

Macrophages: important accessory cells for reproductive function

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Abstract: Macrophages are found throughout reproductive tissues. To determine their role(s), we have studied mice homozygous for a null mutation (*Csfm^{op}*) in the gene encoding the major macrophage growth factor, colony-stimulating factor-1 (CSF-1). Both male and female *Csfm^{op}/Csfm^{op}* mice have fertility defects. Males have low sperm number and libido as a consequence of dramatically reduced circulating testosterone. Females have extended estrous cycles and poor ovulation rates. CSF-1 is the principal growth factor regulating macrophage populations in the testis, male accessory glands, ovary, and uterus. However, analyses of CSF-1 nullizygous mice suggest that the primary reproductive defect is in the development of feedback regulation of the hypothalamic-pituitary axis. Although not correlating with deficiencies of microglia populations, electrophysiological investigations indicate an impairment of neuronal responses. This suggests that microglia, under the influence of CSF-1, act to organize neuronal connectivity during development and that the absence of this function results in a perturbation of the hypothalamic-pituitary-gonadal axis. Macrophages also appear to have functions in the differentiated tissues of the reproductive system, including having a positive influence on steroidogenic cells. These data suggest that macrophages, through their trophic functions, can be considered as essential accessory cells for normal reproductive functioning. *J. Leukoc. Biol.* 66: 765–772; 1999.

Key Words: osteopetrotic mouse · colony-stimulating factor-1 · ovary · testis · uterus · hypothalamic-pituitary-gonadal axis

INTRODUCTION

Macrophages are ubiquitous cells that play central roles in the innate immune response and are important accessory cells for many other immune responses. In addition, during development, these cells are also thought to have trophic roles enacted through their remodeling capabilities and cytokine production. In the mouse, studies of macrophage function have been greatly advanced by the analysis of mice carrying null mutations in genes that encode macrophage growth factors [1–3]. These studies have shown that colony-stimulating factor-1 (CSF-1,

also known as macrophage colony-stimulating factor) is the major, but not the only, growth factor that regulates production of macrophages [4, 5]. Thus, mice homozygous for the null mutation in the CSF-1 gene, osteopetrotic (*Csfm^{op}*) have severely depleted macrophage populations in many tissues [6, 7]. It is interesting that close analysis of macrophage populations suggest that the tissue density of those macrophages that have scavenging/trophic roles is dramatically affected by the absence of CSF-1, whereas those that populate immune organs are relatively less affected (**Fig. 1**) [5]. However, even populations of macrophage whose density is unaffected by the absence of CSF-1 express the CSF-1 receptor, suggesting that their function may be compromised in the absence of CSF-1.

Macrophages are also found abundantly in the reproductive tract of both males and females. These cells are in close proximity to steroidogenic cells in the ovary and testis. In the uterus, they undergo dramatic fluctuations in response to the changing hormonal milieu. Furthermore, there are specialized macrophages, the microglia, found within the region of the central nervous system that controls reproductive function. These observations, together with functional studies, have led to suggestions that macrophages play important roles in reproductive processes [8–13]. We have exploited the macrophage-deficient *Csfm^{op}/Csfm^{op}* mouse to define roles for CSF-1-regulated macrophages in reproduction. The data obtained with this mouse mutant, and reviewed below, show that macrophages play important roles in reproductive function in both males and females.

MACROPHAGES IN THE REPRODUCTIVE TISSUES

Testis

Macrophages form a substantial portion of the interstitial cells (~25%) of the testis but with none found within the seminiferous tubule (**Fig. 2**) [14]. These testicular macrophages form intimate associations with the steroidogenic Leydig cells through a unique form of cell-to-cell communication whereby the microvilli of the Leydig cells fit into coated vesicles on the macrophage surface [15]. Such interactions also occur *in vitro*

