INTRODUCTION

Epithelial/mesenchymal cell interactions play a major role in the development of the epithelial ductal tree throughout embryonic and postnatal mammary gland development (Cunha and Hom, 1996; Robinson et al., 1999). Two different mesenchymal cells are known to be important in the morphogenesis of mammary epithelial ducts (Sakakura et al., 1982). One is the fibroblastic cell closely surrounding the epithelial rudiment, and the other is the fat precursor cell that forms the fatty stroma. The fibroblastic mesenchyma induces the fetal mammary epithelium to develop and differentiate so that it fills the fatty stroma (Sakakura et al., 1982). Similarly, numerous studies have demonstrated that mammary fatty tissue determines the unique morphogenesis of normal mammary parenchyma in both fetal (Sakakura et al., 1976, 1982) and adult (Daniel et al., 1984; Sakakura et al., 1982) mice.

Postnatal growth of the mouse mammary gland is chronologically divided into five periods (Topper and Freeman, 1980): neonatal (1-3 weeks of age), juvenile (3-4 weeks of age), prepubertal (4-5 weeks of age), pubertal (5-7 weeks of age), and mature virgin (7-9 weeks of age). Before the juvenile period, a rudimentary epithelial tree is formed in the fatty stroma and is surrounded by a fibroblastic stroma. With the onset of the prepubertal growth period, accelerated ductal extension commences when large club-shape terminal end buds (TEBs) appear and continues through puberty, to cease when the fat pad is laced with a ductal tree at around 9 weeks of age (Imagawa et al., 1994). During this last mature virgin period, the highly mitotic TEB structures are replaced by mitotically quiescent terminal end ducts (TED) and alveolar buds (Topper and Freeman, 1980; Williams and Daniel, 1983). The overall growth and morphogenesis of the epithelium and the differentiation of the mesenchyma are regulated by systemic hormones including estradiol-17β (E2), progesterone (P4) and glucocorticoids in combination with local growth factors (Imagawa et al., 1994; Topper and Freeman, 1980). A number of growth factors are implicated as autocrine and/or paracrine mediators of epithelial/mesenchymal interactions in the mammary gland including insulin-like growth factor-I and -II, transforming growth factors beta and hepatocyte growth factor/scatter factor (Hovey et al., 1998; Niranjan et al., 1995;
Robinson et al., 1991; Singer et al., 1995; Soriano et al., 1998). Several gene targeting mouse models demonstrate the necessity of epithelial/mesenchymal interactions through these growth factor activities and identify the tissue compartments that receive and execute these signals. For instance, inhibin betaβ is mainly provided by the stroma and acts on epithelial growth (Robinson and Henninghausen, 1997), while PTHR1 was identified as an epithelial signal that is received by the mammary mesenchyma (Wysolmerski et al., 1998) and is associated with ductal growth. Furthermore, some studies have investigated the type of stromal cells that are the source of growth factors by showing the growth-promoting activity of mammary fibroblasts (Berdichevsky et al., 1994; Enami et al., 1983) and adipocytes (Carrington and Hosick, 1985; Levine and Stockdale, 1984) towards the mammary epithelium in culture. Although the participation of mesenchymal components in epithelial growth during mammary gland development is well established, the role of other stromal cells that can affect mammary gland development remains to be investigated.

Macrophages, one of the differentiated cell types of the mononuclear phagocytic lineage, are found in every tissue. In addition to their important role in host defense against pathogens, macrophages also play a role in tissue remodeling during development and in normal tissue homeostasis (Horton, 1996). Several growth factors regulate the proliferation and differentiation of mononuclear phagocytes in vivo. These include interleukin-3, granulocyte-macrophage colony-stimulating factor and the macrophage colony-stimulating factor (CSF1) (Stanley et al., 1994). Studies using mice that have null mutations in the genes encoding these growth factors or their receptors indicate that CSF1 is the major regulator of tissue macrophage production controlling their survival, recruitment, differentiation and growth (Cecchini et al., 1994; Dranoff et al., 1994; Nicola et al., 1996; Nishinakamura et al., 1996). The action of CSF1 is mediated through a cell surface receptor tyrosine kinase, CSF1R, which is the product of the c-fms proto-oncogene (Sherr et al., 1985). CSF1R is present on all cells belonging to the mononuclear phagocyte lineage, including terminally differentiated tissue macrophages, osteoclast, and microglia of the brain (Sherr et al., 1985). The roles of CSF1 in vivo have been explored by analyzing mice homozygous for an inactivating recessive mutation in the CSF1 gene (osteopetrotic: Csfrm1−/− mice) (Marks and Lane, 1976; Wiktor-Jedrzejczak et al., 1990; Yoshida et al., 1990). Phenotypically, Csfrm1−/− mice are osteopetrotic, toothless and have reduced macrophage number in most tissues (Pollard and Stanley, 1996). Csfrm1+/−/Csfrm1−/− females also display reduced fertility evident at the pre- and post-implantation stages of pregnancy (Cohen et al., 1997). Although some Csfrm1−/−/Csfrm1−/− females are able to produce offspring, few nurture any pups and none feed a full litter (Pollard and Henninghausen, 1994). This lactational defect is associated with incomplete mammary gland ductal growth during pregnancy, and a precocious development of the lobuloalveolar system leading to a dense and atrophic mammary gland, suggesting a role of macrophages in normal ductal development (Pollard and Henninghausen, 1994).

In this present work, we explore the hypothesis that macrophages are required for ductal morphogenesis in postnatal mammary gland development. We present evidence consistent with a mechanism by which CSF1-dependent macrophages are essential for the epithelial ductal outgrowth of postnatal mammary gland development, surprisingly in collaboration with eotaxin-dependent eosinophils, the presence of which had not previously been documented in normal mammary tissue.

