GM-CSF Is an Essential Regulator of T Cell Activation Competence in Uterine Dendritic Cells during Early Pregnancy in Mice

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GM-CSF Is an Essential Regulator of T Cell Activation Competence in Uterine Dendritic Cells during Early Pregnancy in Mice

Lachlan M. Moldenhauer,* Sarah N. Keenihan,* John D. Hayball,‡,§ and Sarah A. Robertson*

Uterine dendritic cells (DCs) are critical for activating the T cell response mediating maternal immune tolerance of the semiallogeneic fetus. GM-CSF (CSF2), a known regulator of DCs, is synthesized by uterine epithelial cells during induction of tolerance in early pregnancy. To investigate the role of GM-CSF in regulating uterine DCs and macrophages, Csf2-null mutant and wild-type mice were evaluated at estrus, and in the periconceptual and peri-implantation periods. Immunohistochemistry showed no effect of GM-CSF deficiency on numbers of uterine CD11c+ cells and F4/80+ macrophages at estrus or on days 0.5 and 3.5 postcoitum, but MHC class II+ and class A scavenger receptor+ cells were fewer. Flow cytometry revealed reduced CD80 and CD86 expression by uterine CD11c+ cells and reduced MHC class II in both CD11c+ and F4/80+ cells from GM-CSF-deficient mice. CD80 and CD86 were induced in Csf2+/- uterine CD11c+ cells by culture with GM-CSF. Substantially reduced ability to activate both CD4+ and CD8+ T cells in vivo was evident after delivery of OVA Ag by mating with Act-mOVA males or transcardiac administration of OVA peptides. This study shows that GM-CSF regulates the efficiency with which uterine DCs and macrophages activate T cells, and it is essential for optimal MHC class II- and class I-mediated indirect presentation of reproductive Ags. Insufficient GM-CSF may impair generation of T cell-mediated immune tolerance at the outset of pregnancy and may contribute to the altered DC profile and dysregulated T cell tolerance evident in infertility, miscarriage, and preeclampsia. 

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There are several lines of evidence implicating GM-CSF as a regulator of DCs and macrophages in their ability to activate T cell responses. GM-CSF influences DC and macrophage recruitment into inflammatory sites, with transgenic overexpression of Csf2 in tumor cells eliciting DC recruitment in vivo (18). GM-CSF is also important for effective Ag processing and presenting function in APCs, with in vitro experiments showing that GM-CSF facilitates Ag uptake (19) and expression of MHC class II and the costimulatory molecules CD80 and CD86 (20–22), and this is linked with increased T cell activation (18, 20, 23, 24) and T cell-dependent Ab production (25). Experiments in Csf2-null mutant mice have demonstrated that although macrophage and DC populations are essentially intact when GM-CSF is absent (26, 27), this cytokine influences mature macrophage and DC function to affect the incidence and severity of some inflammatory and autoimmune diseases, including arthritis, multiple sclerosis, glomerulonephritis, and LPS-induced lung inflammation (28). The impact of GM-CSF on the kinetics and outcome of inflammatory and immune responses appears to depend on the tissue site and eliciting stimulus. For example, in T cell-dependent peritonitis, but not in thioglycollate-elicited peritonitis, macrophage activation, phagocytosis, and Ag-presenting function are compromised when GM-CSF is deficient (29).

The uterine response to pregnancy requires induction of a T cell response, with paternal Ag-specific T cells within the maternal T cell repertoire showing evidence of Ag encounter from early in pregnancy, even before embryo implantation occurs (30). This T cell response is activated at conception (31), when paternal Ags present in seminal fluid are presented indirectly by maternal bone marrow-derived Ag-presenting cells to CD4+ and CD8+ T cells in the para-aortic lymph nodes (LNs) draining the uterus (32). In normal healthy pregnancies, activated paternal Ag-reactive T cells do not develop antifetal immunity and instead become functionally anergic or hyporesponsive, leading to tolerance of the conceptus and also tissue grafts expressing paternal Ags (30, 33, 34). The aim of this study was to use Csf2-null mutant mice to determine the physiological role of GM-CSF in the recruitment and/or activation and maturation of DCs and macrophages in the uterus during early pregnancy, and to define whether GM-CSF is required for these uterine APC populations to present paternal Ags to activate the maternal T cell repertoire for pregnancy tolerance. We show that although GM-CSF is not essential for DC and macrophage recruitment into the uterus during the estrous cycle or in the postcoital inflammatory response, these cells express less MHC class II and MR1 in the absence of GM-CSF. This phenotypic change is associated with reduced capacity to present paternal Ags via MHC class II and cross-presentation via MHC class I to Ag-specific CD4+ and CD8+ T cells. Impaired priming of paternal Ag-specific T cells may inhibit generation of maternal immune tolerance for pregnancy, and it is likely to contribute to the reproductive defects seen in Csf2-null mutant mice and potentially in infertile and subfertile women.

