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Fertility Impairment in Granulocyte-Macrophage Colony-Stimulating Factor-Deficient Mice[1]

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ABSTRACT

Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been identified as a potentially important mediator of intercellular communication in the female reproductive tract, with principal target cells being the large populations of myeloid leukocytes in the cycling and pregnant uterus, the preimplantation embryo, and trophoblast cells of the developing placenta. To determine the physiological significance of this cytokine in reproduction, the fertility of genetically GM-CSF-deficient (GM−/−) mice was examined. Implantation rates were normal in GM−/− mice, and viable pups were produced. However, the mean litter sizes of GM−/− × GM−/− breeding pairs were 25% smaller at weaning than those of GM+/− × GM+/− pairs, due to fetal death late in gestation and early in postnatal life, with a disproportionate loss of male pups. On Day 17 of pregnancy, the mean number of resorbing and malformed fetuses was twice as high in pregnant GM−/− females (21%, vs. 11% in GM+/− females); the mean fetal weight and the mean fetal:placental ratio in surviving conceptuses were diminished by 7% and 6%, respectively; and the number of very small fetuses (< 500 mg) was 9-times as high (23% vs. 2.5%). Mortality during the first 3 wk of life was 4.5-times as high in pups born to GM−/− mothers (9%, vs. 2% in GM+/− females), and diminished size persisted in GM−/− pups, particularly males, into adulthood. The detrimental effect of maternal GM-CSF deficiency was less apparent when GM−/− females were mated with GM+/+ males; litter sizes at birth and at weaning were not significantly smaller than in GM+/− matings, and fetal weights and fetal:placental ratios were also comparable. When polymerase chain reaction was used to genotype embryonic tissue in heterozygote matings, GM−/− fetuses from GM−/− females were found to be smaller than their GM+/− littermates and smaller than GM−/− fetuses gestated in GM+/− females. The size and distribution of uterine granulocyte and macrophage populations were normal during the estrous cycle, during early pregnancy, and in midgestation. Analysis of placental structure revealed that the ratio of labyrinthine to spongiotrophoblast areas was reduced by approximately 28% in GM−/− placentae, and the proportion of vacuolated trophoblast "glycogen cells" in the spongiotrophoblast layer was diminished. Compromised placental function as a result of subtle developmental aberrations may therefore partially account for embryonic growth retardation in GM-CSF-deficient mice. Collectively, these studies show that fetal growth and viability are jeopardized in the absence of maternal GM-CSF. The detrimental effects are most clearly evident when the conceptus is also GM-CSF deficient, suggesting that GM-CSF of either maternal or fetal origin is required for optimal growth and survival of the fetus in mice.

INTRODUCTION

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a lymphohemopoietic cytokine with well-defined in vitro effects on the survival, proliferation, and differentiation of myeloid leukocytes and their precursors [1]. The in vivo hemopoietic activity of GM-CSF has been confirmed in murine models by generating GM-CSF transgenic mice [2] and by administering exogenous GM-CSF to mice [3]. In vivo and in vitro experiments indicate that GM-CSF can also modulate the function of mature monocyte/macrophages, granulocytes, and dendritic cells through promoting antigen presentation, chemotaxis and adhesion, phagocytosis and cytotoxicity, and induction of anti-tumor immunity [4]. In hemopoietic cells, GM-CSF exerts its effects at the target cell surface through binding to a high-affinity, heterodimeric receptor complex composed of a GM-CSF-specific α subunit and a signal-transducing β subunit shared by the interleukin (IL)-3 and IL-5 receptors [5]. Non-lymphohemopoietic cells including endothelial cells, oligodendrocytes, certain tumor cells, and placental trophoblast cells can also express GM-CSF receptors and exhibit biological responsiveness to this cytokine [6].

In the female reproductive tract, GM-CSF is synthesized under the regulatory influence of estrogen by uterine luminal and glandular epithelial cells in mice [7], sheep [8], and women [9]. In mice, a further surge in GM-CSF release from estrogen-primed epithelial cells is induced after mating [7], by specific factors in seminal plasma including transforming growth factor (TGF)β1 [10, 11], and is accompanied by a dramatic infiltration and activation of GM-CSF-responsive leukocytes including macrophages, dendritic cells, neutrophils, and eosinophils within the endometrial stroma [10, 12]. A key role for epithelial GM-CSF in mediating this postmating inflammatory response was suggested by findings that recombinant GM-CSF administered into the uterine luminal cavity is sufficient to replicate the cellular changes seen during the postmating period [13] and in the expression of GM-CSF receptor by leukocytes recruited into the endometrium [14]. Although uterine expression of both GM-CSF and its receptor declines in response to progesterone prior to embryo implantation ([10] and unpublished results), abundant GM-CSF-responsive leukocytes, particularly macrophages, remain in the uterus for the duration of pregnancy [15, 16]. The functions of the leukocytes recruited during the postmating inflammatory cascade remain undefined, but roles in mediating both the tissue remodeling and changes to the local immune environment required to accommodate pregnancy have been proposed [17, 18].

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It has also been indicated that GM-CSF is a regulator of the growth and development of the conceptus and conceptus-derived tissues. Murine preimplantation embryos express GM-CSF receptors, attain higher cell numbers, and implant more rapidly in vitro when cultured with GM-CSF [19, 20]. The embryotrophic effects of GM-CSF are more pronounced in humans [21] and in bovine [22], species in which in vitro embryo development is characteristically difficult to achieve. GM-CSF is also implicated in the morphological and functional development of the placenta, as it promotes DNA proliferation, differentiation, and enhanced secretory activity in human and rodent cytotrophoblast cells in vitro [23–25]. In the human, GM-CSF receptor expression has been identified in human villous and extravillous cytotrophoblast cells and choriocarcinoma cell lines [26, 27]. Human placental cell-conditioned medium is a rich source of GM-CSF [28], with Hofbauer cells, other mesenchymal cells in placental villi, and trophoblast cells all potential sites of synthesis [29–31]. Conventional and unique GM-CSF mRNA transcripts have been detected in murine placental tissues associated with a variety of cells including stromal fibroblasts, presumptive leukocytes, endothelial cells, and trophoblast-like cells within the spongiotrophoblast zone of the placenta [32, 33]. In rodent and human maternal tissues, uterine natural killer cells within the decidua contribute to GM-CSF production [31, 34], as do epithelial cells in the undecidualized endometrium [7].

Additional evidence highlighting a role for GM-CSF in pregnancy is provided by experiments in mice showing that when administered in small amounts, exogenous cytokine can dramatically alter pregnancy outcome. Administration of a single dose of GM-CSF protects against lipopolysaccharide (LPS)- or interferon-γ-induced fetal resorption and enhances fetal and placental weights [35]. Furthermore, nanogram amounts of GM-CSF administered during the preimplantation period can reverse the high rate of implantation failure and fetal resorption in mice bearing colony-stimulating factor (CSF)-1-secreting tumors or mice injected with recombinant CSF-1 [36]. A similar treatment reduces the early embryo malformation and embryo loss that occur spontaneously at elevated rates in the CBA/J × DBA/2 mating combination [37].

