph nodes (LN) draining the female reproductive tract (12). A role for seminal fluid in priming the immune response to pregnancy would explain why disrupting the female response to seminal fluid interferes with embryo implantation (32).

Despite these observations, there is no direct evidence that male Ags delivered in seminal fluid can activate Ag-specific T cells in females, and no information on the mechanisms by which seminal fluid Ags might be processed and presented. In this study, we have used a TCR-transgenic model using OVA as a model paternal Ag to evaluate the female CD4+ and CD8+ T cell response to seminal fluid Ags, and to identify the APCs involved in seminal fluid Ag processing and presentation. We show that OVA constitutively expressed in the male reproductive tract and delivered by the ejaculate to the female elicits activation and proliferation of adoptively transferred CD4+ and CD8+ OVA-specific T cells in para-aortic LN. CD8+ T cells are activated by cross-presented seminal fluid OVA mediated exclusively by female bone marrow-derived APCs, using a pathway dependent on TAP.

Materials and Methods
Mice
Mice were housed in a pathogen-free facility on a 12:12 light-dark cycle, and given food and water ad libitum. TCR-transgenic C57BL/6 OT-I mice (33) (generously provided by W. Heath, University of Melbourne, Victoria, Australia) and C57BL/6 OT-II mice were from Animal Resource Centre, Perth, Australia; and F. Carbonne, Melbourne University, Melbourne, Australia) were crossed with B6.SJL-Tprcp/Pep3b/B6.J mice expressing CD45.1 (B6.SJL mice; Agricultural Research Council) to generate OT-I mice expressing CD45.1 (OT-I mice). TCR-transgenic C57BL/6 OT-II mice were from Animal Resource Centre, Perth, Australia; TAP+/− mice with a null mutation in the TAP1 and TAP2 genes (34) were provided by W. Chen (Ludwig Institute, Melbourne, Australia); bm1 mice (H-2Kbm1) (35) and Act-mOVA bm1 mice were provided by W. Heath and F. Carbonne; and C57BL/6 mice expressing an Ac-mOVA transgene (Act-mOVA mice) (36) were provided by M. Jenkins (University of Minnesota, Minneapolis, MN). Act-mOVA TAP+/− mice were generated as the F2 progeny of the respective intercrossed parental lines, and together with C57BL/6 (H-2Kb) (B6) mice were bred in-house. Act-mOVA, Act-mOVA bm1, and Act-mOVA TAP+/− mice were all homozygous for OVA transgene expression were identified by OVA+ peripheral blood cells detected using biotinylated rabbit anti-OVA (Rockland). The University of Adelaide Animal Ethics Committee reviewed and approved all experiments.

Surgical excision of the seminal vesicle gland (SVX) or vasectomy in male mice, and ligation of the oviductal-uterine junction in female mice, was performed under isoflurane anesthesia 2–4 wk before mating, as previously described (33). For experiments evaluating OVA transgene expression were identified by OVA+ peripheral blood cells detected using biotinylated rabbit anti-OVA (Rockland). The University of Adelaide Animal Ethics Committee reviewed and approved all experiments.

Additional groups of intact B6 females in proestrus (defined by external vulvar swelling and the presence of nucleated and keratinized epithelial cells in vaginal smears) (37) were administered OVA (fraction V; Sigma-Aldrich), or seminal fluid from Act-mOVA mice recovered by uterine lavage from mated bm1 females. Females were anesthetized with isoflurane and Ag (0.4–5 μg in 50 μl of PBS), or uterine lavage fluid (50 μl) was instilled into the uterine cervix transvaginally, using a syringe fitted to a vinyl 3.5 Fr Tom Cat catheter (Tyco Healthcare Group).

For analysis of fetal loss rates and late gestation pregnancy parameters, B6 females mated with Act-mOVA administered OT-I or B6 T cells were sacrificed by cervical dislocation at 1000–1200 h on day 17.5 pc. The intact uterus of each female was removed, and total, viable, and resorbing implantation sites were counted. Each viable fetus was dissected from the amniotic sac and umbilical cord, and fetuses and placentae were weighed.

OVA ELISA
The OVA content of uterine luminal lavage fluid, or from homogenized fetal and placental tissue, was determined by ELISA. Goat anti-OVA capture Ab (MP Biomedicals) was incubated overnight at 4°C in Maxisorp 96-well microtiter plates (Nunc). Plates were washed and blocked with 1% BSA/PBS. OVA protein standards and samples were added to wells and incubated for 2 h at 37°C. Bound OVA was detected using biotinylated rabbit anti-OVA (Rockland), followed by streptavidin-HRP (Rockland) and HRP Substrate Fast o-phenylenediamine (Sigma-Aldrich). The OVA concentration of uterine luminal fluid was calculated according to the parameter-logistic curve fit function of SigmaPlot software (Systat).

Adoptive transfer of OVA-reactive OT-I and OT-II TCR-transgenic T cells
Lymphocytes prepared from regional LN of OT-I and OT-II mice were suspended at 106 to 108 cells/ml in RPMI 1640 containing 2 mM L-glutamine, 5 · 10−5 M 2-ME, and antibiotics (complete RPMI 1640), and labeled by addition of CFSE (0.1 μl of 5 mM stock in DMSO) (Molecular Probes) for 10 min at 37°C. After washing, 106 cells in 200 μl of complete RPMI 1640 were administered i.v. into B6 or bm1 female recipients, at 1200 h on specified days of pregnancy (days 0.5, 3.5, 6.5, 10.5, and 14.5 pc) or postpartum (pp) (days 3, 7, 10, 20, 40, and 70 pp). Mice designated days 3.5–14.5 pc were only included in experiments if viable conceptus tissue was present at autopsy.