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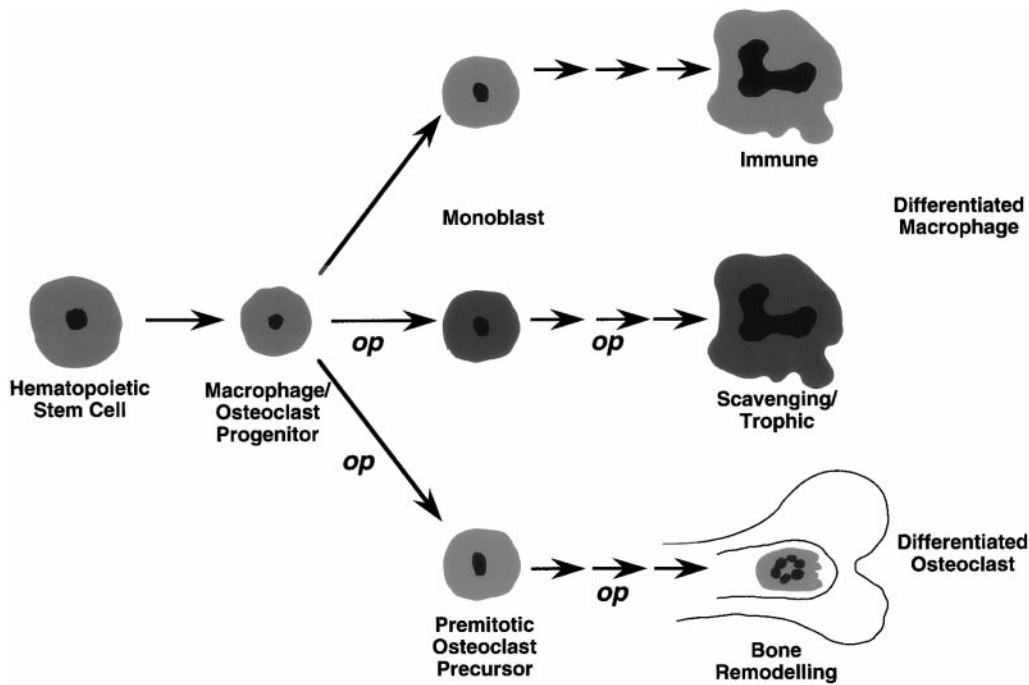
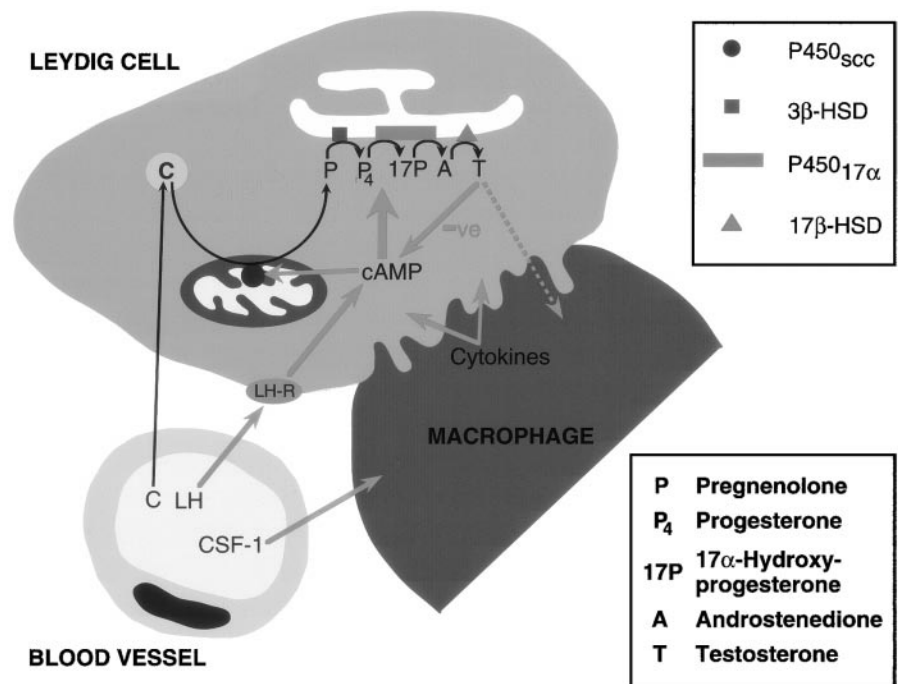


Fig. 1. CSF-1 regulation of cells of the mononuclear phagocytic lineage. Analysis of mononuclear phagocytic populations in CSF-1-deficient mice have revealed populations with variable dependencies on CSF-1. Shown are the common mononuclear phagocytic precursor that gives rise to the osteoclastic lineage and populations of macrophages that have trophic and scavenging roles, both of which are dependent on CSF-1. In contrast, macrophages that populate the immune organs are relatively independent of CSF-1 in terms of tissue density. *op* indicates a stage of differentiation that is affected by the osteopetrotic mutation at the *Csfm* locus.