Two independent groups have now generated genetically GM-CSF-deficient mice by homologous recombination in embryonic stem cells [38, 39]. Interestingly, these mice show no major perturbations of hemopoiesis or in populations of leukocytes in blood, bone marrow, or spleen, indicating that the action of this cytokine in hemopoiesis is largely redundant. However, both lines of GM-CSF-deficient mice have a characteristic nonfatal lung pathology, with local accumulation of surfactant and a high incidence of bacterial and fungal infection resembling human alveolar proteinosis [38, 39]. Although GM-CSF deficiency was not initially associated with any overt reproductive incapacity, closer examination revealed that litter sizes were moderately smaller in GM-CSF-deficient mice [40]. The purpose of this study was to undertake a specific and detailed investigation of the fertility and fecundity of GM-CSF-deficient mice in order to determine the physiological importance of GM-CSF to the growth and survival of the conceptus in murine pregnancy.

MATERIALS AND METHODS

Mice

Mice homozygous for a disrupted GM-CSF gene (GM−/− mice) were generated using gene-targeting techniques in 129 embryonic stem cells and were propagated from founder mice by mating with C57Bl/6 mice, as previously described [38]. After re-derivation by embryo transfer to achieve specific pathogen-free (SPF) status, GM−/− mice were bred from parents proven by polymerase chain reaction (PCR) to be homozygous for the GM-CSF disrupted gene. Control (GM+/+) mice on an equivalent genetic background were derived from F2 offspring of GM−/− females crossed with wild-type 129 males and were identified as homozygous for the wild-type gene by PCR. Heterozygous (GM+/−) mice were bred from GM−/− females mated with GM+/+ males. Mice were provided with food and water ad libitum and were housed within an SPF facility at the University of Adelaide. This study was carried out in accordance with the Guiding Principals for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction.

For breeding experiments, adult females (8–12 wk, GM−/− or GM+/−) were housed 2:1 with adult stud males (GM−/−, GM+/+, or GM+/−) and allowed to mate naturally. The day on which a copulation plug was evident was designated Day 1 of pregnancy. Pregnant females were killed by cervical dislocation on Day 8 of pregnancy for counting of implantation sites, or on Day 17 for fetal and placental weight assessment. The fetal:placental ratio was determined as the ratio of fetal weight to placental weight. Some mice were allowed to proceed to term for determination of litter size at birth and weaning. Additional details of breeding experiments are given below.

For induction of decidualization in vivo, adult mice were ovariectomized through a dorsal incision under Avertin (Winthrop Inc., New York, NY) anesthesia and treated as previously described [41, 42]. Essentially, mice were primed on 2 consecutive days with 100 ng estrogen and then, beginning 3 days later, were injected daily for 6 days with 500 μg progesterone and 10 ng estrogen (100 μl s.c. in peanut oil). On Day 4 of this protocol, anesthetized mice received a deciduogenic stimulus in the form of 10 μl of peanut oil injected into one uterine horn. Mice were killed on Day 8, and the mass of each uterine horn was determined.

PCR for GM-CSF Gene Knockout

GM-CSF genomic status was assessed by PCR of DNA extracted from blood or tail tissue of adult mice, or from trunk tissue of Day 17 fetuses, as previously described [38]. The PCR primers were as follows: GM 5′ primer 5′-CCAGCCTCAGAGACCCAGGTATCC-3′; GM 3′ (complementary) primer 5′-GTTAGAGACGACTTCTACCTCTTC-3′; and M13 (−47) 24-mer sequencing primer 5′-GATGTGCTGCAAGGCTATTAAGTT-3′ (no. 1224; New England Biolabs, Herts, UK). Primers GM 5′ and GM 3′ generate a 1.2-kilobase product from wild-type DNA, and primers GM 5′ and M13 generate a 1.0-kilobase product from DNA containing the correctly integrated target construct.

The PCR amplification employed reagents supplied in a Taq DNA polymerase kit (Bresatec, Adelaide, Australia) and 96-well PCR trays (Hybaid, Teddington, UK), as described previously [42]. Each reaction mixture contained 2 μM of each primer (GM 5′, GM 3′, and M13) and 0.5 μg of DNA. The amplification reaction was allowed to proceed for 40 cycles, with each cycle consisting of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C; a final extension for 7 min at 72°C

was used. Reaction products were analyzed by electrophoresis through 2% agarose gel containing 0.5 μg/ml ethidium bromide in Tris-acetate EDTA buffer, and the size of PCR products was determined using molecular weight markers (Bresatec).

Uterine Luminal Fluids, Endometrial Cell Culture, and GM-CSF Bioassay

Uterine intraluminal fluid (25–50 μl per horn in 500 μl RPMI-fetal calf serum) was harvested from Day 1 pregnant mice as previously described [7]. Endometrial cell cultures composed predominantly of epithelial cells were prepared by enzymatic digestion in trypsin and pancreatin as detailed previously [7]. GM-CSF and IL-6 in uterine luminal fluids, or in supernatants collected at 24 h after the initiation of endometrial cell cultures, were quantified by measuring proliferation of the specifically GM-CSF-dependent cell line FD 5/12 and specifically IL-6-dependent cell line 7TD1, respectively, as described previously [7]. The minimum detectable amount of GM-CSF was 1 U/ml (50 U/ml is defined as the concentration of GM-CSF stimulating half-maximal FD 5/12 cell proliferation). The minimum detectable amount of IL-6 was 5 U/ml (50 U/ml defined as producing half-maximal 7TD1 growth). The specificity of the bioassays was confirmed using cytokine-neutralizing antibodies as previously described [7].

Microbiology

Uteri and lungs recovered from virgin or mated mice were cut into segments and disrupted with a manual glass homogenizer in 2 ml PBS per pair of organs. Bacteriological assessment was conducted by accredited veterinary microbiologists (Vetlab, Institute of Medical and Veterinary Science, Adelaide, Australia) by typing of colonies grown on McConkey's agar and blood agar plates.

Histology and Immunohistochemistry

Uterine tissue from GM−/− and GM+/+ mice collected at 1000–1200 h on the day of estrus or on Day 1 or Day 8 of pregnancy was embedded in OCT compound (Tissue Tek; Bayer Corp., Elkhart, IN) and frozen in isopentane cooled by liquid N_2; tissue was then stored at −80°C until use. Semiserial sections (6 μm) were cut from an area midway between the cervix and oviductal junction in estrous and Day 1 uteri, and from both implantation sites and interimplantation sites in Day 8 pregnant uteri.

Placentae and underlying decidual tissue were dissected from implantation sites in Day 15 pregnant GM−/− and GM+/+ mice mated with males of the same genotypes. After immersion fixation in 4% paraformaldehyde/0.25% glutaraldehyde/2.5% polyvinylpyrrolidone in 70 mM phosphate buffer at 4°C for 3 days, placentae were washed in sterile PBS four times over 2 days, processed, and embedded in paraffin. Midsagittal 7-μm sections were cut through each placenta and stained with Masson's trichrome stain, hematoxylin and eosin (H&E), or periodic acid-Schiff reagent (PAS), both with and without amylase pretreatment, using standard protocols [43]. The areas of the spongiotrophoblast and labyrinthine region were determined by video image analysis using Video Pro software (Leading Edge Software, Adelaide, Australia) with a ×10 objective and 3.3× photo eyepiece. The video image was calibrated to micrometers with the aid of a hemocytometer. Repeated measurements of the spongiotrophoblast area in one section validated the precision of this method (< 5% within-assay variation).