Analysis of OT-I and OT-II T cell proliferation and expression of activation markers
Virgin, pregnant, or pp mice were killed 68 h after OT-I or OT-II T cell transfer, and single-cell suspensions were prepared from excised para-aortic (iliac) LN, cervical LN, mesenteric LN, and the spleen. Cells were labeled with anti-CD8α-PE (OT-I T cells) or anti-CD4-PE (OT-II T cells; BD Pharmingen), anti-CD45.1 and anti-CD25-PE-Cy7, followed by streptavidin-PE-Cy5 (all obtained from BD Pharmingen). Analysis was performed using a FACSCanto with FACSDiva software (BD Biosciences). OT-I T cell proliferation was quantified by measuring CFSE content in the CD8+CD45.1+ population (38) after gating to exclude debris and dead cells. The extent of OT-I and OT-II T cell division was quantified using FACSDiva software, as described (39). In brief, the number of cells in each CFSE generation was used to calculate the ratio of T cells recovered, as follows:

\[ \text{ratio of T cells transferred} = \frac{n \times n_1 + n_2 + \ldots + n_m}{n_{pp}} \]

where \( n \) is cell number, PP = parent peak, G1 = generation 1 peak, G2 = generation 2 peak, etc.

FACS analysis of cytokine expression
Expression of IFN-γ and IL-2 was determined in OT-I T cells recovered from para-aortic LN 68 h after transfer to B6 mice on day 0.5 pc or on day 14.5 pc after mating with Act-mOVA mice. Additional groups of OT-I T cells for cytokine analysis included naïve OT-I T cells, or OT-I T cells activated in vitro or in vivo. To generate in vitro activated OT-I T cells, B6 spleen cells (10 × 106 cells/ml) were pulsed with 1 μM OVApeptide for 2 h at 37°C/5% CO2, complete RPMI 1640 plus 10% FCS, then washed and cultured in a 1:1 ratio at 2 × 106 cells/ml with OT-I T cells for 48 h in complete RPMI 1640 plus 10% FCS. To generate in vivo activated OT-I T cells, B6 female mice were administered 25 μg of OVA protein in 25 μl of PBS injected s.c. into the footpad, accompanied by adoptive transfer of 10 × 106 OT-I T cells i.v., and then popliteal LN cells were harvested 68 h later.

To assess cytokine expression in OT-I T cells activated in vivo or in vitro, para-aortic LN cells, popliteal LN cells, or cultured OT-I T cells (5 × 107 cells/ml) were restimulated by incubation for 4 h at 37°C/5% CO2, in complete RPMI 1640 plus 10% FCS with the following additions: PMA (Sigma-Aldrich; 100 ng/ml), calcium ionophore (Sigma-Aldrich; 1 μg/ml), monensin (eBiosciences; 1/1000 dilution), and a 1:1 ratio of OVApeptide.
peptide-pulsed B6 splenocytes. Cell surface markers (CD8, CD45.1, and CD69) were labeled with fluorochrome-tagged mAbs, as described above. Intracellular cytokine staining was then performed, as previously described (28). Briefly, for IL-2 staining, cells were fixed in 4% paraformaldehyde (1 ml/6 min/4°C), washed in FACS buffer, and incubated with anti-IL-2-PE (eBiosciences; clone JES6-5H4) in saponin buffer (PBS/1% FCS/0.1% saponin (Sigma-Aldrich); 1 ml/45 min/4°C). For IFN-γ staining, cells were fixed, permeabilized, and labeled with anti-IFN-γ-PE (eBiosciences; clone XMG1.2) using reagents supplied with an eBiosciences staining buffer set (catalog no. 00-5523), according to the manufacturer’s instructions.

B3Z T cell hybridoma activation assays

The B3Z response to 1H-2K/OVA_{323-330} complexes expressed by LN or spleen APCs was measured by IL-2-driven βgalactosidase reporter activity, as previously described (40). LN or spleen cells (1 x 10^6 cells) collected on day 14.5 pc were cultured with 5 x 10^6 B3Z cells in 1 ml of complete RPMI 1640 plus 10% FCS. After 18–20 h incubation, wells were washed and fixed in cold 2% formaldehyde/0.2% glutaraldehyde at 4°C for 10 min, then overlaid with 0.5 ml of substrate containing 1 mg/ml X-gal (Sigma-Aldrich), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl_2 in PBS, and incubated overnight at 37°C. The number of blue cells per well was counted by light microscopy.

Preparation of bone marrow chimeras

Donor bone marrow was harvested from the tibia and fibia of female B6.SJL or bm1 mice, and depleted of T cells and NK cells by incubation with anti-Thy 1 (TIB107; American Type Culture Collection), anti-NK1.1 (HB191; American Type Culture Collection), and guinea pig complement. B6.SJL and bm1 females were lethally irradiated with 900 cGy, then administered donor bone marrow from either B6.SJL or bm1 mice (5 x 10^6 cells i.v.), and allowed to recover for 8 wk to allow complete bone marrow reconstitution. Recipients were administered either OVA protein (50 µg) or seminal fluid recovered as uterine luminal lavage from bm1 females mated 8–10 h previously with Act-mOVA males, and immediately given CFSE-labeled OT-I T cells, as above.

Statistical analysis

The effect of treatment group on PI, percentage of CD69 expression, and late gestation pregnancy parameters was analyzed by nonparametric Kruskal-Wallis H test and Mann-Whitney U tests using SSPS 13.0. (SPSS). The effect of treatment on fetal resorption rate was analyzed by χ^2 analysis. Differences between treatment groups were considered significant when p < 0.05.