between these two cell populations, but not between Leydig cells and other macrophage populations [16]. In rats, macrophages first populate the testis at day 19 of gestation and increase in both size and number over the first 50 days of post-natal life [14, 17]. The distinct interdigitations between these cells and Leydig cells occurs between 20 and 30 days post-partum just before the androgen surge marking the

beginning of puberty [15]. This suggests that macrophages may have some role in regulating steroidogenesis in the Leydig cells [9, 18, 19]. Testicular macrophages also display numerous immune properties: they can secrete cytokines, present antigens, and secrete lysozyme but are somewhat immunosuppressed compared to other resident macrophage populations [20–22].

Fig. 2. Macrophage-Leydig cell interactions in the testis. Cholesterol (C) enters the Leydig cell from the bloodstream and is stored in lipid droplets within the cell before being transported to the mitochondria for steroidogenesis. In the mitochondria, the cytochrome P450 side chain cleavage enzyme (P450_{scc}) converts cholesterol to pregnenolone (P) to initiate steroidogenesis. Pregnenolone exits the mitochondria and travels to the cytoplasmic surface of the endoplasmic reticulum where it is then converted to progesterone (P₄) by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD). Progesterone is converted in a two-step process to androstenedione (A), via 17α-hydroxyprogesterone (17P) by the enzyme P450_{17α}. Finally, the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD) converts androstenedione to testosterone (T). The process is regulated by LH acting via cell surface LH receptors (LH-R) that, on LH occupancy, affect a rise in cyclic AMP (cAMP). The Leydig cells are in close association with macrophages whose density is regulated by CSF-1 derived from the circulation. These macrophages influence Leydig cell integrity through cell to cell interactions or through the secretion of cytokines.



Studies using *Csfm^{op}/Csfm^{op}* mice have shown that this testicular macrophage population, together with all of those of the male reproductive tract, are severely depleted in the absence of CSF-1 [23]. Restoration of circulating CSF-1 to the mutant mice from birth resulted in the re-population of the testis with macrophages, showing their dependence on circulating CSF-1 [23]. Although the overall structure of the testis is normal, ultrastructurally the Leydig cells in *Csfm^{op}/Csfm^{op}* mice show striking abnormalities [24]. In wild-type mice, Leydig cells have a profusion of endoplasmic reticulum and tight membranous whorls that occupy a small portion of the cytoplasm [25]. In contrast, Leydig cells in *Csfm^{op}/Csfm^{op}* mice appear to have an unraveling of these whorls and/or dilation of the inter-membrane spaces such that much of the cytoplasm is occupied by these structures [24]. Restoration of testicular macrophages by systemic treatment with CSF-1 resulted in restoration of Leydig cell ultrastructure. Because only macrophages in the testis express the CSF-1 receptor [26], this suggests that macrophage-Leydig cell interactions help maintain the structural integrity of the Leydig cell.

Ovary

In a similar fashion to the testis, the ovaries of all mammalian species examined are well populated with macrophages. These are found in the interstitial tissue while being excluded from the germ cell compartment (**Fig. 3**) [11, 27, 28], except in atretic follicles where macrophage infiltration might contribute to the destruction of the defunct follicle. During oocyte development in mice, rats, and humans, macrophages are recruited into the