Sections of fresh frozen uteri were fixed in 96% ethanol and immunolabeled with monoclonal antibodies (mAbs): rat anti-mouse F4/80, specifically reactive with macrophages [44], and rat anti-mouse RB6–6C5, specifically reactive with neutrophils [45]. To localize endogenous peroxidase cells (eosinophils), slides were incubated in diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, MO) (5 mg/ml in 0.05 M Tris-HCl, pH 7.2) plus 0.02% hydrogen peroxide for 10 min at room temperature. Sections of placentae and decidual tissue were stained with F4/80 and with two mAbs that were specifically reactive with vacuolated glycogen cells in the syncytiotrophoblast layer: mouse anti-human trophoblast (clone L185, provided by B. Kalionis, Flinders Technologies Pty. Ltd., Adelaide, Australia) and mouse anti-human pan cytokeratin (C2562; Sigma). Reactivity was visualized using a Histomouse-SP Kit (Zymed Laboratories Inc., San Francisco, CA), or as previously described [7]. Sections were counterstained in hematoxylin. The number of neutrophils per millimeter of luminal epithelium, or the area of positive staining in the endometrial stroma, decidua, or placenta (expressed as a function of the area of total staining), was determined by video image analysis.

Statistics

Data were analyzed by ANOVA and Bonferroni *t*-tests using the General Linear Models procedures of the Statistical Analysis System (SAS Institute Inc., Cary, NC) or SPSS Software (Chicago, IL). Data expressed as proportions were examined by chi-square analysis, using CHITEST and CHIDIST procedures in Microsoft (Redmond, WA) Excel 5.0. Placental morphology data were analyzed by Mann-Whitney U Test (SPSS Software). Differences between groups were considered to be significant when $p < 0.05$.

RESULTS

Verification of the GM-CSF Status of Reproductive Tract Tissues

GM-CSF synthesis in deficient mice is ablated by deletion of exons 1 and 2 of the GM-CSF gene [38]. Because the GM-CSF-deficient mice used in this study were re-derived by embryo transfer to achieve SPF status, the presence in offspring of the targeted allele was confirmed by diagnostic PCR (Fig. 1A). To confirm that the reproductive tract tissues of GM−/− mice were deficient in GM-CSF bioactivity, the GM-CSF content of uterine luminal fluids and endometrial cell supernatants from Day 1 pregnant GM−/−, GM+/−, and GM+/+ mice (all mated with GM+/+ males) was determined by FD 5/12 bioassay. The amount of bioactivity in supernatants or luminal fluids from GM−/− endometrial cells was beneath the level of detection of the bioassay, whereas the amount of GM-CSF bioactivity present in endometrial cell culture supernatants or in luminal fluids from GM+/+ and GM+/− mice (irrespective of parental strain combination) was significant (Fig. 1B) and was similar to the content of fluids and supernatants prepared from Balb/c F1 mice [7]. In contrast, the IL-6 content of luminal fluids and endometrial cell supernatants was not affected by GM-CSF status and was comparable in both instances to previous findings (not shown).

TABLE 1. The effect of GM-CSF deficiency on fertility, litter size, and pup survival to weaning in mice.

Parameter	Parental genotype: female × male		
	GM+/− × GM+/−	GM−/− × GM−/−	GM−/− × GM+/+
% Fertility (n)[a]	100 (10)	100 (27)	100 (10)
Total number of litters	52	59	46
Number of litters per week[b]	0.21 ± 0.04	0.17 ± 0.05	0.20 ± 0.03
Total number of pups born	417	377	333
Litter size: at birth (day 1–4)[b]	8.0 ± 2.8[d]	6.4 ± 2.8[e]	7.2 ± 3.0[de]
at weaning (wk 3–4)[b]	7.8 ± 2.8[d]	5.9 ± 3.0[e]	7.1 ± 3.0[de]
% Postnatal mortality (n)[c]	2.0 (9)[d]	8.9 (31)[e]	2.1 (7)[d]
Number of male pups/litter[b]	4.2 ± 1.9[d]	3.1 ± 1.7[e]	3.9 ± 2.0[de]
Number of females/litter[b]	3.6 ± 1.9	2.9 ± 1.9	3.2 ± 1.6
% Male pups at weaning[c]	54.3	51.1	55.5

[a] All females produced a litter within 4 wk of caging with male.
[b] Values are means ± SD; data were compared by one-way ANOVA and Bonferroni *t*-test.
[c] Data were compared by chi-square test.
[d,e] Data in the same row with different superscript letters denote significant differences between groups ($p < 0.05$).

Effect of GM-CSF Deficiency on Litter Size, Frequency of Pregnancy, and Sex Ratio

To investigate the effect of parental GM-CSF genotype on breeding parameters including the frequency of litter production, litter size, and sex ratio, GM−/− or GM+/− females were caged with males of the same genotype and allowed to breed naturally for approximately 6 mo. Litters were counted within the first 3 days of birth and again at 3–4 wk when pups were weaned and their sex was determined. The number and sex of pups at birth and at weaning were not influenced by the parity of females (data not shown). There was no effect of GM-CSF genotype on the frequency at which females produced litters. However, GM−/− females mated with GM−/− males produced smaller litters than their GM+/− counterparts (6.4 ± 2.8 vs. 8.0 ± 2.8 at Days 1–4, respectively, $p < 0.05$) (Table 1). The survival rate of pups during the first 3 wk of life was also affected by parental GM-CSF genotype, with 91.1% survival of pups born to GM−/− females as compared with 98.0% survival of pups born to GM+/− females ($p < 0.05$) (Table 1). Interestingly, the reduced litter size appeared to reflect a selective loss of male pups, with approximately one male less per litter weaned from GM−/− females than from GM+/− females ($p < 0.01$) (Table 1). A skew toward female progeny in pups born to the GM−/− breeding colony was maintained over the entire period of the study, but this never attained significance (48.1% males [n = 591] and 52.7% males [n = 804] in GM−/− and GM+/+ colonies, respectively; chi-square test $p = 0.13$).

Effect of GM-CSF Deficiency on Implantation and Decidual Transformation

To investigate whether the effect of parental GM-CSF deficiency on litter size was mediated at implantation, GM−/− and GM+/− females were mated with males of the same genotype, and uteri were examined on Day 8 of pregnancy. There was no effect of genotype on the proportion of mated mice found to be pregnant on Day 8 (16 of 22 and 15 of 20 for GM−/− and GM+/−, respectively), or in the mean number of implantation sites in pregnant mice (mean ± SD = 9.2 ± 2.5 and 9.1 ± 3.3, respectively).