Results

Seminal fluid OVA induces OT-I and OT-II T cell proliferation in para-aortic LNs

Initially, we evaluated whether seminal fluid from Act-mOVA males can induce CD8^+ and CD4^+ T cell responses in mated females, and compared the location and scale of responses with those elicited by OVA-expressing fetal tissues during midgestation pregnancy. Female B6 mice were mated with Act-mOVA males, followed by adoptive transfer of CFSE-labeled OT-I T cells ~12 h later on day 0.5 pc. When OT-I T cells were evaluated 68 h after transfer, CFSE dye dilution showed OT-I T cells undergo extensive proliferation in the para-aortic LN, associated with up-regulated expression of the very early activation marker CD69 and the IL-2R CD25. OT-I T cells did not proliferate or modulate CD69 or CD25 expression after transfer to B6 females mated with B6 males (Fig. 1A) or to virgin B6 mice (data not shown). All of four B6 mice mated with Act-mOVA males showed clearly detectable responses, with (mean ± SEM) PI = 2.0 ± 0.4 (Fig. 3A) and percentage of CD69 expression = 47 ± 6 (Fig. 3B), compared with values in B6 females mated with B6 males of PI = 1.0 ± 0.0 and percentage of CD69 expression = 5 ± 2 (both p < 0.05). No proliferation or modulation of CD69 or CD25 expression was evident in OT-I T cells recovered from other LNs and the spleen of mated mice (Fig. 1A).

This contrasted with the more intense and disseminated response seen when B6 mice mated with Act-mOVA males were given CFSE-labeled OT-I T cells in midgestation pregnancy on day 14.5 pc. Extensive OT-I T cell proliferation as well as CD69 and CD25 expression were evident not only in the para-aortic LN, but also systemically in the cervical LN, mesenteric LN, and spleen (Fig. 1B). In contrast, OT-I T cells did not respond when given in midgestation to B6 females mated with B6 males (Fig. 1B).

The occurrence and location of OVA-specific CD4^+ T cell responses were analyzed by undertaking similar experiments using CFSE-labeled OT-II T cells. In female B6 mice mated with Act-mOVA males and administered CFSE-labeled OT-II T cells ~12 h later, extensive proliferation together with up-regulated CD69 and CD25 expression were evident in OT-II T cells in the para-aortic LN 68 h later. No response was seen in OT-II T cells recovered from other LNs or the spleen (Fig. 2A). OT-II T cells transferred to virgin B6 mice did not proliferate or modulate CD69 expression (data not shown), and similarly, OT-II T cells did not respond in B6 females mated with B6 males (Fig. 2A). Responses were seen in all of four mated B6 mice, with (mean ± SEM) PI = 2.2 ± 0.4 and percentage of CD69 expression = 63 ± 6, compared with values in B6 females mated with B6 males of PI = 1.0 ± 0.0 and percentage of CD69 expression = 8 ± 6 (both p < 0.05). When B6 mice mated with Act-mOVA males were given CFSE-labeled OT-II T cells in midgestation pregnancy on day 14.5 pc, extensive OT-II T cell proliferation, as well as CD69 and CD25 expression, were evident in the para-aortic LN, cervical LN, mesenteric LN, and spleen (Fig. 2B). No OT-II response occurred in midgestation support B6 females mated with B6 males (Fig. 2B).

These data show that OVA Ag delivered to the female reproductive tract in the context of seminal fluid at mating can be processed and presented via both MHC class I and MHC class II pathways to elicit OVA-specific CD8^+ and CD4^+ T cell responses, respectively, characterized by substantial T cell proliferation and expression of activation markers CD69 and CD25. In comparison with responses elicited in CD8^+ and CD4^+ T cells by OVA-expressing fetal tissue in midgestation pregnancy, which were disseminated to peripheral LNs and the spleen, the seminal fluid response was moderately less intense and limited in both cases to local LNs draining the female reproductive tract.

Kinetics of OT-I T cell proliferation after mating, in pregnancy, and after birth

To investigate the kinetics of the CD8^+ response to OVA in pregnancy, OT-I T cell proliferation and CD69 expression were evaluated at defined stages of pregnancy and after delivery of pups in female B6 mice mated with Act-mOVA males. CFSE-labeled OT-I T cells were adoptively transferred to pregnant mice on days 0.5, 3.5, 6.5, 10.5, and 14.5 pc, or to pp mice on days 3, 7, 10, 20, 40, and 70 pp. In each case, OT-I T cell responses were evaluated 68 h later. In the para-aortic LN, the extensive proliferation and up-regulated expression of CD69 observed after mating declined to a low, but still detectable response when OT-I T cells were transferred on day 3.5 pc, at the time of embryo implantation (Fig. 3). In OT-I T cells transferred from day 6.5 pc onward, there was a progressive increase in the intensity of the proliferative and CD69 responses over the course of gestation. Following delivery of pups, the OT-I T cell response diminished, although substantial proliferation and CD69 expression were evident in OT-I T cells transferred as late as 10 days pp (Fig. 3). Although significant increases compared with virgin baseline data were not observed following that time, individual mice occasionally showed evidence of OT-I T cell proliferation as well as up-regulated CD69 expression until day 40 pp. The kinetics of CD25 expression by OT-I T
FIGURE 1. Mating with Act-mOVA males elicits activation and proliferation in OT-I T cells. Female B6 mice were mated with Act-mOVA males, and OT-II T cells were adoptively transferred i.v. on day 0.5 pc in mated females (A) or day 14.5 pc in pregnant females (B). Para-aortic LN (PALN), mesenteric LN (MLN), cervical LN (CLN), and spleen (SPL) were recovered 68 h later, and proliferation, CD69 expression, and CD25 expression were evaluated in each tissue. Additional groups of B6 mice were mated with B6 males and given OT-I T cells on day 0.5 pc or day 14.5 pc, before recovery and analysis of para-aortic LN 68 h later (PALN-NEG). All data shown are representative of n = 4 mice per group. PI, percentage of CD69−, and percentage of CD25+ values are shown in the top left corner of individual histograms and dot plots.
FIGURE 2. Mating with Act-mOVA males elicits activation and proliferation in OT-II T cells. Female B6 mice were mated with Act-mOVA males, and OT-I T cells were adoptively transferred i.v. on day 0.5 pc in mated females (A) or day 14.5 pc in pregnant females (B). Para-aortic LN (PALN), mesenteric LN (MLN), cervical LN (CLN), and spleen (SPL) were recovered 68 h later, and proliferation, CD69 expression, and CD25 expression were evaluated in each tissue. Additional groups of B6 mice were mated with B6 males and given OT-II T cells on day 0.5 pc or day 14.5 pc, before recovery and analysis of para-aortic LN 68 h later (PALN-NEG). All data shown are representative of $n = 4$ mice per group. PI, percentage of CD69$^+$, and percentage of CD25$^+$ values are shown in the top left corner of individual histograms and dot plots.
cells in pregnancy and after pup delivery almost exactly mirrored that of CD69 (data not shown).