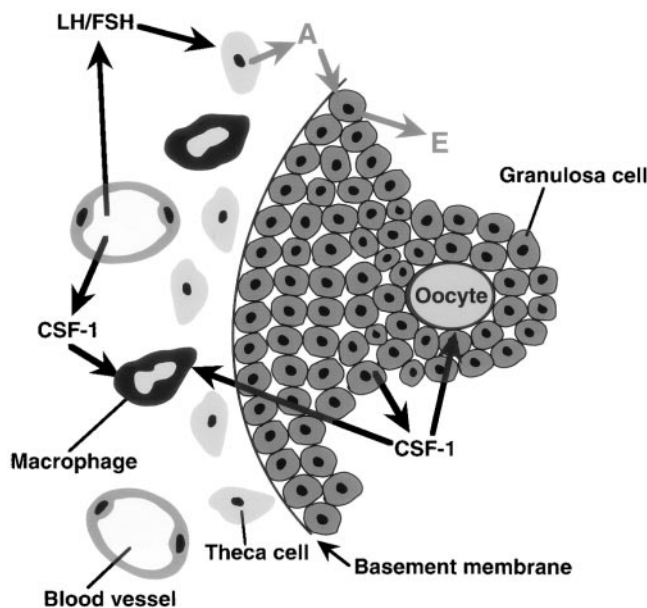


Fig. 3. CSF-1 and macrophages in the ovary. As follicles grow, the number of macrophages associated with theca increases. These macrophages are recruited to this area in response to rising levels of follicular CSF-1 synthesized by the granulosa cells. The macrophages release cytokines and other factors that may influence thecal cell steroidogenesis and that may also facilitate follicular rupture and post-ovulatory repair. Granulosa cell-derived CSF-1 is also targeted to the oocyte, which bears receptors for CSF-1. Blue arrows indicate regulatory pathways, dark and light arrows indicate steroidogenic pathways. A, androstenedione; E, estrogen.

theca layers of the follicle such that their number is greatest just before ovulation [29–31]. Macrophages are also preferentially localized to the cortical surface of pre-ovulatory follicles where they may assist in the process of follicle rupture and/or pre-luteal repair of the ovarian wall [30]. Once the oocyte is released, macrophages contribute a substantial proportion of the cells in the corpus luteum [27, 31, 32]. In the absence of CSF-1, the number of ovarian macrophages is very significantly reduced and virtually none are recruited to the developing follicle [31, 33]. In mice and humans, CSF-1 mRNA can be detected in granulosa cells as the follicle matures and at least in humans, the CSF-1 concentration of follicular fluid is significantly higher than serum [34, 35]. Because CSF-1 is a chemoattractant for macrophages [36], it seems probable that the follicle-synthesized CSF-1 plays at least a part in the recruitment of macrophages proximate to the developing follicle. It is interesting that as the corpus luteum matures after copulation, the number of macrophages in *Csfm^{op}/Csfm^{op}* mice increases until it reaches approximately 35% of the wild-type number [31]. This strongly suggests the presence of another macrophage growth factor/chemoattractant synthesized by the luteal cells [37].

Uterus

Macrophage density in the adult uterine stroma is strongly influenced by the endocrine milieu. Their density is lowest at diestrus during periods of relative estrogen depletion and increases to ~20% of the stromal cells at pro-estrus under the influences of estrogen [38–42]. This population is sustained if the mice copulate, resulting in pregnancy or a pseudopregnant state [42, 43]. CSF-1 is synthesized by the uterine epithelium in response to female sex steroid hormones [44], and in ovariectomized mice uterine CSF-1 mRNA can be acutely induced by estradiol-17 β [45]. Studies with *Csfm^{op}/Csfm^{op}* mice have shown that CSF-1 is the key regulator of the uterine stromal macrophage population because their numbers are very substantially reduced in these mice [42, 46]. Consistent with these observations are experiments whereby the intra-luminal uterine installation of CSF-1 to wild-type mice resulted in macrophage accumulation into the stroma [47]. It is interesting that in granulocyte-macrophage CSF nullizygous mice, there is no effect on these macrophage populations in the uterus despite the uterine synthesis of GM-CSF after copulation [48, 49]. Thus, we can conclude that CSF-1 synthesized by the uterine luminal epithelium under the influence of sex steroid hormones is largely responsible for the regulation of the macrophage populations in the uterine stroma (**Fig. 4**). The exact function of these macrophages is unknown. However, functions have been suggested that range from classical immunological roles of phagocytosis, antigen presentation, and bactericidal activities, to the production of cytokines that promote pre-implantation embryo growth and implantation [13, 40, 43].