**MATERIALS AND METHODS**

**Mice**

Osteopetrotic (Csfrm1+/Csfrm1−) (Marks and Lane, 1976; Wiktor-Jedrzejczak et al., 1990) and littermate (+/Csfrm1−) control mice were obtained from +/Csfrm1− female × Csfrm1+/Csfrm1− male crosses and maintained in a barrier facility at the Albert Einstein College of Medicine as described by Pollard et al. (1994). Offspring of the Csfrm1+/Csfrm1− genotype were identified by the absence of incisors at postnatal day 10 of age. These mice were maintained on a diet of powdered chow ad libitum and infant milk formula (Enfamil, Mead Johnson and Company, Evansville, IN). Animals undergoing CSF1 treatment received from day 2 a daily subcutaneous injection of 106 Units of human recombinant CSF1 (hrCSF1; a generous gift from Chiron Corp., Emeryville, CA), in 0.05 ml 0.9% saline as described by Coccini et al. (1994). Eotaxin-deficient mice were maintained on an inbred 129/SvEv background (Rothenberg et al., 1997). Control 129/SvEv wild-type mice were age- and sex-matched, and mated under identical pathogen-free conditions in a barrier facility at the Albert Einstein College of Medicine. To assess cell proliferation, mice were given a single intraperitoneal injection of bromodeoxyuridine (BrdU), 2 hours before killing. All studies were performed under NIH guidelines for the care and treatment of experimental laboratory rodents.

**Whole-mount mammary gland preparation**

The fourth (abdominal) mammary glands were surgically removed, stretched onto a glass slide and fixed in 75% ethanol/25% acetic acid overnight. They were then washed in 70% ethanol, washed 5 minutes in distilled water, and stained overnight in an alum carmin solution (Sigma, Chemical, St Louis, MO). The following day, mammary glands were dehydrated through a graded series of ethanol solutions, defatted in toluene (Sigma Chemical, St Louis, MO) and stored in methyl salicylate (Sigma Chemical, St Louis, MO). Mammary gland whole-mount preparations were measured from the nipple area to the tip of the 3 longest ducts through the lymph node to determine ductal lengths (in mm). Numbers of branches represent the mean of branching number along the 3 longest ducts from the nipple area to the migration front. Terminal end buds (TEB) were counted in the whole mammary gland. Statistical evaluations were performed with a two-tailed Student’s t-test.

**Animal irradiation and bone marrow transplantation**

The whole bodies of +/Csfrm1− mice were irradiated at day 19 of age (γ-irradiation, 700 rad, 80.7 rad/minute). Mice were killed 10 days after irradiation, and mammary gland development examined as described above. Blood was collected by suprapubic bleeding under metofane anesthesia. Erythrocytes were lysed by adding 10 ml of NH4Cl buffer (150 mM) and after washing in phosphate buffered saline (PBS: 9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, pH 7.4), leukocytes were resuspended in 1 ml PBS and counted using a haemocytometer chamber. Bone marrow transplantation was performed 2 hours after irradiation. Bone marrow cell suspensions were prepared from femurs of 5 +/Csfrm1− control mice. Marrow was flushed into sterile PBS, placed on ice and aspirated through a 25G needle to ensure a single cell suspension before injection of 5.7x106 cells in a 200 μl volume via the lateral tail veins.
Histology and immunohistochemistry

Whole-mount mammary glands were fixed with formalin overnight onto a glass slide and then paraffin wax embedded. 5 μm sections were immunostained with the anti-F4/80 rat monoclonal antibody (Caltag Laboratories, Burlingame, CA) against a murine macrophage-restricted cell surface marker (Austyn and Gordon, 1981) and developed using a peroxidase detection kit (Vector Laboratories, Burlingame, CA) as described by Cecchini et al. (1994). F4/80+ cells associated with TEB areas were counted in a square unit of surface with an area of 0.01 mm². The mean of F4/80+ cells/surface unit was calculated from 3 TEBs per mammary gland sections from 3 mice in each group. Similarly, macrophages and eosinophils were differentially counted per surface unit around TEBs after the anti-F4/80 immunostaining followed by a light hematoxylin/aqueous eosin Y counterstaining. Eosinophils were recognized by their pink cytoplasmic granules and their segmented nucleus. Anti-Mac-3 and CSF1R (Upstate Biotechnology Incorporated; Lake Placid, NY), B220 and Gr1 (Pharmingen, San Diego, CA) immunohistochemistry were performed similarly, except that an incubation for 3 hours at 37°C was performed with CSF1R antibody. The anti-mouse CSF1R antibody was purified for IgG using a protein A sepharose (Sigma, St Louis, MO) chromatographic column prior to immunostaining. The cell proliferation kit from Oncogen Science was used for BrdU immunostaining.

RIA for serum estrogen levels

Serum samples were subjected to RIA analysis for E2 using the Active Estradiol kit from Diagnostic Systems Laboratories, Inc. (Webster, TX).

Estrogen implant transplantation

Mice were ovariectomized at 3 weeks of age, 4 days prior to estrogen implant transplantation. Silastic implants (Cohen and Milligan, 1993) containing 2 μg/ml E2 in peanut oil (Sigma, St Louis, MO) were inserted subcutaneously into the back of mice. This dose was chosen since it completely restores control mammary gland development in ovariectomized mice. Mice were killed 2 weeks later and their mammary gland development analyzed.

RNA preparation and northern blot hybridization

Total RNA from mammary glands of at least 3 mice from each group was isolated by the method of Chomczynski and Sacci (Chomczynski and Sacci, 1987). For positive and negative controls for CSF1R transcripts, total RNA was isolated from the macrophage cell line, BAC.1.2P5, and mouse L-cells, respectively (Morgan et al., 1987). Ten μg of total RNA was separated by formaldehyde-agarose gel electrophoresis, transferred to nylon filters, and probed with a [32P]dCTP-labeled cDNA probe for the CSF1R (Arceci et al., 1989) and eotaxin (ATCC #1463042; GenBank, AA711712) using the methods described by Arceci et al. (1989).