In the mesenteric LN, cervical LN, and the spleen, detectable OT-I T cell proliferation was observed from day 6.5 pc onward, after which time the kinetics of the response was similar to the para-aortic LN, with increasing intensity over the course of gestation, albeit with a moderately lower maximal proliferative response and expression of activation markers CD69 and CD25.

Seminal fluid induces cytokine expression in OT-I T cells

In addition, we evaluated whether OVA in seminal fluid or conceptus tissues in midgestation pregnancy can induce a cytokine response in OT-I T cells. OT-I T cells activated in vitro with OVA\textsubscript{257–264} peptide-pulsed spleen cells, or OT-I T cells activated in vivo by OVA administration to the footpad. Expression of IFN-γ and IL-2 was clearly detectable in OT-I T cells activated in vitro, and in the majority of popliteal LN preparations from footpad-immunized mice (four of five mice; Fig. 4). OT-I T cells recovered from the para-aortic LN after transfer on day 0.5 pc expressed clearly detectable IFN-γ, with (mean ± SEM, n = 6 mice) percentage of IFN-γ\textsuperscript{+} OT-I T cells = 8.6 ± 1.3 (p = 0.002 compared with naive OT-I T cells; Fig. 4C) and percentage of IL-2\textsuperscript{+} OT-I T cells = 3.4 ± 0.5 (p = 0.002; Fig. 4D). In contrast, neither cytokine was consistently induced in OT-I T cells recovered after transfer on day 14.5 pc (Fig. 4, C and D). Expression of IFN-γ and IL-2 in OT-I cells after mating provides further evidence of CD8\textsuperscript{+} T cell activation in response to seminal fluid OVA Ag, consistent with their proliferative response and expression of activation markers CD69 and CD25.

Seminal plasma, but not sperm, is required to elicit OT-I and OT-II T cell proliferation

To investigate the physiological role of seminal fluid constituents in eliciting the CD8\textsuperscript{+} and CD4\textsuperscript{+} response in females, initially we quantified the presence of OVA in different fractions of seminal fluid of Act-mOVA male mice. The mean ± SEM OVA content in seminal fluid flushed from the female tract within 2 h of ejaculation was 6.8 ± 1.6 μg, with ~50% associated with each of the cellular and cell-free fractions of the uterine luminal exudate (Fig. 5A), implying a relatively similar abundance of membrane-bound and soluble OVA in the ejaculate. Comparable amounts of OVA were present in the ejaculates of intact and vasectomized males, indicating that spermatozoa and tests-derived material does not contribute substantially. In contrast, OVA was barely detectable in ejaculates of males from which the seminal vesicle glands were surgically excised. This shows that OVA in seminal fluid principally originates from the male seminal vesicle glands, where the majority of seminal plasma is produced (Fig. 5A). When repeated ejaculates from the same individual intact males were studied, the OVA content ranged from 0.8 to 12.3 μg, and the total OVA deposited in sequential matings from the same individual males varied up to 120% of mean values, with a mean variance of 43% (Fig. 5B). This most likely represents differences in the number of intromissions and amount of seminal fluid deposited per mated female, as well as variation in OVA synthesis, accumulation, and degradation within the male reproductive tract.