To investigate whether GM-CSF status influenced the capacity of the uterus to undergo decidual transformation, GM+/− and GM−/− mice were ovariectomized and maintained on a steroid hormone regimen mimicking that occurring naturally at implantation. Mice were killed 4 days after administration of an intraluminal stimulus of peanut oil to one uterine horn. Significant decidualization was observed in 7 of 10 GM+/− mice and 5 of 9 GM−/− mice; and there was no effect of GM-CSF status on the extent of decidualization, which was highly variable between individuals in both groups (mean ± SD mass of stimulated uterine horn = 165 ± 113 vs. 136 ± 71, respectively).

Effect of GM-CSF Deficiency on Conceptus Viability and Fetal and Placental Weights at Day 17

To determine whether parental GM-CSF status influenced survival of the conceptus during pregnancy, GM−/− or GM+/− females mated naturally with males of the same

TABLE 2. The effect of GM-CSF deficiency on fetal viability at Day 17 of pregnancy in primiparous mice.

Parameter	Parental genotype: female × male		
	GM+/− × GM+/−	GM−/− × GM−/−	GM−/− × GM+/+
Total number of matings	26	32	49
% Pregnant at day 17 (n)[b]	62 (16)	57 (21)	71 (35)
Number of implantation sites[a]	8.7 ± 2.9	9.6 ± 1.9	8.4 ± 2.9
% Viable (n)[b]	89.2 (124)[c]	79.1 (159)[d]	84.2 (203)[cd]
% Resorbing (n)[b]	9.4 (13)[c]	16.9 (34)[d]	13.3 (27)[cd]
% Abnormal (n)[b]	1.4 (2)[c]	4.0 (8)[d]	2.4 (5)[cd]
Fetal weight	655 ± 97[c]	611 ± 135[d]	664 ± 151[c]
Placental weight[a]	82 ± 13	81 ± 14	82 ± 15
Fetal: placental weight ratio[a]	8.1 ± 1.7[c]	7.6 ± 1.5[d]	8.3 ± 2.3[c]

[a] Values are means ± SD, and fetal and placental weights are in milligrams; data were compared by one-way ANOVA and Bonferroni *t*-test.
[b] Data were compared by chi-square test.
[c,d] Data in the same row with different superscript letters denote significant differences between groups ($p < 0.05$).

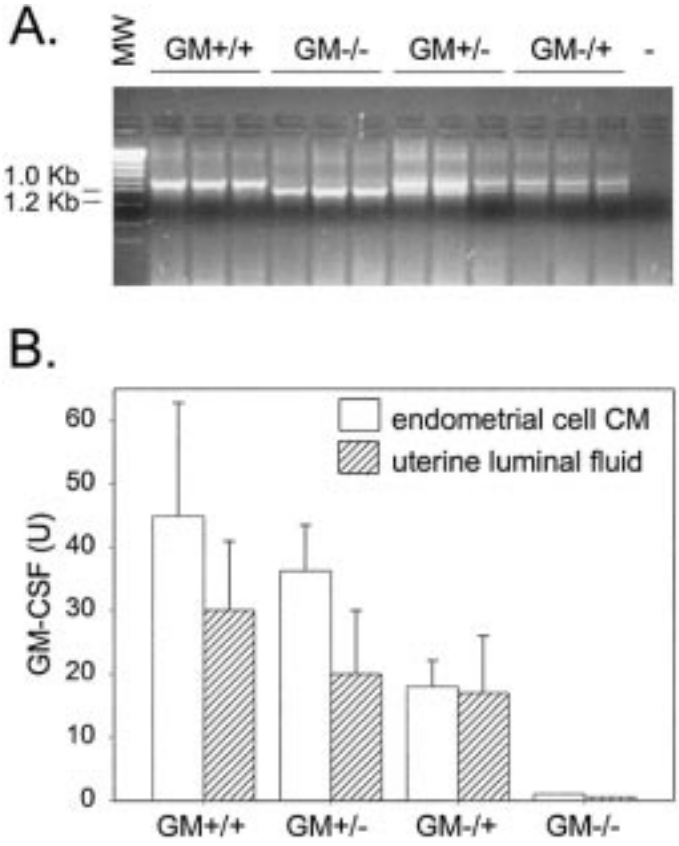

FIG. 1. Confirmation of the GM-CSF status of mice. **A**) Diagnostic PCR of DNA extracted from tail tissue of homozygote pups (GM−/−), heterozygote pups (GM+/−, sired by GM+/+ males; and GM−/+, sired by GM−/− males), and wild-type pups (GM+/+). **B**) GM-CSF content of endometrial cell-conditioned media and uterine luminal fluid prepared from Day 1 pregnant GM+/+, GM+/−, GM−/+, and GM−/− mice (all mated with GM+/+ males), determined by FD 5/12 bioassay. Values are mean ± SD GM-CSF (U/uterus, for uterine luminal fluid; U/10^5 cells per 24 h, for endometrial cell-conditioned medium; n = 3 per group).

genotype were killed on Day 17 of pregnancy. The proportion of mice that had plugs on Day 1 and were pregnant on Day 17, and the total number of implantation sites, were not affected by genotype. However, the proportion of implantation sites found to be resorbing or morphologically abnormal (malformed or anemic fetus and/or necrotic placenta) on Day 17 was higher in GM−/− females (16.9% and 4.0%, respectively) than in GM+/− females (9.4% and 1.4%, chi-square test $p = 0.001$) (Table 2).

To examine whether parental GM-CSF influenced the growth of the placenta or fetus, the weights of viable fetuses and placentae were measured in GM−/− and GM+/− females mated with males of the same genotype and killed on Day 17. Mean placental weight was not affected by GM-CSF deficiency. However, there was a significant reduction in the mean weight of fetuses and in the fetal:placental ratio in GM−/− females compared with GM+/− females (Table 2). The distributions of fetal weight, placental weight, and fetal:placental ratio were all influenced by GM-CSF genotype, with a significant skew to the left for all parameters (chi-square test, $p < 0.0001$) (Fig. 2). Most notably, there was a significantly greater proportion of very small fetuses in the absence of GM-CSF (23.4% and 2.5% of fetuses in GM−/− and GM+/− females, respectively, weighed less than 500 mg; $p < 0.001$). Likewise, there were significantly more