RESULTS

Macrophages and eosinophils home to the developing mammary gland

Immunohistochemistry with a battery of antibodies specific for different hematopoietic lineages was used to analyze the population of hematopoietic cells in the developing mammary gland. The monoclonal antibody anti-F4/80 was used to examine the population of mononuclear phagocyte cells (Austyn and Gordon, 1981). F4/80-positive (F4/80+) cells were found preferentially around terminal end buds (TEBs), the highly proliferative epithelial structures, which are the initial structure for ductal elongation. Surprisingly, on close histological inspection, F4/80+ cells consisted of both...
macrophages and eosinophils. Double staining of the same TEB section with Hematoxylin/Eosin (H/E) (Fig. 1A-C) and subsequently with anti-F4/80 antibody (Fig. 1D-F) revealed this cross-reactivity. Eosin-positive granules containing polymorphonuclear eosinophils (empty arrowhead, Fig. 1B,C) were stained with the anti-F4/80 antibody (empty arrowhead, Fig. 1E,F), as well as the mononuclear macrophages (filled arrowhead, Fig. 1E,F). This cross-reactivity has not been previously observed in tissues, most likely due to the paucity of tissue eosinophils in mice under normal conditions. Nevertheless, previous studies, using flow cytometry analysis, have indicated that bone marrow and peritoneal eosinophils cross-react with the anti-F4/80 antibody (McGarry and Stewart, 1991). High power photomicrographs show distinct morphologies of F4/80+ macrophages and eosinophils and hence indicate their distinct and common locations around TEBs. Macrophages displayed a large and quite round single nucleus (mononuclear cell) within a spread cytoplasm. They were mainly distributed at the neck of the TEB (Fig. 1E) and were generally not found associated with ductal structures distal to the TEBs. A unique location for macrophages within the TEB was also found (Fig. 1G). In this case, macrophage cytoplasm included apoptotic bodies, suggesting strongly their role in phagocytizing dead epithelial cells that are found during the process of TEB outgrowth (Humphreys et al., 1996). The shape of the nucleus of eosinophils is segmented, often forming a ring, with a round and generally smaller cytoplasmic surface, and they are abundantly found around the head of the TEB (Fig. 1C,F). The identification of macrophages around TEBs was also confirmed by immunostaining using the anti-Mac-3 antibody (Fig. 1H). The cross-reactivity of Mac-3 with eosinophils has been reported to be very low (McGarry and Stewart, 1991), and polynuclear cells in mammary glands were indeed negative for Mac-3. Macrophage and eosinophil recruitment in the vicinity of TEBs coincided with TEB presence from about 3 weeks until 8 weeks of age, when these structures disappear and the fat pad is filled entirely with the epithelial tree.

F4/80 immunohistochemistry data also indicated the presence of macrophages and eosinophils in other locations in mammary gland throughout postnatal development. At 2 weeks of age, macrophages are the major population of the F4/80+ cells and colonize the nipple area, surrounding the quiescent epithelial ducts (Fig. 1I). In addition to their potential mammary vascular origin, F4/80+ cells most likely originate from two other places, the connective tissue adjacent to the fat pad and the lymph node localized in the middle of the mammary gland, consistent with intense F4/80 immunostaining in both these locations (not shown). The
connective tissue F4/80+ cells were mainly composed of macrophages, whereas the lymph node included both macrophages and eosinophils. Using other leukocyte-lineage markers such as B220 and Gr1, neither B cells nor neutrophils were detected in the vicinity of epithelial structures in the mammary gland. Nevertheless, the lymph node, as expected, contained many B cells and some neutrophils (not shown).

Our data indicate the occurrence of macrophage and eosinophil homing to the mammary gland throughout postnatal development, while their distinct location around TEBs suggests a potential role in epithelium development.

**Leukocytes are required for postnatal mammary gland development**

To test the hypothesis that leukocytes are important for the development of the mammary gland, we depleted hematopoietic precursors by whole body γ-irradiation of mice and analyzed mammary ductal outgrowth. A sub-lethal γ-irradiation (700 rads) was chosen to kill the highly proliferative and hence irradiation ultrasensitive hematopoietic progenitors without killing the mice within 10 days of irradiation. Mice were irradiated at 19 days of age, prior to TEB formation in the mammary gland. The depletion of leukocytes after 10 days of irradiation was confirmed by counting cells in the blood of mice (0.05178±0.031×10^6 cells/ml in 5 irradiated mice versus 1.687±0.69×10^6 cells/ml in 5 control mice, P=0.0015). A similar dramatic decrease in leukocytes was also found after only 5 days following irradiation (not shown). Ten days after irradiation, outgrowth of the branching tree was inhibited and a reduced rudimentary epithelial tree characterized by the absence of TEBs was found in these mice (Fig. 2A-1). In age-matched control mice that did not undergo irradiation, TEBs have reached the lymph node area (Fig. 2A-2). Importantly, 10 days after irradiation, mammary epithelial and stromal cell proliferation was not affected, consistent with a similar pattern of BrdU incorporation analyzed by BrdU immunohistochemistry in TEDs of the irradiated mice (Fig. 2A-3) or in TEBs of the non-irradiated mice (Fig. 2A-4) as well as in their stromal cells. Since estrogen is the major hormone involved in prepubertal-pubertal mammary gland development, a potential effect of irradiation on estrogen concentrations was tested. Serum estrogen levels were measured in mice 10 days after irradiation and in their age-matched non-irradiated mice. Their levels were similar (57.5±2.87 in irradiated mice n=8, versus 56.62±2.28 in non-irradiated mice n=5, two-tailed Student’s t-test). These data indicate that the causes of the ductal growth arrest in irradiated mice are neither defective epithelial growth nor defective ovarian function induced by irradiation.

In order to show that the impaired ductal outgrowth was due to leukocyte depletion in the mammary gland, we transplanted 6 irradiated mice with a bone marrow cell suspension derived from control mice on the same day as irradiation (day 19). 28 days after irradiation followed by transplantation, all the mice displayed proper ductal development (Fig. 2B-1), characterized by elevated numbers of TEB and branching relative to irradiated but non-transplanted mice (Fig. 2B-3-4). Out of 6 irradiated control mice, that had not undergone bone marrow transplantation, only 2 survived to 47 days of age. Both of these had a strongly impaired epithelial tree development (Fig. 2B-2) with a low branching number corresponding to the

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**Fig. 3.** Delayed and abnormal mammary ductal development in CSF1 null mutant mice. (A) Representative whole-mount preparations of mammary glands from +/-Csfmop (+/-, left panels) and Csfmop/Csfmop (-/-, right panels) virgin mice of 3, 4, 9 or 12 weeks of age as indicated. The photomicrographs of the entire fourth abdominal mammary gland were taken at the same magnification and show the atrophic development in Csfmop/Csfmop mice. Arrowheads indicate TEBs; NP is the nipple area and LN the lymph node of the mammary gland. (B) TEB number, branching number, ductal length and the relative ductal growth in the mammary glands of +/-Csfmop (squares) and Csfmop/Csfmop (triangles) mice. Mice were killed at 2.5 to 12 weeks of age, and the fourth abdominal mammary gland whole mounts were analyzed. Points represent mean±s.d. of at least three mice per time point.
undeveloped epithelial tree of 3 weeks old mice. Nevertheless, TEBs begin to form at the tips of ducts, consistent with the late restoration of leukocyte number in the blood that occurred in these mice (2.74±0.74·10^6 cells/ml). These results indicate that some endogenous hematopoietic progenitors escaped irradiation-mediated death and were still able to proliferate, and over a long time period restore blood leukocyte number.