In view of the finding that different doses of OVA are delivered in different mating events, and to determine whether OT-I T cell proliferation depends on Ag dose, we evaluated the female OT-I T cell response to known doses of OVA administered to the uterus by transcervical catheter. The uterus is the site of seminal fluid deposition in the mouse (41). Female B6 mice identified as showing external signs and vaginal cell composition indicative of proestrus were anesthetized and OVA protein was administered transcervically to the uterus, followed immediately by adoptive transfer of CFSE-labeled OT-I T cells. Extensive OT-I T cell proliferation was observed in the para-aortic LN (Fig. 5C), but not mesenteric LN, cervical LN, or spleen when OT-I T cells were recovered 68 h later (data not shown). The extent of OT-I T cell proliferation observed was dose dependent, but was clearly evident from the lowest dose of OVA administered (0.4 μg). At doses approximating the quantity of OVA deposited by natural mating, substantial OT-I T proliferation was seen (PI = 1.31 ± 0.09 and PI = 1.28 ± 0.07 for 2 and 10 μg of OVA, respectively) (Fig. 5C), although this was lower than the proliferative response induced by mating with intact Act-mOVA males. For comparison, the OVA
FIGURE 4. Mating with Act-mOVA males elicits cytokine expression in OT-I T cells. Female B6 mice were mated with Act-mOVA males, and OT-I T cells were adoptively transferred i.v. on day 0.5 pc in mated females or day 14.5 pc in pregnant females. Para-aortic LN were recovered 68 h later, and expression of IFN-γ (A and C) and IL-2 (B and D) was quantified by intracellular cytokine staining. Additional preparations of naive and activated OT-I T cells were recovered from para-aortic LN of naive mice, popliteal LN of mice immunized with OVA in the footpad, or after OT-I T cell activation in vitro. Representative histograms illustrate percentage of IFN-γ+ OT-I T cells (A) and percentage of IL-2+ OT-I T cells (B) in each group, with percentage of positive cells in the top right corner. The percentage of IFN-γ+ OT-I T cells (C) and percentage of IL-2+ OT-I T cells (D) were quantified for each group. Symbols are data from individual mice, with median values indicated (n = 4–6 mice per group). Treatment effects were analyzed by Kruskal-Wallis H test and Mann-Whitney U tests. Groups with different superscripts (a, b, and c) are significantly different (p < 0.05).

Maternal cells present seminal fluid and conceptus-derived OVA Ag to OT-I T cells

OT-I T cells are restricted to recognition of the immunodominant OVA peptide, OVA257–264, in the context of H-2Kb. In B6 (H-2b haplotype) females mated with Act-mOVA (H-2b haplotype) males, APCs eliciting OT-I T cell proliferation could theoretically be of female or male origin (42). To evaluate the origin of the APCs presenting seminal fluid OVA Ag to OT-I T cells, bm1 females that carry a point mutation in H-2Kb preventing presentation of OVA257–264 to OT-I T cells (35) were used. Additionally, TAP−/− mice were used to determine the necessity for TAP in the Ag-presentation pathway. The bm1, TAP−/−, or B6 females were mated with Act-mOVA males and OT-I T cells were adoptively transferred ~12 h later on day 0.5 pc. When the OT-I T cell response was evaluated 68 h later, neither proliferation nor increased CD69 expression was evident in the para-aortic LN of bm1 mice, in contrast with robust responses in B6 females (Fig. 6A). In contrast, the bm1 mutation or TAP deficiency in Act-mOVA males did not diminish their capacity to elicit a strong proliferative and CD69 response. This result shows that CD8+ T cell proliferation in mated B6 females is dependent on processing and presentation of seminal fluid OVA Ag by female APCs, using a TAP-dependent pathway.

A similar lack of proliferation was evident in bm1 females or TAP−/− females mated with Act-mOVA males when OT-I T cells were adoptively transferred on day 14.5 pc (Fig. 6B), indicating that OVA Ag released from the fetus and placenta must be

content of fetal and placental tissue at day 14.5 pc was also quantified and both tissues contained amounts of OVA that, assuming an ejaculate volume of ~50 μg, would correspond to similar OVA concentrations in seminal fluid and gestational tissues (Fig. 5D).

To determine the relative importance of the sperm and seminal plasma constituents of seminal fluid in eliciting CD8+ and CD4+ T cell proliferation, female B6 mice were mated with intact, vasectomized, or seminal vesicle-deficient (SVX) Act-mOVA males and administered CFSE-labeled OT-I or OT-II T cells 12 h later. When OT-I T cell responses in the para-aortic LN were evaluated 68 h later, extensive proliferation (Fig. 5E) and up-regulated CD69 expression and CD25 expression (data not shown) were evident in all females mated with intact or vasectomized males, but were only detectable at low levels in some females mated with SVX males. Similar results were seen in OT-II T cells (Fig. 5F). When the oviductal-uterine junction was ligated surgically in female mice to prevent conception, but allow uterine exposure to the complete ejaculate, OT-I and OT-II T cell responses were indistinguishable from those seen in females with patent tracts, indicating that conception and formation of embryos are not a prerequisite for either OT-I or OT-II T cell responses (Fig. 5, E and F). These data show that female reproductive tract exposure to the seminal plasma component of seminal fluid is necessary to elicit OVA-specific CD8+ and CD4+ T cell proliferation in the para-aortic LN after insemination, but that sperm and embryos are not required for a response.
processed and presented by cells expressing maternal MHC via a TAP-dependent pathway to elicit CD8$^+$ T cell proliferation in midgestation pregnancy. We then examined whether OT-I T cell proliferation in LNs and spleen is the consequence of Ag presentation occurring locally within those tissues. The abundance of H-2Kb/OVA257–264 complexes expressed by LN and spleen cells in day 14.5 pc pregnant B6 females mated with Act-mOVA males was measured in vitro using B3Z reporter cells. Robust B3Z responses were elicited by cells isolated from the para-aortic LN, cervical LN, mesenteric LN, and spleen (Fig. 6C). This result indicates that fetal and/or placental OVA Ag is processed and presented in each of the LNs and in the spleen, suggesting either that APCs traffic into those tissues from the uterus, or antigenic material shed from the placenta is processed and presented locally in peripheral sites. We were, however, unable to detect in vitro responses in B3Z cells when stimulated by para-aortic LN cells recovered at day 3.5 pc (data not shown), presumably due to insufficient OVA content in the LN at that time.