Materials and Methods

**Mice**

All mice were bred and maintained in a pathogen-free facility at the University of Adelaide Medical School Animal House on a 12-h light/dark cycle and were administered food and water ad libitum. Female and male wild-type C57BL/6 (H-2Kb) mice were bred in-house. Mice with a null mutation in the Csf2 gene (Csf2−/−) mice were generated using gene targeting techniques in 129/OLA-derived E14 embryonic stem cells and were propagated from founder mice by mating with C57BL/6J mice as previously described (26). Csf2−/− breeding pairs were provided by A. Dunn (Ludwig Institute for Cancer Research, Melbourne, Australia). The genotype of Csf2−/− and wild-type control mice were confirmed by PCR of DNA extracted from blood or tail tissue of breeding pairs and offspring. TCR transgenic C57BL/6 OT-I mice (37) (provided by W. Heath, Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia, and F. Carbone, Melbourne University, Melbourne, Australia) were crossed with B6.SJL-PtprcaPep3b/BoyJ mice and TCR transgenic C57BL/6 Tg-mOVA mice (provided by M. K. Jenkins (University of Minnesota, Minneapolis, MN). Act-mOVA male mice heterozygous for OVA transgene expression were bred from B6 × Act-mOVA intercrosses and identified by the presence of OVA protein detected by flow cytometry on peripheral blood cells using biotinylated rabbit anti-OVA Ab (Rockland Immunocchemicals, Gilbertsville, PA). For experimental matings, adult (6–12 wk old) female wild-type or Csf2−/− mice were placed one to three per cage with individual stud B6 or Act-mOVA males of proven fertility. The day of sighting of a vaginal plug (checked between 0800 and 1000 h) was designated day 0.5 postcoitum (pc). Mated females were removed from males and housed in groups of two to five females per cage.

Some mated female mice were administered OVA323–339 or OVA233–339 delivered into the uterus transcervically at 0900–1100 h on day 0.5 pc. The vaginal plug was removed under isofluorothane-induced anesthesia, and the peptide was administered transcervically using a 3.5 Fr Tom Cat catheter (Tyco Healthcare Group, Mansfield, MA) inserted through the cervix and into the uterus, positioned with the aid of a lubricated anoscope (Heine Optotechnik, Herrsching, Germany). The University of Adelaide Animal Ethics Committee approved all experiments.

**Abs**

Rat mAbs 2F8 (reactive with scavenger receptor class A [MSR1, CD204]), PA11 (reactive with macroscalin), and 3D6 (reactive with sialoadhesin) were provided as tissue culture supernatants by S. Gordon (University of Oxford, Oxford, UK). Rat mAbs as tissue culture supernatants generated by Dr. M. K. Jenkins (University of Minnesota, Minneapolis, MN). Act-mOVA intercrosses and identified by the presence of OVA protein detected by flow cytometry on peripheral blood cells using biotinylated rabbit anti-OVA Ab (Rockland Immunocchemicals, Gilbertsville, PA). For experimental matings, adult (6–12 wk old) female wild-type or Csf2−/− mice were placed one to three per cage with individual stud B6 or Act-mOVA males of proven fertility. The day of sighting of a vaginal plug (checked between 0800 and 1000 h) was designated day 0.5 postcoitum (pc). Mated females were removed from males and housed in groups of two to five females per cage.

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**Immunohistochemistry**

Immunohistochemistry was performed as previously described (6). Briefly, pregnant females were freshly killed extracorporeally, day 0.5 pc, or day 3.5 pc mice were perfused intracardially with PBS, and transverse sections (5 mm) were fixed in ethanol and labeled sequentially with mAbs, biotin-conjugated rabbit anti-rat Ig, and HRP-conjugated streptavidin. Within an experiment, uterine sections from all mice were processed together and control sections were incubated in PBS with the primary normal mouse serum or isotype-matched mAbs. Bound HRP was visualized using diaminobenzidine tetrachloride (DAB) (Sigma-Aldrich, St. Louis, MO). After counterstaining with Gill’s hematoxylin (Sigma-Aldrich), sections were dehydrated in absolute ethanol,
cleared in Safsolvent (Ajax Chemicals, Auburn, Australia), and mounted in DePeX (BDH Laboratory Supplies, Poole, U.K.). Full-section high-resolution images were made using a NanoZoomer slide scanner (Hamamatsu Photonics, Hamamatsu City, Japan). DAB-labeled cells in the endometrial stroma of duplicate sections from each uterus were quantified by video image analysis using VideoPro software (Faule Imaging, Adelaide, Australia). The mean percentage positivity value (mean area of DAB stain/mean area of hematoxylin + DAB stain) × 100) of ≥10 fields per section was calculated. Eosinophils were identified as endogenous peroxidase+ cells present in sections stained with DAB in the absence of primary Ab or detection reagents.