In the blood of transplanted mice, the ongoing restoration of leukocyte number was confirmed 12 days after the irradiation/bone marrow transplantation. The leukocyte number was significantly greater in the group of transplanted mice (6.301±4.55·10^5 cells/ml) compared to the non-transplanted control mice (0.2812±0.297·10^5 cells/ml, \( P = 0.0169 \)). At this stage of recovery, 2 transplanted mice were killed to analyze their mammary gland development. Neither of them displayed TEB formation, and the ductal tree was still at its rudimentary state despite the partial rescue of leukocytes. 4 weeks after bone marrow transplantation, the ductal outgrowth rescue in transplanted mice was associated directly with a restoration of leukocyte number in blood (1.23±0.62×10^6 cells/ml), and a dramatic recruitment of F4/80+ cells in the vicinity of TEBs (Fig. 2C, left panel). In contrast, in irradiated mice F4/80+ cells were absent around the TEDs of the underdeveloped epithelial tree (Fig. 2C, right panel). No immunostaining was detected with a B lymphocyte marker (B220) or a neutrophil marker (Gr1) around epithelial ducts and TEBs in transplanted mice (not shown).

The restoration of mammary gland development upon rescue of the leukocyte population in the mammary gland provides evidence for the necessity of leukocytes, and more precisely the F4/80+ macrophages and eosinophils, in ductal outgrowth. To test this hypothesis further, we used two mouse models deficient in one or the other cell types, and analyzed mammary gland development.

**Deficiency in the macrophage growth factor CSF1 results in abnormal postnatal mammary gland development**

In order to investigate the role of macrophages during postnatal

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**Note:** The diagram and table (Fig. 4) are not transcribed due to their complex visual content.
mammary gland development, the effect of a recessive null mutation in the CSF1 gene (Csfmop) on this development was analyzed. CSF1 is the major growth factor required for macrophage proliferation, differentiation and recruitment. Consequently Csfmop/Csfmop mice display reduced macrophage numbers in most tissues (Pollard and Stanley, 1996). To determine the effect of this macrophage deficiency, we examined mammary ductal outgrowth in Csfmop/Csfmop and +/+ control mice by analyzing whole-mount preparations of the fourth abdominal mammary glands from 2.5 to 12 weeks of age (Fig. 3). The heterozygous +/+Csfmop mice were chosen as control mice since the serum level of CSF1 was similar in these mice to those in +/+ wild-type mice, and no difference in macrophage density could be detected in any tissues studied (Pollard and Stanley, 1996).

TEB formation was delayed in Csfmop/Csfmop mice (Fig. 3). At 2.5 weeks of age, mammary glands of control and mutant mice displayed the same rudimentary branching tree (not shown). In control mice, TEBs first appeared at 3 weeks and increased in number reaching a peak at 5 weeks of age when the fat pad was the widest in the lymph node area, followed by a decline until they have disappeared at 9 weeks of age. However, in mutant mice, the onset of TEB formation was delayed by one week, and although also increasing to a peak at 5 weeks, the number was reduced over 2.5 fold compared to control mice. In addition, certain major ducts in mutant mice displayed a tapered end compared to the TEBs that end all ducts at the epithelial migration front in control mice. Consequently, the mammary gland of Csfmop/Csfmop mice has fewer ducts than in control mice.

The more extensive ductal tree in the mammary gland of +/+Csfmop mice compared to mutant mice was shown by a consistently greater branching number and ductal length (Fig. 3B) as well as a larger fat pad size throughout postnatal development (Fig. 3A). Between 8 and 9 weeks, the ductal tree of +/+Csfmop mice has filled the whole fat pad, TEBs have disappeared and some secondary ducts resulting from the hormonal estrus cycle influence were formed. In contrast, the ductal tree was still growing in Csfmop/Csfmop mice at 9 weeks and some TEBs were present. In addition, the orientation of ducts in mutant mice was disorganized. Ducts did not always migrate towards the tip of the fat pad, but instead they were curved and migrated in a lateral or opposite direction (not shown). Finally at 12 weeks of age, the ductal tree of Csfmop/Csfmop mice almost filled the atrophic fat pad, nevertheless secondary branches remained rare compared to the well-branched epithelial tree of control mice. To ensure that the atrophic epithelial tree in mutant mice was not only due to the result of a small fat pad, we measured the ductal growth relative to the growth of fat throughout postnatal development (Fig. 3B). In both control and mutant mice, this ratio tends to converge to one, indicating that the ductal tree reaches the tip of the fat pad. However, the ductal growth relative to the fat growth from 3 weeks to 11 weeks of age was significantly greater in control mice than mutant mice. This difference was particularly noticeable at 3-4 weeks. At this stage, the ductal outgrowth was delayed in mutant mice. Only a few TEBs were seen and the increase in ductal length or branching number has not yet occurred. These data confirm that the defective ductal growth is not only the result of a smaller fat pad but also of a
defect in epithelial tree development. Taken together, these data show a delay in mammary gland development in CSF1 null mutant mice that leads to the formation of an abnormal atrophic adult mammary gland, characterized by fewer ducts and minimal branches.

**CSF1-dependent macrophage recruitment is correlated with the formation of branches and TEBs**

Since CSF1 is the major growth factor for macrophages, we next addressed the question of whether the defective ductal tree in Csfmop/Csfmop mice was indeed associated with a macrophage population deficiency in the mammary gland. We analyzed macrophage distribution during prepubertal mammary gland development in the CSF1 null mutant mice and their littermate controls, by performing immunohistochemistry with the anti-F4/80 antibody. Since the anti-F4/80 antibody cross-reacts with eosinophils, we distinguished eosinophils from macrophages at high power magnification (1000x) by their nuclear and cytoplasmic shape as previously described (Fig.1), and by counterstaining with H/E to highlight the characteristic eosin-positive cytoplasmic granules of eosinophils.