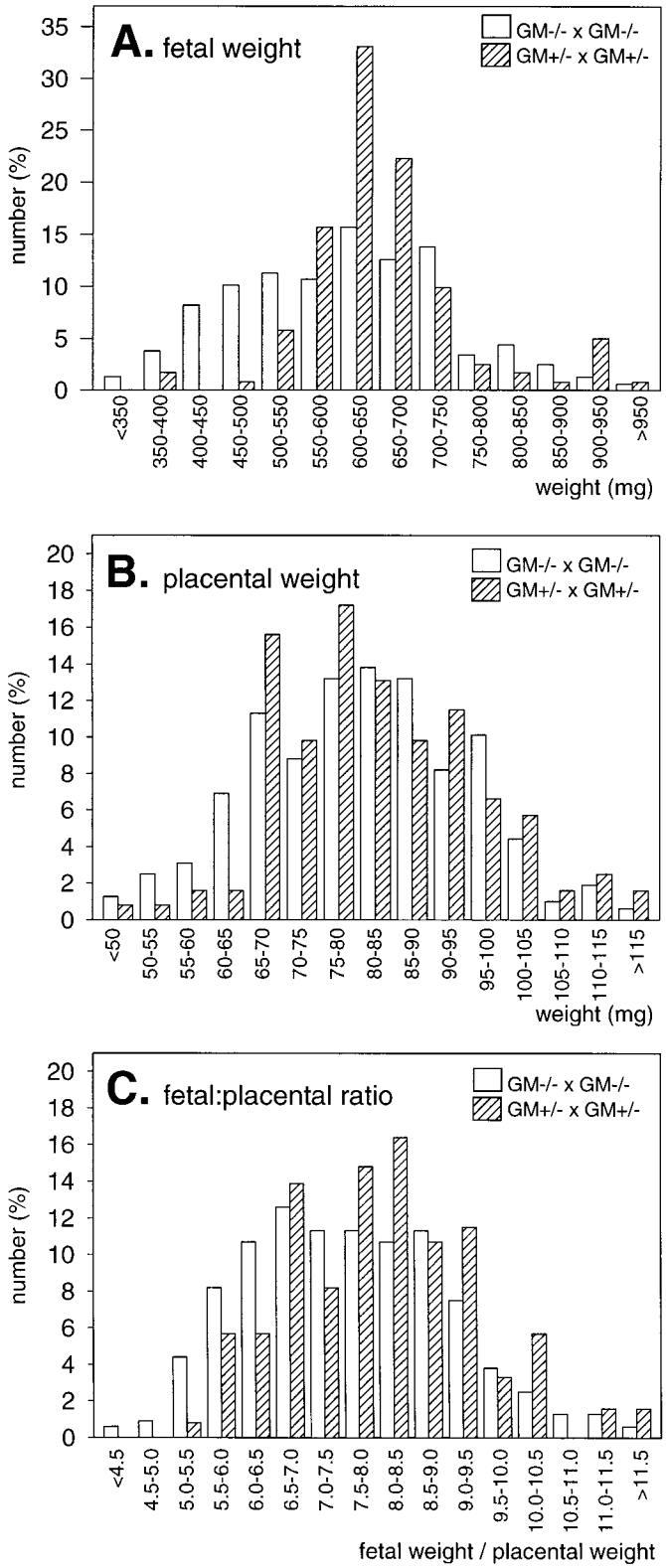

FIG. 2. The effect of GM-CSF deficiency on the distribution of **A**) fetal weights, **B**) placental weights, and **C**) fetal:placental ratio in viable implantation sites on Day 17 of pregnancy, from GM+/− × GM+/− matings (n = 122 pups) and from GM−/− × GM−/− matings (n = 158 pups). The distributions of fetal weight, placental weight, and fetal:placental ratio were all influenced by GM-CSF genotype, with a significant skew to the left for all parameters (chi-square test, $p < 0.0001$).

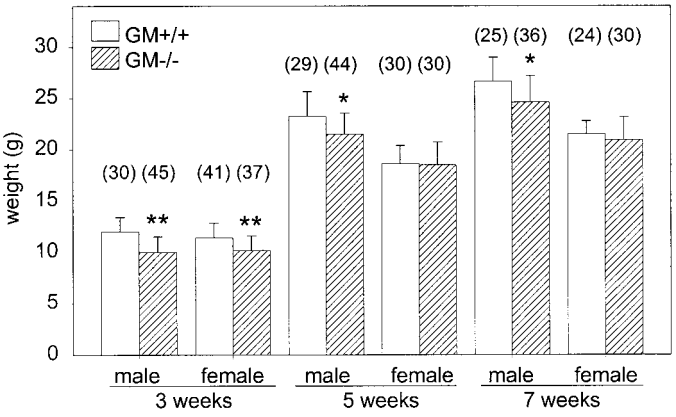

FIG. 3. The effect of GM-CSF deficiency on the weights of male and female pups, born to GM+/+ and GM−/− females mated with males of the same genotypes, at 3 wk, 5 wk, and 7 wk of age. Values are mean ± SD, and the numbers of observations are given in parentheses. Data were compared by one-way ANOVA followed by Bonferroni *t*-test (** $p < 0.002$; * $p < 0.02$).

implantation sites with a very low fetal:placental ratio in GM-CSF-deficient females (14.1% and 6.5% of fetuses in GM−/− and GM+/− females, respectively, had a ratio of less than 6.0; $p < 0.035$).

To determine whether this reduction in fetal size persisted after birth, pups born from GM−/− and GM+/+ females mated with males of the same genotype were weighed at 3 wk, 5 wk, and 7 wk of age. At 3 wk of age, male and female GM−/− pups were 17% and 11% smaller, respectively ($p < 0.002$), than age and sex-matched GM+/+ pups (Fig. 3). The smaller body weight of males, but not females, persisted into adulthood; male GM−/− mice were 8% smaller than male GM+/+ mice at 5 wk and at 7 wk of age ($p < 0.02$).

Effect of Fetal Versus Maternal GM-CSF Deficiency on Litter Size, Fetal Viability, and Fetal and Placental Weights

To investigate whether the effect of GM-CSF deficiency on litter size was a function of maternal as opposed to fetal GM-CSF deficiency, GM−/− females were mated with GM+/+ males. Reproductive outcome was improved in these mice as compared with GM−/− females mated with GM−/− males. Although litter sizes still tended to be smaller than control values, there were no significant differences between the numbers of pups born to and weaned from these females in comparison to GM+/− females mated with GM+/− males. The postnatal mortality rate and sex ratio of pups at weaning were also comparable to those seen in GM+/− matings.

To examine the relative importance of fetal and maternal GM-CSF for fetal viability and fetal and placental size, GM−/− females were mated with GM+/+ males and killed at Day 17. Resorption and abnormality rates (13.3% and 2.4%, respectively) were midway between (and not significantly different from) the values obtained for GM+/− females mated with GM+/− males and the values obtained for GM−/− females mated with GM−/− males. However, the fetal weights and the fetal:placental ratio in surviving implantation sites were significantly higher than in GM−/− females mated with GM−/− males and were comparable with those of GM+/− females mated with GM+/− males ($p < 0.05$) (Table 2).