Flow cytometric analysis of uterine DCs and macrophages

Uteri were collected aseptically on day 0.5 pc and were trimmed of mesentery and fat, slit longitudinally, and minced extensively using fine scissors. The tissue fragments of individual uteri were stirred gently at room temperature for 1 h in 2 ml of RPMI 1640 (Sigma-Aldrich), filtered through 70-μm mesh, and labeled with fluorescein-conjugated Abs to detect CD4 (OT-II T cells), followed by PE-Cy5–conjugated strepavidin. All uterine tissue was collected aseptically on day 0.5 pc and was trimmed of extraneous fat, mesentery and mesometrial triangle regions of the uterus also increased immediately subjacent to the luminal and stromal epithelium, and appeared of uterine DCs and macrophages was evident. In wild-type mice, with increases of 2.0-fold and 1.9-fold, respectively, at day 0.5 pc compared with wild-type mice (Figs. 1G, 1H, 2B). MHC class II+ cells were also elevated, regardless of GM-CSF presence or absence (2.1-fold and 4.0-fold in wild-type mice and accumulated predominantly in the stromal tissue immediately subjacent to the luminal and stromal epithelium, and a comparable 2.0-fold increase was seen in Csf2−/− mice (Figs. 1G, 1H, 1F). There was no effect of GM-CSF deficiency on the number of either cell lineage (Fig. 2A, 2B). In contrast, MHC class II+ cells were reduced by 43% in Csf2−/− mice (p = 0.02) (Figs. 1I, 1J, 2C). Cells expressing macrophilin, MSR1, or sialoadhesin were comparable in abundance and distribution to F4/80+ macrophages, whereas Csf2−/− was expressed by very few cells. There was no effect of GM-CSF deficiency on the distribution or abundance of cells expressing these markers or on the population of eosinophils, which were detected as endogenous peroxidase+ cells (Fig. 2H). Cells expressing these markers were also present in the myometrial and mesometrial triangle regions of the uterus of both wild-type and Csf2−/− mice but were generally fewer in number than in the endometrium (data not shown).

After coitus on day 0.5 pc, the numbers of CD11c+ cells were increased 3.0-fold in the uterine endometrium of wild-type mice and a similar 2.1-fold increase was seen in Csf2−/− mice (Figs. 1C, 1D, 2A). F4/80+ macrophages were elevated 1.4-fold in wild-type mice and accumulated predominantly in the stromal tissue immediately subjacent to the luminal and stromal epithelium, and a comparable 2.0-fold increase was seen in Csf2−/− mice (Figs. 2G−2H). MHC class II+ cells were also elevated, regardless of GM-CSF presence or absence (2.1-fold and 4.0-fold in wild-type and Csf2−/− mice, respectively) (Figs. 1K, 1L, 2C). Endometrial MSR1+ and sialoadhesin+ cells were substantially elevated in wild-type mice, with increases of 2.0-fold and 1.9-fold, respectively, at day 0.5 pc compared with estrus, but neither increase occurred in Csf2−/− mice (Figs. 1O, 1P, 2E, 2F). MSR1 expression was most notably affected by GM-CSF deficiency, with a 47% decrease in MSR1+ cells at day 0.5 pc in Csf2−/− mice compared with wild-type mice (p < 0.01) (Fig. 2E). Macrophilin was unchanged after coitus in either wild-type or GM-CSF–deficient mice (Fig. 2D). The most strikingly change after coitus was seen in CD86+ cells, which increased regardless of Csf2 status (3.6-fold and 2.0-fold increase in wild-type and Csf2−/− mice, respectively) (Figs. 1S, 1T, 2G). For each of these markers, labeled cells were seen to accumulate in the superficial endometrium immediately subjacent to luminal epithelial cells after coitus (Fig. 1I). Whereas eosinophils were not changed after mating in wild-type mice, their numbers increased >2-fold in Csf2−/− mice (Fig. 2H). Numbers of F4/80+ and MSR1+ cells in the myometrium and mesometrial triangle regions of the uterus also increased on day 0.5 pc, albeit not as dramatically as in the endometrium, whereas numbers of class II MHC+, CD11c+, and CD86+ cells in these tissue compartments remained similar to estrus (data not shown).

On day 3.5 pc, a further marked change in the number and appearance of uterine DCs and macrophages was evident. In wild-
type mice, the numbers of cells expressing CD11c remained similar to day 0.5 pc, whereas F4/80+ macrophages declined to levels seen in estrus mice, and Csf2−/− mice showed similar staining patterns (Fig. 2A, 2B). In contrast, whereas the numbers of MHC class II+ cells remained similar from day 0.5 pc to day 3.5 pc in wild-type mice, in Csf2−/− mice MHC class II+ cells declined by day 3.5 to a level 57% lower than numbers in wild-type mice on day 3.5 pc (p < 0.05) (Fig. 2C), and the remaining MHC class II+ cells showed a reduced intensity of MHC class II staining (data not shown). Macrosialin+ cells, MSR1+ cells, and sialoadhesin+ cells in the endometrium all declined by between 50 and 80% compared with day 0.5 pc, and this occurred regardless of Csf2 genotype status (Fig. 2D–F). In the case of both MSR1 and macrosialin, the density of positive cells on day 3.5 pc was below

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**FIGURE 1.** Effect of GM-CSF deficiency on DC and macrophage localization in uterine endometrial tissue. Sections of uterine tissue collected from wild-type or Csf2−/− mice at estrus or on day 0.5 pc were labeled with mAb reactive with CD11c (A–D), F4/80 (E–H), MHC class II (I–L), scavenger receptor class A (MSR1) (M–P), or CD86 (Q–T). Images are representative of n = 5 mice/group. DAB and hematoxylin, original magnification ×20. En, endometrium; LE, luminal epithelium.
that seen at estrus. Most notably, the density of CD86+ cells decreased by >75% between day 0.5 pc and day 3.5 pc ($p = 0.01$), and this also occurred in $\text{Csf2}^{-/-}$ mice (Fig. 1F). Interestingly, cells expressing MSR1 and CD86 in the day 3.5 pc uterus were generally located in the deep endometrium, often adjacent to the myometrium, and unlike earlier stages they frequently occurred in clusters (data not shown). The density of F4/80+ and MSR1+ cells in the mesometrial triangle and the myometrium decreased from day 0.5 pc to day 3.5 pc, whereas MHC class II+ and CD86+ cells were similar in number (data not shown). The distribution and morphology of eosinophils and cells expressing other activation markers in the endometrium were comparable between genotypes, and no differences were evident in the mesometrial triangle or myometrium on day 3.5 pc (data not shown).