Very early in postnatal development of the mammary gland, at 2.5 weeks of age, macrophages were the main F4/80+ cells and they were spread abundantly around the nipple area in +/Csfmop mice (Fig. 4A-1). In contrast, F4/80+ cells in Csfmop/Csfmop mice are almost completely absent (Fig. 4A-2). At 3 weeks of age, when the first TEBs appear in +/Csfmop mice, F4/80+ cells were recruited around all TEBs at the density of 12.94±0.46 cells/surface unit (Fig. 4A-3,B). In contrast, in Csfmop/Csfmop mice, very few F4/80+ cells were found around the rudimentary tips of ducts that, at this time, have not yet formed into TEBs (Fig. 4A-4,B). At 5 weeks of age, the density of total F4/80+ cells associated with TEBs in control mice increased significantly (19.67±2.43 versus 12.94±0.46 cells/surface unit at 3 weeks of age, p=0.0092) (Fig. 4A-5,B). Interestingly, at this later age, F4/80+ cells are also recruited around the TEBs that eventually develop in Csfmop/Csfmop mice (Fig. 4A-6,B). In both genotypes, approximately 50% of F4/80+ cells are macrophages, while the other 50% are eosinophils (Fig. 4B). However, the total F4/80+ cell density surrounding TEBs remains significantly lower in mutant mice compared to control mice (10.33±0.979 in Csfmop/Csfmop mice versus 19.67±2.43 cells/surface unit in +/Csfmop mice, P=0.0074). Of these F4/80-positive cells, both macrophage and eosinophil numbers were significantly reduced in mutant mice compared to control mice. These data indicate that macrophage and eosinophil recruitment is closely related to TEB formation, and their total number is significantly reduced in Csfmop/Csfmop mice.

**Restoration of macrophage density rescues the TEB and branching defect in Csfmop/Csfmop mice**

In order to determine whether CSF1-dependent macrophages are necessary for ductal outgrowth, we treated mutant mice from birth with a daily injection of 106 IU of human recombinant CSF1. This dose was previously designed to at least maintain circulating concentrations of CSF1 (Cecchini et al., 1994). Following daily CSF1 treatment, we examined mammary gland development in Csfmop/Csfmop mice at 5 weeks of age. Although the total number of F4/80+ cells surrounding TEBs did not reach the number in untreated control mice (11.92±1.33 versus 19.67±2.43 cells/surface unit), the macrophage number in the CSF1-treated Csfmop/Csfmop mice was completely restored to the untreated control level (9.15±0.95 versus 9.25±1.5 cells/surface unit) (Fig. 4B). Most of the F4/80+ cells in CSF1-treated mutant mice were identified as macrophages, the numbers of eosinophils being decreased and significantly different to the macrophage numbers (P<0.026; Fig. 4B).

In parallel to the determination of macrophage number, the right abdominal mammary gland whole mounts were analyzed as described in Fig. 3. CSF1 treatment rescued the TEB number and the branching number in Csfmop/Csfmop mice (Fig. 4C), consistent with the rescue of macrophage density around TEBs (Fig. 4B). Ductal length remained low and did not reach the levels seen in +/Csfmop mice, although it significantly increased compared to the untreated mutant mice. Similarly, the fat pad size was still reduced in Csfmop/Csfmop mice after CSF1 treatment (not shown), suggesting a close correlation between the control of fatty stroma size and ductal length.

CSF1R-bearing cells were identified following immunohistochemistry using an anti-CSF1R antibody (Fig. 5A). The CSF1R-expressing cells were mononuclear cells displaying a spread cytoplasmic shape with a similar distribution to F4/80+ macrophages in the mammary gland. Epithelial and other stromal cells including eosinophils were consistently negative. In +/Csfmop mice, the CSF1R-bearing macrophages were abundant around TEBs, in the lymph node, as well as in the connective tissue surrounding the fat pad (Fig. 5A). These immunohistochemistry results confirm the distribution of F4/80+ macrophages in the mammary gland and discriminate them further from eosinophils. In Csfmop/Csfmop mice, CSF1R protein was expressed on the few macrophages localized around TEBs (Fig. 5A) and in lymph node (not shown), but CSF1R immunostaining was virtually absent in the connective tissue adjacent to the fatty stroma (Fig. 5A), consistent with the data from F4/80 immunostaining. In Csfmop/Csfmop mice, treated with CSF1 from birth, CSF1R-bearing macrophages were found around the TEBs and were recruited to the border between the fatty stroma and connective tissue (Fig. 5A).

Analysis by northern blotting experiments of total mammary gland RNA from 5-week-old mice showed a significant level of 4.0 kb CSF1R transcripts in +/Csfmop mice but a barely detectable level in Csfmop/Csfmop mice (Fig. 5B). This is the same size transcript as determined for a macrophage cell line (BAC 1.2F5) shown to express the CSF1R (Morgan et al., 1997), it was necessary to ensure that the defective ductal outgrowth was not due to a perturbation of estrogen production.
Leukocyte regulation of postnatal mammary gland

Direct measurements of estradiol-17β (E2) were performed by RIA in Csfmop/Csfmop (-/-) mice and their littermate control +/+ Csfmop mice (+/+) from 2 to 8 weeks of age (Fig. 6A). Mutant and control mice showed similar increased levels of estrogen through prepuberty and puberty. Furthermore, in order to analyze the epithelial growth response to physiological concentrations of estrogen, we transplanted E2 implants (2 μg/ml) subcutaneously in 3-week-old ovariectomized mutant and control mice. Two weeks after ovariectomy, the ductal epithelial tree resembled the rudimentary tree seen before 3 weeks of age in both +/+ Csfmop and Csfmop/Csfmop (-/-) mice, confirming the major role of estrogen in postnatal epithelial growth (Fig. 6B-1,-2). The restoration of estradiol-17β by implants in ovariectomized mice rescues completely the development in +/+ Csfmop mice (Fig. 6B-3). In Csfmop/Csfmop mice however, although the ductal outgrowth was rescued, the defective development was still apparent (Fig. 6B-4). Consequently, defective estrogen production cannot be the explanation for the relative failure of ductal outgrowth observed during postnatal development in Csfmop/Csfmop mice. However, the extended estrus cycles may account for the relative lack of secondary branching observed in adult Csfmop/Csfmop mice.

Altogether, these data provide evidence for a CSF1-dependent macrophage requirement in TEB and branching development, with these cells being recruited around the growing ducts of the mammary gland.