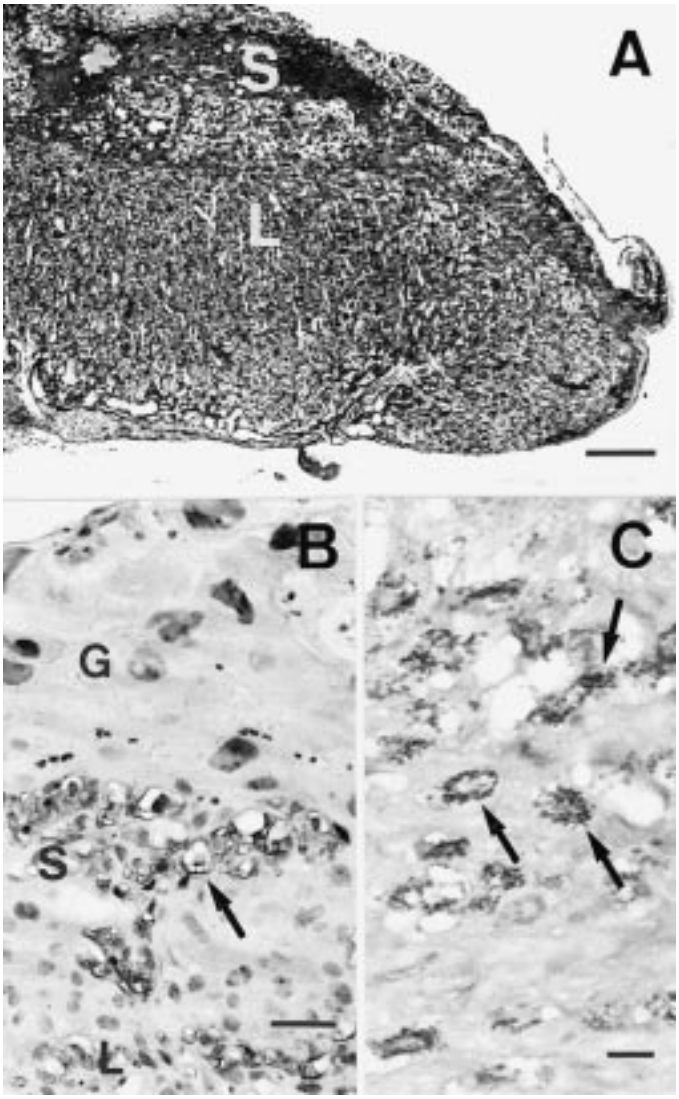

FIG. 4. Photomicrographs of implantation sites on Day 15 of pregnancy in GM−/− mice. **A**) Placenta stained with Masson's trichrome to delineate spongiotrophoblast (S) and labyrinth (L) regions. The area of spongiotrophoblast in placentae from GM−/− mice was increased by 22% compared with that in GM+/+ placentae, while that of the labyrinth was decreased by 13% (both $p = 0.008$) (Table 4). Bar = 250 μm. **B**) A nest of vacuolated trophoblast "glycogen cells" (arrow) in the spongiotrophoblast region, localized immunohistochemically with mAb L815. The proportion of L185-immunoreactive cells per mm² of spongiotrophoblast was diminished by 20% in GM−/− placentae ($p = 0.048$) (Table 4). G, Trophoblast giant cell layer. Bar = 50 μm. **C**) Mesometrial triangle region of the decidua stained with PAS to localize uterine natural killer cells (arrows), which were comparable in size, distribution, and frequency in GM−/− and GM+/+ mice. Bar = 20 μm.

To further evaluate the interaction between maternal and fetal GM-CSF genotype, and particularly to determine whether fetal GM-CSF genotype was a major determinant of fetal size, GM−/− and GM+/− females were mated with GM+/− studs and killed on Day 17. Fetuses and placentae were weighed, and the genotype of the conceptus was determined by PCR of DNA extracted from embryonic tissue using primers specific for the wild-type GM-CSF gene and transgene construct. Fetal weights were significantly less for GM−/− pups in GM−/− than in GM+/− mothers, whereas the weight of GM+/+ or GM+/− pups was not affected by maternal GM-CSF. There was no effect of the genotype of the conceptus or of the mother on the weight of the placenta,

TABLE 3. The effect of maternal and fetal GM-CSF deficiency on fetal and placental weights and fetal : placental weight ratio on Day 17 of pregnancy in mice (mean ± SEM).

Female	Male	Fetuses[a]	n	Fetal weight (mg)	Placental weight (mg)	Fetal : placental ratio
GM−/−	GM+/−	GM−/−	21	589 ± 76[b]	81 ± 15	7.5 ± 1.7[b]
		GM+/−	28	708 ± 164[c]	79 ± 19	9.4 ± 3.1[c]
GM+/−	GM+/−	GM−/−	12	703 ± 134[bc]	80 ± 17	9.4 ± 3.8[cd]
		GM+/−, +/+	58	646 ± 114[bc]	82 ± 14	8.0 ± 1.5[bd]

[a] Genotype of fetuses was determined by PCR.
[b,c] Data were compared by one-way ANOVA and Bonferroni *t*-test; data in the same row with different superscript letters denote significant differences between groups ($p < 0.05$).

but the fetal:placental ratio in GM−/− mothers was significantly smaller for GM−/− fetuses than for GM+/− fetuses (Table 3).

To investigate the effect of GM-CSF genotype on embryo survival in utero, the PCR-derived genotype data from the above experiment were combined with PCR data from Day 14 to 17 embryos in an additional cohort of heterozygote pregnancies. Although the proportion of embryos with a GM−/− genotype was smaller than the expected Mendelian ratio, the difference did not reach significance (GM−/−, GM+/−, GM+/+ = 22%, 48%, 30%, respectively, n = 114 implantation sites). Similarly, a moderate but insignificant reduction in the expected proportion of GM−/− pups was observed in GM−/− females mated with GM+/− males (GM−/−, GM+/− = 45%, 55%, respectively, n = 82 implantation sites).

Effect of GM-CSF Deficiency on Uterine Morphology and Macrophage Populations during the Estrous Cycle and Early Pregnancy

GM-CSF expression in the endometrial epithelium during early pregnancy is maximal during the 24-h period after mating and is associated with the presence of abundant populations of myeloid leukocytes in the endometrium [10]. To investigate the effect of GM-CSF deficiency on uterine and endometrial morphology, and to ascertain whether uterine macrophage and granulocyte populations were depleted in GM-CSF-deficient mice, sections of uteri were taken from estrous and Day 1 pregnant GM−/− and GM+/+ mice (n = 6 mice per group). Sections were stained with H&E or with DAB to visualize endogenous peroxidase-positive cells (eosinophils) and were labeled immunohistochemically with F4/80 and RB6–6C5 mAbs to identify macrophages and neutrophils, respectively. There were no unusual features or overt differences in the morphology of uteri from GM−/− mice compared with uteri from GM+/− mice, either at estrus or on Day 1 of pregnancy, with no histological evidence of infection or lymphocytic infiltration. GM-CSF status did not influence the numbers of macrophages, neutrophils, or eosinophils in the uterine endometrium or myometrium, and the spatial locations of these cells were comparable between genotypes and similar to those described previously for other strains of mice [10, 12]. The mean numbers of RB6–6C5-positive cells present in the luminal epithelium on the day after mating were diminished but were not significantly lower in GM−/− compared with GM+/− uteri, and both values were within the range reported previously for Balb/c F1 mice [10]. F4/80-positive macrophages but few eosinophils or neutrophils were present in implantation sites of both GM+/+ and GM−/− mice on Day 8 of pregnancy, where they were located predominantly in the mesometrial triangle and at the myometrial-decidual boundary, regardless of maternal genotype.