**Effect of GM-CSF deficiency on uterine endometrial DC and macrophage number and activation phenotype**

Since the immunohistochemical study indicated that the effect of GM-CSF deficiency was most notable during the postcoital inflammatory response, we sought to further investigate the effect of GM-CSF deficiency on expression of activation markers by uterine DCs and macrophages at this time. Individual uteri from wild-type mice and $\text{Csf2}^{-/-}$ mice were recovered on day 0.5 pc, enzymatically digested to generate single-cell suspensions, and assessed by flow cytometry.

CD11c+ cells and F4/80+ macrophages were assessed for MHC class II, MSR1, CD80, and CD86 expression (Figs. 3 and 4, respectively), and activation marker expression was further classified as low or high. The proportion of CD11c+ cells expressing MHC class II was decreased by 22% in $\text{Csf2}^{-/-}$ mice (Fig. 3C), and there was a decrease in the intensity of MHC class II expression, with a 42% reduction in the percentage of CD11c+ MHC class II+hi cells in $\text{Csf2}^{-/-}$ mice (Fig. 3D). There was no effect of GM-CSF deficiency on the proportion of CD11c+ cells expressing MSR1 (Fig. 3E,3F). Within the CD11c+ population there was an 8% reduction in CD11c+ macrophages expressing MHC class II+hi (Fig. 4D) and expression was less intense, with a 48% reduction in CD11c+ CD80+ cells (Fig. 3H). CD86 also showed decreased intensity of expression with 68% fewer CD11c+ CD86+hi cells in $\text{Csf2}^{-/-}$ uterine tissue following coitus (Fig. 3J).

The proportion of F4/80+ macrophages expressing MHC class II+hi was reduced by 50% in $\text{Csf2}^{-/-}$ mice (Fig. 4D). There was no
effect of GM-CSF deficiency on expression of MSR1, CD80, or CD86 in F4/80+ macrophages (Fig. 4 E–J).

To further investigate whether expression of activation markers in uterine CD11c+ and F4/80+ cells is regulated by GM-CSF, uterine cells were recovered by enzymatic digestion from day 0.5 pc Csf2<sup>−/−</sup> females and cultured with or without recombinant GM-CSF for 24 h. MHC class II, MSR1, CD80, and CD86 expression by cultured CD11c<sup>+</sup> and F4/80<sup>+</sup> cells were assessed by flow cytometry. There was no change in MHC class II or MSR1 expression in CD11c<sup>+</sup> or F4/80<sup>+</sup> cells (data not shown). CD11c<sup>+</sup> cells cultured in the presence of 80 ng/ml GM-CSF showed a 31% increase in CD80<sup>hi</sup> expression (Fig. 5 A) and a 36% increase in CD86<sup>hi</sup> expression (Fig. 5 B). CD86 expression was also increased in the F4/80<sup>+</sup> population, with a 40% increase in F4/80<sup>+</sup> CD86<sup>hi</sup> cells in the presence of 40 ng/ml GM-CSF compared with control cells cultured without GM-CSF.

**FIGURE 3.** Effect of GM-CSF deficiency on MHC class II, scavenger receptor class A (MSR1), CD80, and CD86 expression by uterine CD11c<sup>+</sup> cells. Uterine cells prepared from WT mice or Csf2<sup>−/−</sup> mice on day 0.5 pc were labeled with mAb reactive with CD11c, MHC class II, MSR1, CD80, and CD86 and analyzed by flow cytometry. Representative histograms of CD11c<sup>+</sup> cells (A) or isotype-matched negative controls (B) are shown, with gates set to discriminate between MHC class II<sup>lo</sup> and MHC class II<sup>hi</sup>, MSR1<sup>lo</sup> and MSR1<sup>hi</sup>, CD80<sup>lo</sup> and CD80<sup>hi</sup>, or CD86<sup>lo</sup> and CD86<sup>hi</sup> cells. The numbers at the top of histograms are the percentages of cells within each gate. C–J, Percentages of CD11c<sup>+</sup> cells expressing MHC class II<sup>+</sup> (C), MHC class II<sup>hi</sup> (D), MSR1<sup>+</sup> (E), MSR1<sup>hi</sup> (F), CD80<sup>+</sup> (G), CD80<sup>hi</sup> (H), CD86<sup>+</sup> (I), and CD86<sup>hi</sup> (J). Data are means ± SEM from wild-type mice (open bars, n = 7–21) and Csf2<sup>−/−</sup> mice (hatched bars, n = 10–22). The effect of Csf2 genotype was determined by Mann-Whitney U test. *p < 0.05, significant effect of Csf2 genotype. WT, wild-type.
Effect of GM-CSF deficiency on MHC class II-restricted and MHC class I-restricted Ag presentation