Microglia in the hypothalamus

Cells that are positive for the mononuclear phagocyte marker, F4/80, are first detected in the brain as early as embryonic day 16 (e16) around blood vessels and in the choroid plexus, one of

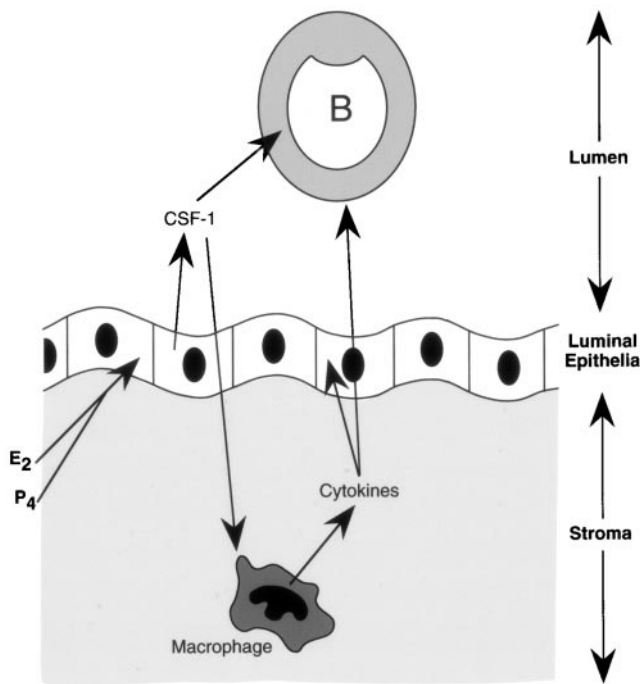


Fig. 4. CSF-1 mediates the response of uterine macrophages to female sex steroid hormones. Uterine epithelial cells secrete CSF-1 in response to fluctuations in circulating steroid hormones (estrogen, E_2 , and progesterone P_4), resulting in the recruitment of macrophages to the endometrial stroma. These macrophages may release cytokines that can influence the interactions between the stroma, epithelium, and blastocyst (B). In addition, CSF-1 is also directed toward the trophoblast of the blastocyst and can promote trophoblastic proliferation and embryonic development.

the major entry sites for blood-bone monocytes [50]. At this time, however, the cells are somewhat rounded and do not appear, at least morphologically, to be microglia. The numbers of $F4/80^+$ cells increases steadily up until birth, when they begin to take on the more complex morphological features characteristic of mature microglia [51]. By adulthood, the population of microglia in the brain equals that of liver macrophages (based on relative organ weights), an organ containing one of the highest macrophage populations in the body [52]. Furthermore, the surface area of mature microglia is up to seven times larger than that of Kupffer cells, indicating the significant distribution of macrophage cell volume through the brain.

Studies in adult $Csfm^{op}/Csfm^{op}$ mice have shown that microglial cell numbers are reduced by 30–50% in the cortex and corpus callosum and are also morphologically altered by the absence of CSF-1 [53]. However, no change in microglia number or distribution were observed by Chang et al. or Blevins and Federoff [54, 55]. This is in line with our own unpublished studies, and suggests that the effects of CSF-1 deprivation on brain microglia density in adult mice are restricted to specific regions of the brain and are relatively minor. However, in a study of retinal development, the acquisition of microglia in $Csfm^{op}/Csfm^{op}$ mice was delayed [6], indicating that further studies on the developmental profile of microglial recruitment are warranted.

REPRODUCTIVE ROLES OF MACROPHAGES AS REVEALED BY STUDIES ON $Csfm^{op}/Csfm^{op}$ MICE

Males

The severe depletion of macrophages in many reproductive tissues in mice homozygous for the null mutation in the CSF-1 gene enabled us to determine the effects of macrophage depletion on reproductive function. Both male and female $Csfm^{op}/Csfm^{op}$ mice show reproductive defects compared to their wild-type counterparts (Table 1). Thus, when homozygous mature mice are mated together, pregnancies rarely ensue [46]. However, when male $Csfm^{op}/Csfm^{op}$ mice are mated to normal females, the major defect is manifested by low libido and consequent infrequent mating [26]. This is due to a serum concentration of testosterone that is only ~10% of wild-type concentrations, which is in turn caused by a lowered biosynthetic capacity of the Leydig cells in the homozygous mutant mice [24, 26]. Further analysis of the steroidogenic pathway in $Csfm^{op}/Csfm^{op}$ males showed a depletion in steroidogenic enzyme protein concentrations and activity, with the cholesterol side chain cleavage enzyme displaying the greatest reduction in activity in $Csfm^{op}/Csfm^{op}$ compared to wild-type mice [24]. This would suggest that Leydig cell function is severely compromised in the absence of their normal communication with macrophages. However, since luteinizing hormone (LH) was able to rescue the steroidogenic defect both *in vivo* and in the cultured Leydig cells, we measured circulating LH concentrations and found them to also be reduced by 90% compared to control mice [24]. To determine whether this was due to a primary defect in the pituitary, we treated $Csfm^{op}/Csfm^{op}$ mice with the GnRH agonist, histrelin, and found that this could largely rescue the circulating LH concentrations [24]. This suggests that $Csfm^{op}/Csfm^{op}$ male mice have a primary defect in their hypothalamic axis. Indeed, treatment of mice with either testosterone or by castration (to remove all endogenous testosterone), resulted in only small changes in serum LH concentrations in a manner that is reversed from normal feedback regulation [24]. Thus, there appears to be a developmental defect in the establishment of the hypothalamic-pituitary-gonadal axis in male $Csfm^{op}/Csfm^{op}$ mice.