Effect of GM-CSF Deficiency on Decidual and Placental Structure and Function

GM-CSF has been implicated as a regulator of the growth and function of trophoblast cells in the placenta [23–25]. The effect of GM-CSF status on placental structure at Day 15 was examined at the light microscope level. Duplicate sections of placentae taken from 6 healthy implantation sites in 3 GM+/+ mice, and 7 healthy implantation sites in 4 GM−/− mice, were stained with H&E or with Masson's trichrome to differentiate between fetal mesenchyme, trophoblast cells, and maternal red blood cells in the placenta. There were significant differences in the structure of placentae from GM-CSF-deficient mice; most notably, the proportionate areas of spongiotrophoblast and labyrinthine regions were altered by GM-CSF deficiency. The area of spongiotrophoblast in placentae from GM−/− mice was increased by 22% compared with GM+/+ placentae, while that of the labyrinth was decreased by 13% (both $p = 0.008$) (Table 4 and Fig. 4).

Monoclonal antibodies L185 and C2562, raised against human first-trimester trophoblast cells and cytokeratin, respectively, were found to be specifically reactive with nests of vacuolated trophoblast cells in the spongiotrophoblast region of placentae (Fig. 4). These cells were identified as ''glycogen cells'' [46] on the basis of their intense, amylase-sensitive reactivity with the carbohydrate-specific stain PAS. The proportion of glycogen cells in the spongiotrophoblast zone was diminished in placentae of GM-CSF-deficient mice, with 20% less L185 immunoreactivity per

TABLE 4. The effect of GM-CSF deficiency on placental structure in GM+/+ and GM−/− mice.

Parameter	GM+/+[ab]	GM−/−[b]	*p* Value
Total area (mm^2)	2.08 ± 0.29	2.03 ± 0.48	NS
Proportion spongiotrophoblast	0.36 ± 0.03	0.44 ± 0.06	0.008
Proportion labyrinth	0.64 ± 0.03	0.56 ± 0.06	0.008
$L185^+$ 'glycogen cells' (% positivity)	63.5 ± 13.7	51.1 ± 15.2	0.048

[a] Mice were mated with males of the same genotype.
[b] Values are means ± SD. Data were compared by Bonferroni *t*-test.

square millimeter of spongiotrophoblast in GM−/− placentae ($p = 0.048$) (Table 4 and Fig. 4). In contrast, no overt effect of GM-CSF deficiency on the size or location of decidual or placental macrophage populations, or on the morphology, size, or distribution of the uterine natural killer cell populations residing in the mesometrial triangle, were observed in additional sections of implantation sites stained with F4/80 and with PAS, respectively (Fig. 3).

Effect of GM-CSF Deficiency on Uterine Microbial Flora

Subclinical lung infections are characteristic of GM-CSF-deficient mice, with microbiologic or histologic infection present in 84% of animals [38–40]. To investigate the incidence of microbial infection of the reproductive tract, uterine tissue (including luminal fluids) was recovered from virgin GM+/− and GM−/− mice (n = 6 per genotype) or mice on the first, second, and third days after mating (n = 3–5 per genotype per day). The capacity of the uterus to clear bacteria after mating was not compromised in GM-CSF-deficient mice, since bacteria (most commonly *Pasteurella pneumotropica* or *Proteus vulgaris*) were recovered from all Day 1 tissues but not from Day 2 or Day 3 tissues, irrespective of genotype. In contrast, virgin GM-CSF-deficient mice were found to have a higher incidence of subclinical bacterial colonization of the uterus, with *Pasteurella pneumotropica* recovered from uteri of 3 of 6 GM−/− mice but 0 of 6 of GM+/− mice. Microbial infection of the lung was also more common in GM-CSF-deficient mice, with fungal and bacterial agents isolated from 10 of 17 GM−/− mice compared to 5 of 17 GM+/− mice.

On four occasions over the course of the mating and histological experiments, recently mated or parous mice were found to have chronic uterine abscesses, from which *Pasteurella pneumotropica* organisms were identified as the causative agent. Abscesses were never seen in GM+/− or GM+/+ females.

DISCUSSION

Initial indications were that the fertility of GM-CSF ''knockout'' mice was normal [38, 39], but a subsequent larger study reported that deficiency in this cytokine leads to 15% smaller litter size at birth [40]. The experiments described in the current study substantiate and extend this recent finding. In summary, we have confirmed that GM-CSF deficiency in mice is associated with a moderate impairment in fertility, manifested as a 25% reduction in litter size by the time of weaning. We have shown that diminished reproductive performance appears to be the consequence of a gradual loss in fetal viability during the second half of gestation, in the perinatal period, and in the first 3 wk of life. Surviving fetuses were also affected; the slower growth kinetics operating in utero appear to persist after birth, giving rise to substantially smaller pups at weaning. In males, this diminished body mass is retained into adult life.

The findings of comparable numbers of implantation sites at Day 7 of pregnancy in GM-CSF-replete and -deficient mice suggest that the events leading to higher resorption rates at Day 17 of pregnancy occur after implantation. The mechanism for postimplantation loss is not clear, but likely contributing factors can be surmised from data gathered on surviving conceptuses. These include the increased numbers of very small and abnormal fetuses, diminished fetal:placental ratio, and histological evidence of altered placental structure. These findings together suggest that impaired placental function may be a major cause of fetal demise in GM-CSF-deficient mice.

The differential susceptibility of individual litters, and particularly of individual implantation sites within a litter, is consistent with the notion that genetic variation within the essentially random outbred population may contribute to the severity of the detrimental effect. Genetic disparity between the mother and the fetus as a determining factor for fetal survival is also suggested by the finding that male fetuses were disproportionately affected, in terms of both their survival to weaning and their growth kinetics during the postnatal period.

Interestingly, the detrimental effects of GM-CSF deficiency were most striking when both the mother and the fetus were GM-CSF deficient. Litter sizes in GM-CSF-deficient mothers were less severely affected in pregnancies sired by wild-type males, and resorption rates, fetal weights, and the fetal:placental ratio were more comparable to normal values. Conversely, fetal weights and fetal:placental ratios were not reduced in GM-CSF-deficient embryos gestating in GM-CSF-replete mothers. These findings suggest that GM-CSF of maternal origin is sufficient for normal growth and development of the placenta and conceptus, and that GM-CSF of embryonic origin can to a large extent compensate for deficiency in the mother.

Reduced placental capacity to deliver nutrients and oxygen to the fetus is a major cause of fetal growth retardation, as well as increased postnatal morbidity, in humans and in animal species [47]. The effect of GM-CSF deficiency was manifested as a reduction in the size of the labyrinthine layer of the placenta, which could potentially limit the surface area for nutrient exchange between the maternal and fetal circulations. In addition, the proportion of vacuolated trophoblast ''glycogen cells'' in the spongiotrophoblast layer was significantly reduced in GM-CSF-deficient mice. These cells are thought to be the murine correlate of the multipotent progenitor trophoblast cells of the human placental basal plate that retain substantial proliferative capacity and give rise to all the populations of intermediate trophoblast (extravillous cytotrophoblast) that migrate deep into the decidua [46, 48]. Together, these data suggest that there may be subtle abnormalities in the regulation of trophoblast cell proliferation and/or differentiation in GM-CSF-deficient mice. It is also tempting to speculate that the placenta attempts to compensate for the lack of GM-CSF by increasing the proportion of the placenta devoted to trophoblast cell generation, at the expense of the area for exchange. Further studies are in progress to address these issues, and in particular to determine whether there are differences in the surface area of trophoblast or the mean barrier thickness in the placental labyrinth that may further contribute to the compromised placental function in GM-CSF-deficient mice.