Previously we showed that maternal bone marrow-derived APCs are critical for both the MHC class I-restricted cross-presentation and direct MHC class II-restricted presentation of paternal seminal fluid Ags at the time of conception (32). To evaluate the effect of GM-CSF deficiency on the capacity of uterine DCs and macrophages to present Ag and activate T cells, we used a model paternal Ag system employing male mice that produce transgenic OVA ubiquitously under an Actb promoter, which results in OVA secretion into the seminal fluid (32). After mating females with Act-mOVA males to expose the uterus to seminal fluid OVA,

FIGURE 4. Effect of GM-CSF deficiency on MHC class II, scavenger receptor class A (MSR1), CD80, and CD86 expression by uterine F4/80+ macrophages. Uterine cells prepared from WT mice or Csf2−/− mice on day 0.5 pc were labeled with mAb reactive with F4/80, MHC class II, MSR1, CD80, and CD86 and analyzed by flow cytometry. Representative histograms of F4/80+ macrophages (A) or isotype-matched negative controls (B) are shown, with gates set to discriminate between MHC class IIlo and MHC class IIhi, MSR1lo and MSR1hi, CD80lo and CD80hi, or CD86lo and CD86hi cells. The numbers at the top of histograms are the percentages of cells within each gate. C–J, Percentages of F4/80+ macrophages expressing MHC class II+ (C), MHC class IIhi (D), MSR1+ (E), MSR1hi (F), CD80+ (G), CD80hi (H), CD86+ (I), and CD86hi (J). Data are means ± SEM from wild-type mice (open bars, n = 7–21) and Csf2−/− mice (hatched bars, n = 10–22). The effect of Csf2 genotype was determined by Mann-Whitney U test. *p < 0.05, significant effect of Csf2 genotype. WT, wild-type.
The effect of GM-CSF deficiency on MHC class II-restricted presentation of Ags in the uterus. Wild-type mice or Csf2−/− mice were mated with Act-mOVA males, or alternatively were mated with B6 males and then administered OVA323–339 peptide transcervically on day 0.5 pc. Both groups received CFSE-labeled OT-II T cells on day 0.5 pc, and OT-II T cell proliferation in para-aortic LNs harvested 68 h later was quantified by flow cytometry and the PI was calculated. Representative histograms from a wild-type (A) and a Csf2−/− mice (B) mated with Act-mOVA males are shown, with PI values given in the top left corner. C and D, Mean ± SEM PI values (from 7–14 mice/group) from WT (open bars) and Csf2−/− (hatched bars) mated with Act-mOVA males (C) or mated with B6 males and then administered OVA323–339 peptide transcervically (D). The effect of Csf2 genotype was determined by Mann-Whitney U test. *p < 0.05, significant effect of Csf2 genotype.

FIGURE 5. Effect of recombinant GM-CSF on activation marker expression by uterine CD11c+ cells and F4/80+ cells in vitro. Uterine cells prepared from Csf2−/− mice on day 0.5 pc were cultured with 0, 20, 40, or 80 ng/ml recombinant GM-CSF for 24 h, then harvested and labeled with mAb reactive with CD11c, F4/80, CD80, and CD86 and analyzed by flow cytometry. Data are the means ± SEM percentage of CD11c+ cells classified as CD86hi (A) or CD86hi (B) and the percentage of F4/80+ cells classified as CD86hi (C) (n = 3–6 wells/treatment group, from n = 6 mice). The effect of GM-CSF concentration was analyzed by Mann-Whitney U test. "p < 0.05, significant differences between treatment groups.

OVA-specific CD4+ OT-II T cells or CD8+ OT-I T cells were CFSE-labeled and transferred into mated females. The extent of Ag presentation was determined by quantifying the number of cycles of proliferation, as indicated by CFSE dye dilution, in Ag presentation was determined by quantifying the number of CFSE-labeled and transferred into mated females. The extent of Ag presentation was determined by quantifying the number of CFSE-labeled and transferred into mated females. The extent of Ag presentation was determined by quantifying the number of CFSE-labeled and transferred into mated females.

FIGURE 6. Effect of GM-CSF deficiency on MHC class II-restricted presentation of Ags in the uterus. Wild-type mice or Csf2−/− mice were mated with Act-mOVA males, or alternatively were mated with B6 males and then administered OVA323–339 peptide transcervically on day 0.5 pc. Both groups received CFSE-labeled OT-II T cells on day 0.5 pc, and OT-II T cell proliferation in para-aortic LNs harvested 68 h later was quantified by flow cytometry and the PI was calculated. Representative histograms from a wild-type (A) and a Csf2−/− mice (B) mated with Act-mOVA males are shown, with PI values given in the top left corner. C and D, Mean ± SEM PI values (from 7–14 mice/group) from WT (open bars) and Csf2−/− mice (hatched bars) mated with Act-mOVA males (C) or mated with B6 males and then administered OVA323–339 peptide transcervically (D). The effect of Csf2 genotype was determined by Mann-Whitney U test. *p < 0.05, significant effect of Csf2 genotype. WT, wild-type.