**Eotaxin is necessary and sufficient for eosinophil recruitment around TEBs**

Expression analysis in the mammary gland of chemokines involved in leukocyte homing indicated a high level of eotaxin transcripts in mammary glands of control mice during postnatal development. Eotaxin is a powerful and specific attractant for eosinophils inducing their local mobilization into tissue (Baggiolini et al., 1997). Around 3 weeks of age, eotaxin transcript levels in control mice were low but they increased at 5 weeks of age (Fig. 7A), consistent with the increased number in prepubertal and pubertal mice. Direct measurements of estradiol-17β (E2) were performed by RIA in Csfmop/Csfmop mice (-/-) and their littermate control +/+ Csfmop mice (+/+) from 2 to 8 weeks of age. Points represent mean±s.e.m. of 5 (+/+) and 10 (-/-) mice per time point. (B) Representative whole-mount preparations of mammary glands from +/+ Csfmop (1,3) and Csfmop/Csfmop (2,4) after ovariectomy only (1,2) or 2 weeks after subcutaneous E2 implant transplantation following ovariectomy (3,4).

**Fig. 6.** Normal estrogen response in mammary glands of Csfmop/Csfmop mice. (A) Similar levels of serum E2 in control +/+ Csfmop (+/+) and Csfmop/Csfmop (-/-) mice from 2 to 8 weeks of age. Points represent mean±s.e.m. of 5 (+/+) and 10 (-/-) mice per time point. (B) Representative whole-mount preparations of mammary glands from +/+ Csfmop (1,3) and Csfmop/Csfmop (2,4) after ovariectomy only (1,2) or 2 weeks after subcutaneous E2 implant transplantation following ovariectomy (3,4).

**Fig. 7.** Eotaxin is sufficient and necessary for eosinophil recruitment around TEBs in mammary glands. (A) Eotaxin mRNA transcript levels were measured in mammary glands of control mice at 3 and 5 weeks of age. Northern blots containing 10 μg total mammary gland RNA per lane from 3 mice per age group was probed with a radiolabeled cDNA encoding eotaxin. Uniformity of RNA loading is shown by ethidium bromide staining of 28S rRNA (bottom panel). (B) Longitudinal sections of TEBs from control (+/+ and eotaxin-deficient (-/-) mice, 5 weeks old, stained lightly with Hematoxylin/Eosin. Right panels are high power views of boxed area in the left panels. Note the abundant eosinophil population in the vicinity of TEBs in the control mice, whereas they are absent in the eotaxin -/- mice. Original magnification: left panels, 400 x, right panels 1000 x. Arrowheads indicate eosinophils identified by their eosin-pink cytoplasmic granules. (C) Quantification of eosinophils per surface unit around TEBs in control and eotaxin -/- mice at 5 weeks of age. Note that eosinophils are rarely seen around TEBs in eotaxin -/- mice and they represent only 2% of the normal population in control mice. (**P<0.0001, two-tailed Student’s t-test).
Fig. 8. The lack of eosinophils around TEBs leads to defective branching morphogenesis in eotaxin −/− mice. (A) Representative mammary gland whole mounts from control (+/+) and eotaxin-deficient (−/−) mice. The photomicrographs in the upper panels were magnified to show the lack of TEBs at the tip of certain major ducts in eotaxin-deficient mice (lower panels). Filled arrowheads indicate TEBs and empty arrowheads the terminal end ducts (TEDs) seen in eotaxin −/− mice. (B) Branching number, TEB number and ductal length were determined in 5 control (+/+ ) and mutant (−/−) mice at 5 and 7 weeks of age as described previously. Histograms represent means±s.d. of 5 mice per time point. (Significant variations from values obtained for eotaxin −/− mice. *P=0.027, ***P=0.0006).

DISCUSSION

It is well established that epithelial/mesenchymal interactions are important for postnatal development of the mammary ductal tree and its differentiation during pregnancy into a milk producing structure (Robinson et al., 1999). The mesenchyme contains a heterogeneous group of cells (Sakakura et al., 1982) and several of these, such as fat cells and fibroblasts, are capable of producing factors that can promote the growth of epithelial cells (Berdichevsky et al., 1994; Carrington and Hosick, 1985; Enami et al., 1983; Levine and Stockdale, 1984). In this study, we identified two migrant stromal cells recruited around the growing ducts, macrophage and eosinophil, and show that these cells play an important role in the development of the mammary gland.

Leukocytes are necessary for ductal outgrowth

Detailed immunohistochemical analysis using antibodies to hematopoietic-specific markers revealed two cell types, macrophages and eosinophils, whose recruitment to the postnatal mammary gland closely paralleled the formation and outgrowth of TEBs. These cells were found in different but also overlapping positions around the TEBs, with eosinophils mostly around the cap, and macrophages along the shaft immediately adjacent to the TEB. TEB formation is essential for ductal elongation in mammary gland development, since the undifferentiated cells located in the outer layer of the TEB structure, known as ‘cap cells’, are able to give rise to the intermediate, luminal and myoepithelial cells of the advancing ducts (Pitelka and Hamamoto, 1977; Williams and Daniel, 1983). The TEBs also divide into heart shaped structures from the ductal outgrowth, therefore we next explored the presence of macrophages in mammary glands of eotaxin −/− mice. Interestingly, the number of macrophages in the vicinity of TEBs in eotaxin −/− mice was not modified significantly compared to those in control mice (12.05±1.5 cells/surface unit in 4 mutant mice versus 9.3±2.1 cells/ surface unit in 3 +/+ mice).

Lack of eosinophil recruitment around TEBs results in aberrant branching morphogenesis

The eotaxin null mutant mice constitute a very useful mouse model for studying the role of eosinophils in mammary ductal outgrowth without interfering with macrophage functions. To determine this role, we analyzed mammary gland whole mounts of 5- and 7-week-old eotaxin −/− mice lacking eosinophils around ducts and their age-matched background controls. Branching numbers were significantly reduced in eotaxin −/− mice, although ductal length was not affected (Fig. 8A,B). The abnormal branching was associated to some extent with a TEB defect in eotaxin −/− mice (Fig. 8B). TEB numbers were not significantly different between the control and mutant mice at 5 weeks of age, but by 7 weeks of age, eotaxin −/− mice displayed a significantly reduced number of TEBs. This defect in TEB formation is illustrated in Fig. 8A, where some ducts do not display the large club-shape TEB at the tip of each major duct seen in control mice, but rather they have thin under-developed end buds. Thus, the absence of eosinophils around TEBs results in a reduction in branching number and defective TEB formation.
Exposure to irradiation (Radford and Murphy, 1994). In our mouse model, a single dose of 800 rad does not induce epithelial cell death in mice. This is consistent with previous work showing that X-ray irradiation does not affect the number of mammary gland epithelial cells in the blood 5 days after irradiation. At this dose, we did not detect any effects on mammary gland epithelial cell proliferation 10 days after irradiation, as assessed by the incorporation of BrdU in irradiated compared to non-irradiated mammary glands. The division-doses up to 800 rad do not induce epithelial cell death of mouse mammary gland epithelial cells.