Maternal APCs are BM derived

The TAP dependence and systemic location of OVA presentation suggest that DCs are the maternal APCs (43). To confirm a bone marrow lineage for female APCs involved in processing and presentation of seminal fluid OVA Ag, we examined OT-I T cell proliferation in para-aortic LNs of bone marrow chimera mice. Successful engraftment of bm1 female mice with donor B6.SJL bone marrow was confirmed by the expression of CD45.1 congenic marker on all peripheral blood leukocytes 8 wk after bone marrow transfer. Because ovarian ablation due to irradiation in bone marrow chimeras prevents natural mating with Act-mOVA males, Ag in the form of OVA protein or seminal fluid from Act-mOVA males was delivered to the uterine cavity by transcervical catheter. CFSE-labeled OT-I T cells were then administered,
FIGURE 6. Female cells expressing H-2K\(^b\) and TAP are required for seminal fluid OVA Ag processing and presentation to OT-I T cells. Female B6, bm1, or B6 TAP\(^{-/-}\) mice were mated with Act-mOVA, Act-mOVA.bm1, or Act-mOVA.TAP\(^{-/-}\) males, and OT-I T cells were adoptively transferred i.v. on day 0.5 pc in mated females (A) or day 14.5 pc in pregnant females (B). Para-aortic LN were recovered 68 h later, and proliferation and CD69 expression were evaluated in each tissue. Data shown are representative of \(n = 4-6\) mice per group. PI and percentage of CD69\(^+\) values are shown in the top left corner of individual histograms and dot plots. C. APCs bearing H-2K\(^b\)/SIINFEKL complexes were present in para-aortic LN (PALN), mesenteric LN (MLN), cervical LN (CLN), and spleens (SPL) of day 14.5 pc pregnant B6 females mated with Act-mOVA males when measured in vitro using B3Z hybridoma reporter cells. The data shown are mean ± SEM of triplicate wells and are representative of two independent experiments.
Female H-2Kb-expressing cells required for seminal fluid OVA Ag processing and presentation are bone marrow derived. Bone marrow chimeras of bm1 female mice with donor B6.SJL or bm1 bone marrow, as well as B6.SJL mice with donor B6.SJL or bm1 bone marrow, were administered OVA protein (A and C) or Act-mOVA seminal fluid (B and D), and OT-I T cells were adoptively transferred i.v. immediately afterward. Para-aortic LN were recovered 68 h later, and proliferation and CD69 expression were evaluated in OT-I T cells (A and B). PI and percentage of CD69$^+$ values are shown in the top left corner of individual histograms and dot plots. The mean ± SEM PI of OT-I T cells in mice administered OVA protein (C) or Act-mOVA seminal fluid (D) is given for n = 4 mice per group. Treatment effects were analyzed by Kruskal-Wallis H test and Mann-Whitney U tests. Groups with different superscripts (a, b, and c) are significantly different (p < 0.05).
and the response was evaluated 68 h later. In B6.SJL female mice engrafted with bm1 BM, neither OT-I T cell proliferation nor increased CD69 expression was evident in the para-aortic LN after administration of OVA protein (Fig. 7, A and C) or Act-mOVA seminal fluid (Fig. 7, B and D). In contrast, para-aortic LN cells recovered from bm1 female mice engrafted with B6.SJL bone marrow showed the characteristic pattern of OT-I T cell responses. Control groups of B6.SJL and bm1 mice given autologous bone marrow transplants showed the expected responses to both OVA protein (Fig. 7, A and C) and Act-mOVA seminal fluid (Fig. 7, B and D). This result indicates the necessity for H-2Kk female bone marrow-derived APCs to present peptide Ag from seminal fluid-derived OVA protein to OT-I T cells.

**OT-I T cell activation and proliferation in vivo do not alter pregnancy outcome**

To evaluate whether administration of OT-I T cells and their subsequent activation in early pregnancy had any impact on fetal viability, B6 females were mated with Act-mOVA males, and naive OT-I or B6 T cells were administered ~12 h later on day 0.5 pc. Recipient mice were autopsied at day 17.5 pc, and the number of viable and resorbing implantation sites and fetal and placental weights were recorded. There was no effect on the proportion of mice that achieved successful pregnancy (4 of 5 and 7 of 7 in OT-I and B6 groups, respectively), on the number of total implantation sites in pregnant recipient mice (mean ± SEM = 6.8 ± 0.1 and 6.6 ± 0.5 in OT-I and B6 groups, respectively), or resorption rate (7 and 2% in OT-I and B6 groups, respectively). Fetal and placental weights were comparable in both groups (data not shown). This result indicates that OT-I T cells activated in vivo in response to seminal fluid OVA do not exert any obvious detrimental or beneficial effect on conceptus viability and pregnancy progression.

**Discussion**

The events that generate paternal Ag-specific tolerance in early pregnancy are not clear, and this is a constraint in designing therapeutic strategies to treat miscarriage and other pathologies of pregnancy that arise when tolerance is deficient. The current study shows that exposure to a model paternal Ag OVA in seminal fluid at conception constitutes the initial Ag delivery event driving activation and proliferation of OVA-reactive CD4+ and CD8+ T cells during the pre- and peri-implantation period of pregnancy, and that CD8+ T cell activation requires cross-presentation of seminal fluid OVA Ag by maternal BM-derived APCs. This indicates that seminal fluid Ags can activate T cells, as evidenced by proliferation and expression of CD25, CD69, and cytokines, and implies these events may be instrumental in initiating the maternal immune response to pregnancy. The findings provide proof-of-principle that reasonably should extend to natural Ags in seminal fluid, which include unique differentiation Ags on the surface of spermatozoa, as well as cell-associated and soluble allo-Ags associated with somatic cells and seminal plasma (21). Several of these Ags are later expressed from paternal genes by the conceptus (22), including classical class Ia and nonclassical class Ib MHC Ags and minor Ags such as H-Y (23, 44, 45). T cell cohorts activated during the preimplantation period would therefore exhibit reactivity with MHC and other Ags expressed by fetal cells and placental trophoblast cells following embryo implantation (18, 19).