Discussion
Uterine DCs and macrophages are important participants in the events of embryo implantation and establishing pregnancy. Their roles include processing and presentation of Ags from seminal fluid and the conceptus to activate the T cell-mediated immune tolerance vital for progression of gestation (32, 33). Variation in the size and phenotype of these populations is linked with infertility, miscarriage, and disorders of pregnancy such as preeclampsia (3–5). In this study we have identified GM-CSF as a key cytokine controlling macrophage and DC populations in the uterine tissue, in particular their expression of activation markers MHC class II and the scavenger receptor, MSR1, CD80, and CD86. We show that the changes in APC populations due to GM-CSF deficiency severely compromise the functional capacity of the uterus to activate a T cell-mediated immune response to reproductive Ags.

Regulation by GM-CSF appears to be most critical during the controlled inflammatory response elicited by male seminal fluid at coitus (12, 13), when GM-CSF expression by epithelial cells followed by CFSE-labeled OT-II T cells. When OT-II T cells were recovered from the para-aortic LNs 68 h later, proliferation was significantly reduced in Csf2−/− mice compared with wild-type mice, corresponding to a 67% reduction in the average number of cell divisions (Fig. 6D). Similarly, to determine whether GM-CSF deficiency alters MHC class I-mediated presentation of OVA peptide to OT-I T cells, OVA257–264 peptide was administered to the uterine cavity of mated wild-type or Csf2−/− mice, and CFSE-labeled OT-I T cells were transferred. When OT-I T cells were recovered from the para-aortic LNs 68 h later, proliferation was significantly reduced in Csf2−/− mice compared with wild-type mice, corresponding to a 78% reduction in the average number of cell divisions (Fig. 7D).
Whitney

Cervically (males and then administered OVA257–264 peptide transcervically on day 0.5 pc. Both groups received CFSE-labeled OT-I T cells on day 0.5 pc, and OVA1 cell proliferation in para-aortic LN harvested 68 h later was quantified by flow cytometry and the PI was calculated. Representative histograms from wild-type (A) and a Csf2−/− mice (B) mated with Act-mOVA males are shown, with PI values given in the top left corner. C and D, Mean ± SEM PI values (from 8–14 mice/group) from WT (open bars) and Csf2−/− mice (hatched bars) mated with Act-mOVA males (C) or mated with B6 males and then administered OVA257–264 peptide transcervically (D). The effect of Csf2 genotype was determined by Mann-Whitney U test. *p < 0.05, significant effect of Csf2 genotype. WT, wild-type.

Lining the female reproductive tract is strongly induced (40). The periconceptive period is a crucial time in the cascade of events driving maternal adaptation to pregnancy, since the first exposure to paternal Ags occurs in the context of seminal fluid. Activation of T cells reactive with paternal Ags occurs within the 72 h after coitus (31, 32) and is associated with expansion of the Treg cell population that confers immune tolerance when the embryo implants during this period causes allogeneic pregnancy to fail (35) and some 4 d later (36). Disruption of the immune adaptation initiated within individual APCs likely explain their deficient ability to drive CD4+ T cell and CD8+ T cell proliferation. This indicates that maternal GM-CSF is required for optimal presentation of DCs through the preimplantation period. CD11b+CD8α− DC precursors have been shown to be differentially responsive to GM-CSF-regulated expansion and maturation into MHC class II+ APCs through the preimplantation period. CD11b+CD8α− DC precursors have been shown to be differentially responsive to GM-CSF-regulated expansion and maturation into MHC class II+ APCs (45).

The presence of otherwise normal numbers of DCs and macrophages in the GM-CSF–deficient uterus during early pregnancy is explained by the array of other chemokines and cytokines expressed in the uterus, including CSF1, RANTES, MIP1α, MIP1β, and MCP1 (47–49). These data are consistent with studies in other tissues of Csf2−/− mice showing that in steady-state conditions, the numbers of myeloid and DC cell populations in LNs and peripheral organs are essentially normal (27, 50).

Using OVA as a surrogate paternal Ag, we showed in vivo experiments that induction of T cell responses in the uterus is severely compromised in GM-CSF–deficient mice. OVA Ag present in seminal fluid or delivered transcervically during the postcoital inflammatory response showed a very limited ability to drive CD4+ T cell and CD8+ T cell proliferation. This indicates that maternal GM-CSF is required for optimal presentation of seminal fluid Ag via both the MHC class II- and MHC class I-restricted pathways. Because there was no major change in the absolute numbers of DCs and macrophages, we conclude that impaired Ag capture, processing, and/or presentation pathways within individual APCs likely explain their deficient ability to activate T cells.

Our finding of substantially reduced T cell activation in the uterus contrasts with other reports showing no effect or only a modest reduction in APC competence to generate T cell responses in Csf2−/− mice. Ag-specific T cell responses to methylated BSA administered to the peritoneal cavity developed normally in Csf2−/− mice; however, macrophages from peritoneal exudates of immunized mice elicited a lower proliferative response in alloimmune splenic T cells (29). In contrast, APCs from Csf2−/− mice activated comparable responses to wild-type APCs in wild-type CD4+ T cells, and an effect of Csf2-null mutation on CD4+ T cell proliferation in response to keyhole limpet hemocyanin immunization was attributed to a defect in Csf2−/− T cells as opposed to APCs (51). Similarly, s.c. immunization with OVA in Csf2−/− mice did not cause any detectable change in CD4+ OT-II proliferation or the production of OVA-specific Ig, unless mice were also Flt3-ligand deficient (52). Taken together, these observations suggest that the extent of APC functional deficiency in Csf2−/− mice depends on the site of Ag delivery and the nature of the local immunohistochemistry was not supported by flow cytometry data. The reduction in MSR1 staining may be due to diminished cytoplasmic MSR1 within DCs and macrophages in the postcoital uterus, rather than less surface MSR1 expression, or possibly fewer cells expressing MSR1 but not F4/80 or CD11c.