These data suggest that the primary cause of the low circulating testosterone in male $Csfm^{op}/Csfm^{op}$ mice is the low

TABLE 1. Reproductive Defects in $Csfm^{op}/Csfm^{op}$ Mice

Males	
Low testosterone biosynthetic capacity	
Reduced libido	
Low sperm number	
Diminished circulating LH concentrations	
Impaired hypothalamic-pituitary feedback response to testosterone	
Females	
Extended estrous cycle	
Delayed puberty	
Low ovulation rate	
Reduced litter size	
Poor lactational ability	
Disrupted positive and negative feedback loops in the hypothalamus and pituitary	

serum LH. This is consistent with our observations in CSF-1-treated nullizygous mice that, despite restored testicular macrophage populations leading to restored Leydig cells morphology and significantly increased steroidogenic enzyme content, the mice still display a very much reduced serum testosterone biosynthetic capacity that is directly proportional to the circulating LH concentration [24]. Regardless of these observations, there is a substantial literature suggesting that macrophages can influence Leydig cell function [9, 15, 17, 56–61]. For example, pharmacological removal of macrophages has severe consequences on Leydig cell function and testicular macrophage conditioned media can influence Leydig cell steroidogenesis in culture [9, 62]. Clearly, from our data, macrophages influence Leydig cell morphology and steroidogenic enzymatic content, suggesting that they can contribute to the local regulation of Leydig cell function superimposed upon a larger mechanism that regulates the entire hypothalamic-pituitary-gonadal axis.

Females

Female *Csfm^{op}/Csfm^{op}* mice show reproductive defects at several levels. They have extended estrous cycles of approximately 14 days compared to the normal 5-day cycle, reduced ovulation rates and, even if they do become pregnant, have smaller litter sizes and a relative failure to nurture their young (Table 1) [31, 46, 63]. Analysis of the causes of the reproductive defects in these mice is complicated by the observation that the CSF-1R, in addition to being expressed in cells of the mononuclear phagocytic lineage, is also expressed in oocytes, decidual cells surrounding the embryo, and in trophoblast (Fig. 5) [44, 64–66]. For example, the ovulation defects appear to be caused by the failure of CSF-1 signaling to the oocyte rather than through effects on macrophages surrounding the ovary [Nishimura and Pollard, unpublished observations] (Fig. 3), whereas

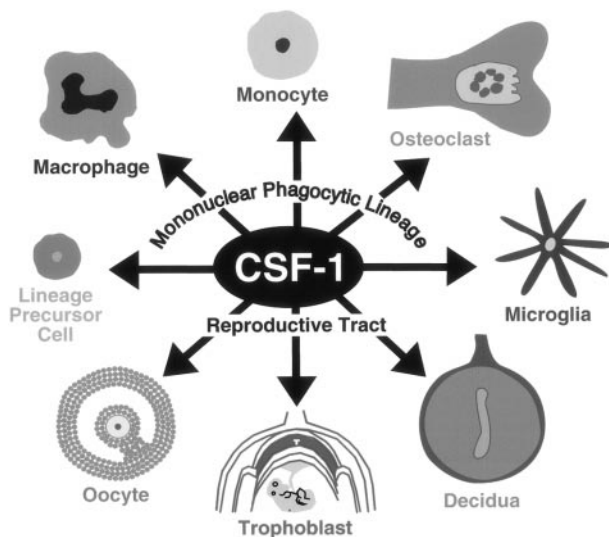


Fig. 5. CSF-1 receptor-bearing cells. CSF-1 receptor is expressed in cells of the mononuclear phagocytic lineage including lineage precursor cells of the bone marrow, monocytes, macrophages, osteoclasts, and microglia. Cells of the reproductive tract also express the CSF-1 receptor and these are the oocyte, the trophoblast of the placenta, and the decidual cells of the peri-implantation uterus.