A caveat on this interpretation is whether adoptive transfer of large numbers of TCR-transgenic T cells accurately reflects natural immune responses, especially those involving low frequencies of endogenous Ag-reactive T cells (46, 47). This concern is less applicable to allo-Ags because alloimmune responses involve up to 10% of the T cell repertoire (48). Furthermore, we noted qualitatively comparable proliferation responses when 10-fold fewer transgenic T cells were transferred (data not shown). It could be argued that OVA in seminal fluid of Act-mOVA mice exists at higher concentrations than Ags present naturally. Although there are limited studies on the MHC content of mouse seminal fluid, human seminal fluid contains soluble HLA in high concentrations (~1 μg/ml) (49), comparable to the lowest concentration of OVA (0.4 μg) that elicited a T cell response in this study. Cell-associated HLA in seminal fluid is associated with the 10 million leukocytes, immature germ cells, and epithelial cells in each ejaculate (50), and spermatozoa also express class I and class II HLA (23). Whether MHC or other Ags in seminal fluid elicit comparable responses to OVA requires experimental confirmation.

This study is the first to demonstrate cross-presentation of male seminal fluid Ags by female APCs. The requirement for TAP indicates that phagocytosis is the dominant route for uptake of seminal fluid OVA Ag by maternal APCs (43). Proteins within phagosomes are passed into the cytosol by means that are not fully understood, whereby proteasome-mediated degradation results in peptides that are then transported by TAP to complex with nascent MHC class I molecules within the endoplasmic reticulum (ER), before export to the plasma membrane for presentation. Alternatively, noncanonical TAP-dependent cross-presentation occurs following fusion of the phagosome with the ER and exogenous proteins are trafficked to the cytosol using ER-derived Ag processing and presentation molecules (51). In contrast, TAP-independent cross-presentation occurs when exogenous peptides are bound by recycled MHC class I after cleavage in the endocytic compartment following uptake by pinocytosis or endocytosis (34, 52).

The uterine mucosa is richly populated with DCs at ovulation (53, 54). Although the majority of CD11c+ MHCII+ cells in the uterus are CD8α− DCs, after mating and during early pregnancy a substantial proportion expresses CD8α+ (54). These DCs express markers indicative of a tolerogenic phenotype (55), which is likely to be maintained by the GM-CSF, IL-10, and IL-4-dominated cytokine environment of the decidual tissue (56). Proliferation of OT-I transgenic T cells in vivo is dependent on OVA presentation by CD8+ DEC205+ DCs (57). These DCs are recognized for their specialized capacity for cross-presenting nonreplicating Ags to MHC class I-restricted T cells (58, 59). Their enhanced ability to selectively phagocytose dead and dying cells might facilitate their processing of Ags associated with seminal fluid cells as well as soluble material after coitus (60). Importantly, we show that male APCs are not capable of eliciting detectable OT-I T cell responses to seminal fluid OVA, despite their presence in seminal fluid (50) and ability to infiltrate the female reproductive tract mucosa and draining LNs after mating (42).

Ag encounter is essential for induction of Treg cells and other forms of Ag-specific tolerance (8, 9). This study provides clear evidence that seminal fluid delivered at mating can provide the antigenic signals necessary for both CD4+ and CD8+ Treg cells to achieve full functional competence in early pregnancy. The interval of several days between insemination and development of the blastocyst-stage embryo would be sufficient to allow expansion of Treg cell populations before embryo implantation, when the conceptus first becomes vulnerable to immune attack. Such a role is consistent with demonstration that cross-presentation can lead to peripheral tolerance of tissue-associated Ags in other model systems, conditional upon the cytokine environment (61–63). The presence in seminal fluid of potent immune-deviating substances including high concentrations of TGF-β and PGE (24, 25) would promote tolerance as opposed to immunity (64). Consistent with this, we have recently reported that seminal fluid is essential for
expanding the CD4\(^+\)CD25\(^+\) Treg cell pool during early pregnancy, with both sperm and particularly seminal plasma contributing to the response (12). The ontology of CD8\(^+\) Treg cells in pregnancy is less well described, but like CD4\(^+\) Treg cells, they became expanded in the preimplantation period (15, 16), and this could be driven by seminal fluid Ags.

The OVA-reactive T cell response is activated by exposure to the plasma fraction of the ejaculate, and occurs independently of sperm. Neither are conception and formation of an embryo required, because uterine ligation did not impair the response. This accords with our previous observations on the T cell response to mating (28) and reflects that OVA Ag mainly originates within the accessory gland secretions, not the testes, because OVA levels were comparable in ejaculates from vasectomized and intact males. T cell activation would be facilitated by proinflammatory signals in seminal plasma that activate cytokine and chemokine expression (65), leading to recruitment and activation of macrophages and DCs in the uterine endometrial tissue (66). Reduced activation and proliferation of OVA-reactive CD4\(^+\) and CD8\(^+\) cells in the absence of seminal plasma can be attributed to the lower Ag dose, and/or fewer numbers or altered activation phenotype in uterine APCs. The capacity of seminal plasma to recruit and activate APCs would partly explain the qualitatively greater response to OVA in seminal fluid compared with OVA protein administered by catheter. The presence of membrane-bound as well as soluble OVA in the ejaculate would also drive a stronger T cell response (67).