Previously we reported that the accumulation of DCs in the implantation site in part stems from immature CD11b+ monocyte precursors recruited at estrus and during the postcoital inflammatory response, which differentiate into CD11b− MHC class II+ cells (6). The present study indicates that GM-CSF is a key cytokine in regulating this phenotypic maturation, with absence of the postcoital GM-CSF surge causing a persisting deficit in MHC class II+ APCs preimplantation period. CD11b+CD8α− DC precursors have been shown to be differentially responsive to GM-CSF-regulated expansion and maturation into MHC class II+ DCs (45). Their altered phenotype in the absence of GM-CSF could be expected to compromise DC function during the postimplantation period when DCs begin to process Ags released by invading placental trophoblasts (32, 33) and have an essential role in regulating the vascular changes that ensure an adequate decidual response and placental cell invasion (2, 46).

The periconceptive period is a crucial time in the cascade of events driving maternal adaptation to pregnancy, since the first exposure to paternal Ags occurs in the context of seminal fluid. Activation of T cells reactive with paternal Ags occurs within the 72 h after coitus (31, 32) and is associated with expansion of the Treg cell pool that confers immune tolerance when the embryo implants some 4 d later (36). Disruption of the immune adaptation initiated during this period causes allogeneic pregnancy to fail (35) and is the mechanism underpinning predisposition to fetal loss in abortion-prone CBA × DBA/2 mice (41).

In the absence of GM-CSF, there were no substantial changes in the kinetics of inflammatory cell recruitment and activation after coitus. The greatest change was seen in cells expressing the scavenger receptor MSR1, which were reduced in number by 50% in the absence of GM-CSF. The postcoital inflammatory response abates in wild-type mice by the time of embryo implantation on day 3.5 pc. A comparable decline in uterine leukocytes was seen in Csf2−/− mice at this time, but MHC class II+ cells were abnormally diminished and were 2-fold lower in GM-CSF-deficient mice by the time of implantation on day 3.5 pc.

In contrast to the immunohistochemical analysis, flow cytometry revealed that the proportion of both DCs and macrophages expressing MHC class II, as well as their intensity of MHC class II expression, was reduced in the absence of GM-CSF on day 0.5 pc. This is consistent with the interpretation that GM-CSF deficiency causes a population-wide reduction in the intensity of surface MHC class II expression on a per cell basis in both DCs and macrophages. More than 40% of CD11c+ cells within the uterine tissue at day 0.5 pc are MHC class II+, and it is conceivable that a proportion of the CD11c+ cells may be macrophages or neutrophils. Alternatively, these cells could be precursors to conventional DCs, analogous to the CD11c− MHC class II+ cells reported in bone marrow and lymphoid tissues (42–44).

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inflammatory environment, and they indicate that APCs in the uterus may be unusually dependent on this cytokine. Since uterine GM-CSF synthesis is controlled by ovarian steroid hormones as well as seminal fluid factors (10, 11), this provides a mechanism through which local APCs can respond to the changing functional requirements of this dynamic tissue, according to stage of the reproductive cycle and pregnancy. GM-CSF is likely to act in concert with other APC-regulating cytokines, modulated by the direct actions of ovarian steroid hormones on macrophages and DCs (53, 54).

This effect on Ag-presenting function, together with the observation of reduced MR1 expression in Csf2−/− mice, suggests that GM-CSF may influence Ag capture mediated by scavenger receptors. MR1 is part of the larger scavenger receptor superfamily, comprised of pattern recognition receptors that mediate internalization of an array of proteins and other materials (55). Msr1 is upregulated in mature and differentiated CD11c+ DCs by GM-CSF (56); however, we are unaware of previous reports of scavenger receptor dysregulation in Csf2−/− mice. Blocking scavenger receptor superfamily members prevents the activation of OVA-reactive CD4+ T cells, indicating that MHC class II-restricted Ag presentation relies upon Ag capture by these molecules (57). It has been suggested that scavenger receptor family members are also important in Ag cross-presentation by DCs (58); however, a specific requirement for MR1 is contentious since DCs from Msr1-deficient mice can cross-present as effectively as DCs from wild-type mice (59, 60).

To evaluate whether a defect in Ag uptake and/or processing might explain the reduced T cell response to OVA Ag in seminal fluid, we used OVA peptides that do not require intracellular processing for presentation to OT-II and OT-I TCRs. However, administration of OVA peptides to the uterus failed to overcome the compromised Ag presentation seen with whole OVA protein in GM-CSF–deficient mice, indicating that Ag capture and/or intracellular processing do not fully explain the defect.