Removal of the hematopoietic system by γ-irradiation results in a dramatic curtailment of ductal development; TEBs are completely absent at the tips of the ducts and the mammary gland retains a neonatal appearance. To confirm that this effect on mammary development was due to leukocyte depletion, syngeneic bone marrow was transplanted into mice to specifically reconstitute the leukocyte population. This restoration was complete in that the circulating leukocyte numbers returned to those of normal mice and the lymph nodes in the mammary gland regained their cellularity. This treatment also restores completely mammary gland development such that all the treated mice develop to the pubertal state 4 weeks after transplantation. This data also indicates that the mammary epithelial cells are unaffected by irradiation and thus, in itself, this could not be the cause of the interrupted development. Since estrogen is the major hormonal component of the postnatal mammary gland development, we confirmed that irradiation has no impact on serum estrogen levels. The restoration of ductal growth coincides with the recruitment of macrophages and eosinophils, but not other hematopoietic cells (neutrophils, B-lymphocytes), around the TEBs. These data strongly suggest that these two cell types play an essential role in ductal morphogenesis.

Recruitment of macrophages and eosinophils to the mammary gland requires CSF1 and eotaxin respectively

To test the specific involvement of macrophages and eosinophils in mammary gland development, we turned to mice carrying null mutations in the genes for CSF1 and eotaxin (Marks and Lane, 1976; Rothenberg et al., 1997; Wiktor-Jedrzejczak et al., 1990; Yoshida et al., 1990). These cytokines are targeted specifically to macrophages and eosinophils respectively, and their absence results in tissues that are depleted in the respective cell type.

CSF1 has been identified both in vitro and in vivo studies as the major growth factor regulating cells of the mononuclear phagocytic lineage (Stanley et al., 1994). Consequently, most but not all tissues of mice homozygous for a null mutation in the CSF1 gene are significantly depleted of macrophages (Cecchini et al., 1994; Naito et al., 1991). Despite the extensive analysis of tissues from $Csfm^{op}/Csfm^{op}$ mice, the mammary gland has never been examined. In control mice (+/+Csfm$^{op}$ mice), macrophages are recruited around the rudimentary ductal tree at 2.5 weeks of age, before TEB appearance. At 3 weeks of age, when TEBs are first discernable, macrophages become abundant around the growing TEBs, where they persist until development is complete. In contrast, in the absence of CSF1, macrophages are almost completely missing from the mammary gland of $Csfm^{op}/Csfm^{op}$ mice prior to TEB formation. At 5 weeks of age, even though macrophages are found around TEBs, their density is significantly reduced. These data show that the macrophages in the mammary gland stroma are largely CSF1 dependent, and in the absence of CSF1, are severely depleted.

CSF1 acts through a single high-affinity tyrosine kinase transmembrane receptor that is the product of the $c-fms$ proto-oncogene and expressed on all cells of the mononuclear phagocyte lineage (Sherr et al., 1985). In human, it is expressed...
in pregnant and lactating mammary epithelial cells (Sapi et al., 1998), as well as in breast tumor cells (Scholl et al., 1994). Therefore, it was important to determine the cell type(s) expressing CSF1R in the mouse mammary gland during development. Our immunohistochemical data shows that only macrophages express CSF1R, excluding expression in epithelial cells and eosinophils, the latter of which have been previously shown to be negative for CSF1R (Byrne et al., 1981). Consequently, macrophages are the exclusive targets for CSF1 signaling in mammary gland during postnatal development. Our findings of a lack of expression in the mammary epithelium in mice confirm other work showing that non-lactating human mammary epithelium does not express detectable levels of CSF1R (Kaciniski et al., 1991; Sapi et al., 1998). Consistent with this restricted expression of CSF1R to macrophages, CSF-1R transcripts are barely detectable in mammary glands of Csfmop/Csfmop mice compared to control mice. In addition, CSF1R transcript levels are strongly induced in mutant mice after CSF1 treatment, corresponding to the resultant increased macrophage recruitment into the mammary gland.

Eotaxin is a powerful chemoattractant for eosinophils but not for mononuclear cells or neutrophils. (Garcia-Zepeda et al., 1996; Sabroe et al., 1999). It acts to mobilize eosinophils and their progenitors from bone marrow into the blood, and their subsequent recruitment into sites of allergic inflammation (Baggiolini et al., 1997; Ganzalo et al., 1996; Palframan et al., 1998; Rothenberg et al., 1997). Beside its role in inflammation, eotaxin is also required for maintaining the physiological baseline trafficking of eosinophils in the jejunum during healthy states (Matthews et al., 1998). Eotaxin mRNA has been previously detected in the mammary gland (Rothenberg et al., 1995), and in our study, eosinophil recruitment to the mammary gland was co-incident with a significant elevation in eotaxin mRNA transcript levels. In mammary glands of mice homozygous for a null mutation in the eotaxin gene, the number of eosinophils around the TEBs dropped to only 2% of the level found in control mice. These data indicate that eotaxin is the major, if not the only, factor required for eosinophil recruitment around the TEBs during ductal outgrowth.

Macrophage and eosinophil functions are required for branching morphogenesis

Having established that mutations in the CSF1 or eotaxin genes significantly deplete the mammary gland populations of macrophages or eosinophils respectively, we analyzed the effects of these depletions upon postnatal mammary gland development. In CSF1 null mutant mice (Csfmop/Csfmop), analysis of mammary gland development showed a severe defect in TEB formation. The TEB number throughout postnatal development was strongly affected, being reduced 2.5 fold in mutant mice at 5 weeks of age when the TEB number in control mice was at its highest level. In addition, there was a delay of 1 week in the onset of TEB formation. Furthermore, the development of the full ductal tree in Csfmop/Csfmop mice is delayed. TEBs persist until 12 weeks of age at which time the fat pad is eventually filled, compared to the 8 weeks it takes in wild-type mice (+/Csfmop mice). The defect in TEB formation and branching pattern leads to a poorly developed atrophic adult gland in Csfmop/Csfmop mice. This is consistent with previous observations that showed limited outgrowth of the ductal tree during pregnancy leading to a dense, atrophic mammary gland (Pollard and Hennighausen, 1994). This branching defect during development is directly related to the recruitment of macrophages, since daily CSF1 treatment rescues the branching number in parallel with the rescue of macrophage population around the TEBs.