GM-CSF could promote placental development and function in a variety of ways. In humans, villous and extravillous cytotrophoblast cells of the developing placenta express GM-CSF receptors [26, 27], and in vitro studies have suggested that the proliferation, differentiation, and secretory function of these cells may be promoted by GM-CSF in both humans and rodents [23–25]. Certainly it seems plausible that GM-CSF of both maternal and embryonic origin, synthesized in the decidua and the placental mesenchyme, respectively, could target trophoblast cells at the maternal-placental interface. Thus the concentrations of cytokine available to trophoblast cells of GM-CSF-deficient

placentae may not be substantially diminished when the mother is GM-CSF replete.

GM-CSF may act indirectly to promote placental growth and invasion, through effects on the uterus or on the maternal immune response to fetal and placental antigens. Uterine development in the absence of GM-CSF appears to be normal with no discernible effect on the process of decidualization. The process of induction of immune responses is influenced by this cytokine, which acts most notably through promoting the activation, maturation, and trafficking of antigen-presenting cells, particularly dendritic cells, at sites of antigen exposure. While dendritic cells from GM-CSF-deficient mice have been shown to function as competent antigen-presenting cells, their capacity to interact with T lymphocytes is attenuated in an antigen-specific manner. Specifically, their ability to evoke IL-2 responsiveness in regulatory T lymphocytes appears to be retarded, since CD4+ T cells from GM-CSF-deficient mice have an impaired proliferative response, and the kinetics of IgG production are slowed [49]. An immune-mediated action of GM-CSF in pregnancy may explain the beneficial effect of very small doses of exogenous GM-CSF during placental development [35–37]. The doses given in these experiments were considered too small to influence placental growth directly, and an indirect route of action, exerted via maternal immune networks, was postulated. Furthermore, it is reasonable to expect that any damaging consequence of an insufficient induction of ''permissive'' maternal immune responses might correlate with the extent of genetic disparity between the mother and the fetus.

The possibility of direct beneficial effects of GM-CSF on the growth and development of the embryo cannot be excluded. Although adult GM-CSF-deficient mice born to GM−/− mothers appear to have normal hemopoiesis and peripheral blood leukocyte counts, it is possible that GM-CSF may have a facilitatory role in embryonic hemopoiesis or other aspects of embryonic development. Since some maternal cytokines, including granulocyte-CSF [50], can cross the placenta and enter the fetal circulation, the effect of embryonic GM-CSF depletion may be partially rescued when GM−/− fetuses gestate in GM-CSF-replete mothers.

Although the effects of GM-CSF deficiency are manifested late in gestation, there is increasing evidence to suggest that events occurring during the early preimplantation stages can dramatically influence fetal and placental development and ultimately compromise the health of the newborn individual. Even small perturbations in the maternal tract environment can have marked effects on fetal size and viability that do not become apparent until later in gestation or after birth [51]. Among the changes seen in preimplantation embryos subject to such perturbations is a diminished allocation or survival of cells in the inner cell mass in the blastocyst [52, 53]. GM-CSF, which is abundantly present in the tract during these early stages, has been identified as an embryotrophic factor, with survival-promoting effects on the inner cell mass of embryos grown in vivo or in vitro [20, 21]. Effects on the developing preimplantation embryo may thus partially account for the effects of GM-CSF deficiency on fetal development. However, since GM-CSF is not expressed in the embryo until after implantation, it would be expected that any detrimental effect of lack of exposure to GM-CSF in the maternal tract prior to implantation would impact equally on embryos irrespective of their genotype. Since the data reported herein show that GM-CSF-replete embryos are proportionately better off than their cytokine-deficient littermates, this route of action of GM-CSF may be less important than effects mediated after implantation.

Recent experiments in GM-CSF-deficient mice support a role for this factor in the functional maturation of macrophages and granulocytes in peripheral tissues, particularly during episodes of inflammation or infection. In vitro experiments have shown that macrophages from GM-CSF-deficient mice are refractory to activation by bacterial LPS, with diminished nitric oxide synthesis and attenuated production of interferon-γ, IL-1, and IL-6 [54]. Compromised macrophage activation, together with failure of ''emergency'' myelopoiesis, is believed to cause diminished peritoneal inflammation and exacerbate bacterial proliferation in *Listeria monocytogenes*-infected GM-CSF-deficient mice [55].

In the uterine endometrium, acute but short-lived inflammation-like responses occur at mating in response to specific stimuli, including TGFβ, present in seminal plasma [20], and may also be associated with the process of parturition. A surge of GM-CSF released from the uterine epithelium has been implicated as a causative agent in this response [13, 14]. However, GM-CSF deficiency did not have any significant effect on the numbers or spatial distribution of the abundant populations of macrophages, eosinophils, or neutrophils found in the uterine endometrium and luminal epithelium after mating, or in the decidua during midgestation. Factors other than this cytokine, most notably the chemokine family of molecules, are now implicated as additional regulators of leukocyte trafficking into the endometrium that may fully compensate for any chemotactic activity of GM-CSF [56, 57]. However, our preliminary observations suggest that endometrial macrophages and dendritic cells in GM-CSF-deficient mice may have functional deficiencies ([56] and unpublished results), in particular a reduced capacity to express major histocompatibility complex class II (Ia) and B7–2, suggesting that an additional and less redundant role of epithelial GM-CSF may be to promote the antigen-processing and -presentation activities of endometrial leukocytes.

Diminished functional capacity of uterine macrophages and dendritic cells could have consequences not only for pregnancy outcome but also for protection of the uterine mucosal surface from bacterial infection. This may explain our observation of increased prevalence of opportunistic bacterial contamination of the usually sterile uterine environment in GM-CSF-deficient mice. The reduced ability of GM-CSF-deficient mice to deal with persistent infection may thus be a systemic characteristic, evident in mucosal tissues in addition to the lung. Interestingly, clearance of bacterial organisms from the reproductive tract after mating appeared to occur normally in GM-CSF-deficient mice, perhaps because the acute inflammatory response associated with mating is dependent primarily upon seminal cytokine induction rather than stimuli provided by microorganisms [11].

Collectively, these studies show that GM-CSF is not essential for successful pregnancy in mice, providing a further illustration of the extraordinary flexibility and redundancy of the cytokine network governing pregnancy. Importantly, however, the mechanisms compensating for GM-CSF deficiency are incomplete, since fetal growth and viability are compromised in the absence of GM-CSF. These data suggest that close examination of gene-targeted knockout mice may be required to reveal specialized roles of particular cytokines in reproductive events. Moreover, the potential significance of the cost to fetal health of even subtle phe-

notypes should not be underestimated, as there is growing evidence that the adverse consequences of a less-than-optimal uterine environment on fetal growth can be amplified in adult life. Epidemiological studies indicate that even moderately impaired fetal growth is correlated with the development of hypertension, non-insulin-dependent diabetes, hyperlipidemia, altered cholesterol and fibrinogen metabolism, coronary heart disease, and obesity [58].

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