the estrous cycling defects are related to a developmental defect in the hypothalamic-pituitary-ovarian axis. Thus, in a similar fashion to that observed in male *Csfm^{op}/Csfm^{op}* mice, female *Csfm^{op}/Csfm^{op}* mice also show a diminished negative feedback response to estradiol depletion with the result of a low serum LH in ovariectomized *Csfm^{op}/Csfm^{op}* mice compared to control mice. In addition, *Csfm^{op}/Csfm^{op}* mice fail to show a LH surge (a measure of positive feedback) under experimentally induced conditions. This appears to explain the defect in estrous cycling. Female *Csfm^{op}/Csfm^{op}* mice also exhibit delayed puberty compared to wild-type mice as assessed by vaginal opening or onset of cyclicity. It is interesting that these defects can be largely corrected by subcutaneous treatment of neonatal *Csfm^{op}/Csfm^{op}* female mice with human recombinant CSF-1 administered daily for the first 2 weeks of life. Together, these experiments strongly argue that CSF-1 has a role in the development of the hypothalamic-pituitary axis in both male and female mice.

The hypothalamic-pituitary-gonadal axis

The major reproductive defect in *Csfm^{op}/Csfm^{op}* mice appears to relate to poorly developed hypothalamic-pituitary-gonadal axis. This is a surprising result given that there is little if any precedent for the involvement of macrophages in this axis. However, as pointed out earlier, there is a substantial population of macrophages, the microglia, in the brain, including the hypothalamus. These cells express the CSF-1 receptor and respond to CSF-1 in culture by proliferation, synthesis of cytokines, and changes in morphology [67–69]. Our hypothesis, therefore, is that CSF-1-regulated microglial functions play a role in establishing functionality to the hypothalamic-pituitary axis. Many roles of microglia are consistent with this, for example, in the posterior pituitary microglia remodel the terminal arborizations of the magnocellular neuronal projections, thereby modulating the secretion of oxytocin and vasopressin into the circulation [70]. Furthermore, the repair of ischemic damage in the brain is significantly impaired in *Csfm^{op}/Csfm^{op}* mice and this is correlated with a reduction in recruitment of microglia to the damaged area and a relative failure of microglial proliferation [51, 71–73]. In our studies, we have demonstrated severe circuitry problems in the brains of *Csfm^{op}/Csfm^{op}* mice as revealed by intra-cortical recordings of visual evoked potentials [74]. Further analyses of multiple unit activity, an index of net ensemble action potentials, in these mutant mice revealed alterations in both inhibitory as well as stimulatory circuits in the cortex. It is interesting that pharmacological studies with bicuculline methiodide, a GABA_A antagonist, further demonstrated that *Csfm^{op}/Csfm^{op}* mice have alterations in neuronal circuitry that balances excitation and inhibition and segregate responses [74]. These data also strongly implicate defects in the GABAergic system of *Csfm^{op}/Csfm^{op}* mice. It is noteworthy that the GABAergic system plays an important role in the regulation of the sex steroid feedback response in the hypothalamus [75].

We hypothesize, therefore, that during development CSF-1-regulated microglia produce trophic substances that influence the neuronal circuitry in the hypothalamus such that in their absence, the correct regulation of the GnRH neurons is not