The data indicate functions for macrophages in ductal development, possibly acting through their well-documented roles in supplying trophic factors for epithelial cell growth or matrix remodeling but also through their phagocytic activity (Horton, 1996). In fact, a unique location for macrophages inside the body of well-formed TEBs was systematically found in control mice and these cells were absent in Csfmop/Csfmop mice. In this particular case, apoptotic bodies were included in the macrophage cytoplasm, suggesting strongly a role in phagocytosing apoptotic epithelial cells during ductal development. Indeed, epithelial apoptosis is a well-established mechanism required for proper ductal morphogenesis (Humphreys et al., 1996). Because macrophages engulfing apoptotic cells are often restricted to the proximal area to the lumen, macrophages might participate in the lumen formation which requires epithelial apoptosis at similar locations within the TEB (Hogg et al., 1983; Humphreys et al., 1996). Beside their phagocytic functions of apoptotic cells, macrophages are also able to themselves induce apoptosis in normal cells in vivo, for example during the programmed capillary regression of the pupillary membrane (Diez-Roux and Lang, 1997). Consequently the absence of this particular macrophage function might in part explain the compromised ductal outgrowth. The specific localization of macrophages to the neck of TEBs also suggests that these cells regulate ductal outgrowth through their ability to produce proteases and angiogenic or growth factors (Polverini, 1997; Welsgus et al., 1992).

CSF1 mRNA is detected at only very low levels in the mammary gland (not shown) suggesting that the depletion of macrophages in the Csfmop/Csfmop mouse is due largely to the low number of circulating monocytes. To restore the monocyte population, we gave daily injections of human recombinant CSF1 in a regimen that has been shown previously to restore circulatory concentrations (Cecchini et al., 1994). This treatment corrects the circulating monocyte number and the macrophage population in most tissues. The mammary gland appears to fall mainly into this category since the number of macrophages returns to normal after CSF1 injections. Nevertheless, we cannot exclude the action of another macrophage chemoattractant, since some macrophages are still found in the mammary gland of un-treated Csfmop/Csfmop mice, although their recruitment is delayed. CSF1 treatment also resulted in the rescue of TEB and branching numbers in Csfmop/Csfmop mice. This rescue was partial because ductal lengths and fat pad sizes were still reduced in CSF1-treated Csfmop/Csfmop mice. Two explanations for this incomplete mammary gland morphogenesis are possible. CSF1 is synthesized primarily as a glycosylated and proteoglycanated species, and this form is found abundantly in the serum (Price et al., 1992). Thus, reconstitution with a soluble bacterially synthesized recombinant form may be inefficient because it cannot be targeted appropriately to the mammary gland stroma.
Alternatively, the very low level of CSF1 mRNA in the mammary gland, from 3-5 weeks of age, may be translated into locally available CSF1 that is required to correctly locate the macrophages or alter their local function to provide a trophic effect upon ductal length and fat pad growth.

The detection of eosinophils co-localizing with macrophages around TEBs was surprising. Eosinophils are generally regarded as cells recruited to tissue as a host defense against parasites or during allergic responses (Gleich and Adolphson, 1986; Rothenberg, 1998; Weller, 1991). They are also detected in a variety of tissues, where their functions are however not defined. For example, eosinophils reside normally in the jejunum and thymus of mice (Matthews et al., 1998), and in the uterus they are recruited abundantly to the stroma at estrus, under the influence of estrogen (Tchernitchin et al., 1974). Similarly, during postnatal development of the mammary gland eosinophils could be also recruited in response to estrogen that is required for TEB formation. They occupy a unique position around the head of TEBs. Because eosinophil recruitment in mammary glands is coincident with the onset of mammary gland development, we studied this process in eotaxin-deficient mice that lack eosinophils around TEBs. In these mice, the branching number was reduced significantly and TEB formation affected although to a lesser extent. However, in contrast to CSF1-deficient mice, ductal elongation was not defective in eotaxin−/− mice, indicating a more targeted role of eosinophils on the definition of the branching pattern. Prior to this study, physiological roles of eosinophils, besides the immune response, had not been documented. Our finding provides the first report of a beneficial role for eosinophils in a physiological process.

In summary, we provide evidence that macrophages and eosinophils constitute two novel stromal components of the mammary gland essential for the proper epithelial ductal development (Fig. 9). Macrophages are recruited around TEBs immediately upon their formation, most of them are CSF1−/− dependent, and they colocalize with eosinophils whose presence relies completely on the locally synthesized chemokine eotaxin. Depletion of these cells results in reduced numbers of TEBs and a ductal tree with significantly reduced branching complexity. These hematopoietic cells share common locations around TEBs but they preferentially locate to specific areas, suggesting both overlapping and distinct roles in ductal outgrowth. Macrophages are concentrated at the neck of TEBs where they may provide proteinases involved in the matrix remodeling upon ductal invasion into the fat pad, or other factors necessary for epithelial proliferation or angiogenesis. Macrophages localized in the middle of TEBs are involved in the phagocytosis of apoptotic epithelial cells that is essential for ductal outgrowth. Eosinophils are located mainly around the head of TEBs. They can secrete growth factors, for example TGFβ1, and actions of these factors could explain the role of eosinophils in determining the branching spacing. These data establish for the first time novel physiological and developmental functions for macrophages and eosinophils in the process of branching morphogenesis in the mammary gland.

We thank Dr Thomas Graf and Dr Todd Evans for their extremely helpful comments upon experimental procedures and the manuscript, as well as Dr Elaine Lin and Andrew Nguyen for their encouragement and helpful discussions. This work was supported by grants from the National Institutes of Health ROI# DK48960, from the Einstein Cancer Center grant P30-13330 and from USAMRMC DOD# 17-97-1-7153 and a DOD post-doctoral fellowship to Valérie Gouon-Evans. J. W. P is the Betty E. Feinberg Senior Faculty Scholar in Cancer Research.

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