A key marker of APC activation and maturity is MHC class II, and failure to upregulate MHC class II could reasonably also contribute to defective Ag presentation to CD4+ T cells through insufficient availability of MHC molecules to form MHC–OVA complexes and engage in TCR ligation. Within a range of in vitro model systems, exogenous GM-CSF has been shown to induce surface MHC class II expression, through both transcriptional and posttranscriptional mechanisms (22, 61), particularly in CD11b+ CD11c+ DCs where GM-CSF induces translocation of cytoplasmic MHC class II to the cell surface (45). However, earlier studies on MHC class II expression in Csf2−/− mice did not show any difference in MHC class II expression, in Ag-elicited peritoneal cells (29), in skin-draining LNs (52), or in spleen APCs (62). We have previously reported that GM-CSF–deficient mice have less MHC class II expression on macrophages and DCs within the ovary (63), suggesting that MHC class II expression on female reproductive tract DCs may be particularly responsive to this cytokine.

GM-CSF is known to upregulate DC expression of costimulatory molecules CD80 and CD86 (21, 22), which are critical for stabilizing the peptide–MHC–TCR interaction and generating strong Ag-specific T cell responses. By immunohistochemistry we did not detect an effect of GM-CSF deficiency on CD86 in the endometrium, consistent with data on costimulatory molecules CD40, CD80, or CD86 in DCs from skin-draining LNs (52) or spleen (62). However, by flow cytometry, expression of CD80 and CD86 was clearly decreased in the uterine CD11c+ population from Csf2−/− mice, and this phenotype could be rescued by ex vivo culture with GM-CSF. Interestingly, Csf2-null mutation is reported to perturb the cytokine expression profile of spleen CD11c+ DCs, with reduced production of IL-6 and IL-23 required to drive Th17 T cell differentiation (62), indicating that exploration of GM-CSF–regulated cytokine synthesis in uterine DCs is warranted.

GM-CSF is an essential cytokine for normal reproduction, with defects in Csf2−/− mice ranging from altered ovarian function (63, 64), compromised preimplantation embryo development (65), dysregulated placental morphogenesis (16, 66), and increased incidence of fetal growth restriction and death in the late gestation and perinatal period (16, 17). The present study suggests that one potential cause for these reproductive anomalies might be failure to establish an optimal T cell response to pregnancy, due to impaired presentation of reproductive Ags to the T cell repertoire.

Our data using the surrogate paternal Ag OVA indicate that indirect presentation via both the MHC class II pathway to CD4+ cells, as well as MHC class I-mediated cross-presentation to CD8+ T cells, is substantially impaired in the absence of GM-CSF. This would compromise adaptation in both the CD4+ T cell compartment required to expand the CD4+ Treg cell pool (67) and in the CD8+ T cell compartment where generation of regulatory cells with suppressive function (68) as well as anergic responses (34) occur. Deficient T cell tolerance fits with the observation that in pregnant Csf2−/− mothers, male fetuses are more likely to die or become growth restricted in utero compared with female progeny (16), since male fetuses comprise a greater antigenic challenge than do females. The interpretation that GM-CSF facilitates an appropriate T cell response to pregnancy is consistent with experiments showing that administration of exogenous GM-CSF in the preimplantation period can normalize fetal loss rates in abortion-prone CBA × DBA/2 mice and is associated with expansion of CD8+ T cells with suppressive function (68). Administration of bone marrow–derived DCs from normal fertile mice to abortion-prone CBA × DBA/2 mice similarly reduces the miscarriage rate, implying that GM-CSF–mediated rescue of fetal loss is operating through a DC-mediated mechanism (69).

As well as generating T cell tolerance, DCs and macrophages are important for the extensive tissue remodeling and angiogenic changes necessary for the uterine decidual response and for placental invasion and development (2, 46). Secretion of vascular endothelial growth factor by decidual DCs is essential for the development of an adequate blood supply to the developing placenta (2). Although this study has focused on the Ag-presenting function of uterine DCs and macrophages, the additional possibility that GM-CSF influences DC or macrophage capacity to support vascular development or otherwise remodel reproductive tissues cannot be excluded.

Collectively, the data presented in this study indicate that GM-CSF is critically important for uterine DCs and macrophages to reach maturity and attain competence to present Ag and activate T cells during the periconceptual phase of early pregnancy, when maternal immune tolerance of reproductive Ags is induced. APCs in the uterus and elsewhere in the female tract appear to be distinguished by the extent of their dependence on this cytokine, perhaps because of their ontogenic relationship with monocyte precursors recruited during the controlled inflammatory response to seminal fluid. Their functional deficiency in the absence of GM-CSF is likely to be the consequence of reduced expression of surface MHC class II and scavenger receptor MR1. These results raise the question of the significance of Csf2 expression in regulating DCs in the human uterus. Our findings underscore the importance of evaluating whether GM-CSF contributes to the altered numbers and phenotypes of DCs seen in women experiencing reproductive pathologies (3–5). Because in women, as in mice, GM-CSF synthesis by reproductive tract epithelial cells is responsive to male seminal
fluid factors as well as ovarian steroid hormones (9, 70), it will be relevant to investigate how male factors and/or perturbation in ovarian steroid synthesis influence uterine DC phenotype and function, as well as the impact of infection, inflammation, and hormonal infertility treatments on these parameters.

Disclosures

The authors have no financial conflicts of interest.

References


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