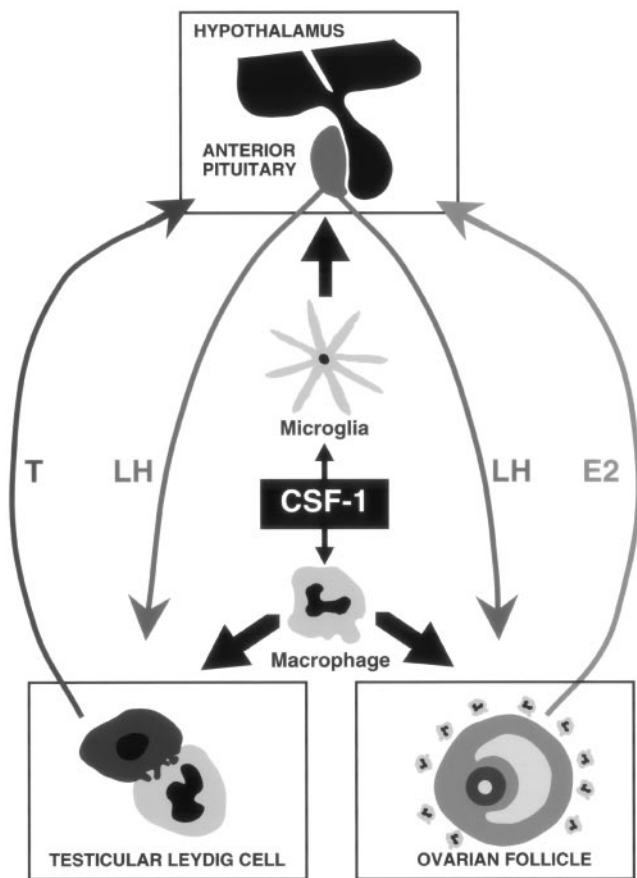


Fig. 6. A model for the role of mononuclear phagocytes in mammalian reproductive function. CSF-1 regulates the function and recruitment of gonadal macrophages during early reproductive development. In the testis, these macrophages form intimate associations with steroidogenic Leydig cells and their number remains fairly constant from puberty onward. These macrophages are thought to regulate both the differentiation of adult Leydig cells as well as their steroidogenic function post-puberty. In the females, macrophages are associated with the thecal compartment of developing follicles and their numbers increase steadily during folliculogenesis. These macrophages may participate in events leading up to follicular rupture and ovulation, but might also regulate follicular oogenesis earlier on during follicular growth. After ovulation, macrophages are involved in repairing the ovary wall and preventing infection and have a positive influence on Luteal cell steroidogenesis. In the ovary, CSF-1 also regulates oocyte growth through CSF-1 receptors located on the oocyte surface. In the hypothalamus, we hypothesize that CSF-1 acts through microglia to regulate neuronal remodeling during fetal and prepubertal development. These changes in neuronal circuitry are essential for the neuroendocrine changes that occur during sexual development and puberty and are thus vital for reproductive function in the adult. Absence of CSF-1 therefore, results in a loss of normal feedback regulation of the hypothalamus by sex steroid hormones in both male and female mice.

established. Consistent with this are the observations that CSF-1 is a neurotropic factor in culture working through non-neuronal cells to enhance neuronal viability and dendrite outgrowth and this response is attenuated in embryonic cultures derived from the *Csfm^{op}/Csfm^{op}* mouse brain [74].

SUMMARY

Macrophages are pervasive cells found in every tissue. Their population densities change according to developmental and

physiological state. Reproductive organs are rich in macrophages and their close proximity to steroidogenic and germ cells as well as fertilized gametes during their development suggest roles in regulating reproductive functions. The inactivating null mutation in the CSF-1 gene results in homozygous mutant mice lacking this important macrophage growth factor and thus, these mice are severely deficient in many tissue populations of macrophages, including those in the reproductive organs. This provided an opportunity to determine the biological roles for CSF-1-regulated macrophages particularly in reproduction. These studies have shown that the recruitment of macrophages into the gonads and uterus is CSF-1 dependent. However, it is surprising to note that when reproduction was analyzed in detail, the major defect in both male and female CSF-1-deficient mice was in the early development of a functioning hypothalamic-pituitary-gonadal axis. The hypothalamus along with most areas in the brain is populated with specialized macrophages known as microglia. These cells are CSF-1 responsive and we hypothesize that CSF-1-regulated microglia play a role in establishing the neuronal circuitry required for proper functioning of the GnRH neurons (**Fig. 6**). Despite this dominant effect of a malfunctioning hypothalamus, the data obtained in the *Csfm^{op}/Csfm^{op}* mouse also suggests local responses to macrophages in the gonads, influencing the function of steroidogenic cells such as Leydig cells in males and corporea luteal cells in females. Further analysis of *Csfm^{op}/Csfm^{op}* mice will undoubtedly continue to reveal novel roles for CSF-1 in a variety of important reproductive processes.

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