Our results contrast with a previous report of absence of CD8\(^+\) T cell priming against male MHC class I or H-Y Ags after mating (68), although different transgenic T cells and Ags were used. A confounding issue in that study was the hormone status of the experimental mice; all mated mice were stimulated with gonadotropins to induce ovulation and receptivity to mating. This treatment can induce supraphysiological levels of circulating estrogens and alterations in the estrogen:progesterone ratio that suppress CD8\(^+\) T cell responses in the female reproductive tract (68, 69). Superovulation profoundly alters expression of uterine GM-CSF, LIF, and other cytokines known to influence APC function, and changes the abundance and phenotypes of macrophage and DC populations (70, 71) (S. Robertson, unpublished observations).

Additionally, the previous study evaluated Ag delivery to the vaginal mucosa, not the uterus, where the majority of seminal fluid is deposited in mice (41), although because the para-aortic LNs also drain the cervix, vagina, and vulva, seminal fluid Ags from the vaginal plug as well as the uterus potentially contribute to the para-aortic LN response. The vagina and uterus are dissimilar in the nature of their epithelial surfaces, leukocyte populations, and responsiveness to ovarian hormones, so that the fate of Ag is not equivalent in each site (72).

The current data concur with previous observations on OVA-specific CD8\(^+\) and CD4\(^+\) responses in pregnant mice gestating fetuses sired by Act-mOVA males (11). These authors did not examine responses before day 7.5 pc or after birth, but during midgestation, T cell proliferation was first evident in para-aortic LNs and the spleen, before disseminating to s.c. LNs and strengthening progressively until late gestation (11). Cross-presentation of OVA Ag by maternal cells was essential, and placental trophoblast cells were incapable of activating T cells through maternal MHC.

These authors concluded that OVA Ag presentation begins in midgestation, most likely in response to shedding of apoptotic blebs formed by endovascular trophoblast cells invading maternal arterioles. We now show that OVA Ag presentation in pregnancy is biphasic, with the first exposure at mating, before a nadir at embryo implantation and resurgence in midgestation.

Our study confirms and expands upon other aspects of the Erlebacher et al. study (11). First, we show that at least for seminal fluid OVA, the maternal cells required for cross-presentation of paternal Ags to CD8\(^+\) T cells are of bone marrow origin and functionally depend on TAP expression. Hence, they almost certainly are DCs, consistent with the known key role of these cells in cross-presentation (34), and any contribution of nonhemopoietic cells such as endothelial or epithelial cells can be excluded. In agreement with the previous report (11), we found that maternal cross-presentation of placental Ags is also essential for OT-I T cell activation in midgestation pregnancy, and whereas we did not confirm a bone marrow origin for cells cross-presenting Ag after implantation, their central importance in early pregnancy points to a likely ongoing role. Second, we show that substantial OVA Ag presentation continues for at least 1 wk after birth, and potentially at low levels for several weeks beyond that time. This accords with the persistence of trophoblast cell-derived OVA Ag on follicular DCs in the spleen for several weeks after birth (11), as well as emerging information on fetal cell microchimerism of the maternal tissues well beyond delivery (73). The time course of decline in OT-I reactivity concurs with the presence of GFP\(^+\) fetal cells in maternal lungs and elsewhere for up to 3 wk after birth (73).

Depending on the context of OVA Ag encounter, OT-I T cells can differentiate into potent cytotoxic effector cells in vivo (74, 75). No fetal death resulted from CD8\(^+\) OT-I T cells activated by seminal fluid OVA Ag in vivo, suggesting this response does not generate T cells capable of exerting fetal attack. Prevention of OT-I cytotoxic attack could be due to phenotype skewing of proliferating OT-I cells into anergic or regulatory cells, or deletion of reactive T cells due to placental trophoblast cell expression of apoptosis-inducing Fas ligand (76) and programmed death ligand 1 (77). In future experiments, we will investigate whether manipulating the OT-I effector phenotype influences the impact of OT-I T cells on fetal viability.

The prospect of female immune priming to seminal fluid Ags has implications for human health. There are some existing data suggesting that cervicovaginal exposure to seminal fluid elicits an immune response including T cell activation in women. Human seminal fluid activates inflammatory cytokine expression in cervical epithelial cells (78) and elicits recruitment of APCs into the ectocervical epithelium (D. Sharkey and S. Robertson, manuscript submitted). A significant peripheral alloimmune response to partner’s leukocytes compared with third-party leukocytes occurs in sexually active women (79). A physiological benefit of prior seminal fluid exposure for reproductive outcome is indicated by epidemiological studies showing that seminal fluid priming to partner’s Ags confers resistance to pathologies of pregnancy involving shallow placentation development (80, 81).

In summary, the experiments reported in this study provide the first compelling evidence that Ag-specific CD4\(^+\) and CD8\(^+\) T cell activation can occur after coitus in response to male seminal fluid Ags, via Ag cross-presentation in female APCs. This provides a pathway through which seminal fluid exposure at conception is linked to tolerance of paternal Ags required for pregnancy, potentially by Ag-driven activation of CD4\(^+\) and CD8\(^+\) Treg cell populations. The prospect of tolerance induction to seminal fluid Ags raises the question of how Ags associated with sexually transmitted pathogens are presented, and future studies will address whether all Ags in seminal fluid are processed similarly. A clear understanding of these events will allow development of targeted therapies to modulate adverse immune responses in women experiencing infertility or repeated miscarriage.
Disclosures
The authors have no financial conflict of interest.

References


