Mobilization of Bone Marrow Stem Cells by Granulocyte Colony-Stimulating Factor Ameliorates Radiation-Induced Damage to Salivary Glands

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ABSTRACT

One of the major reasons for failure of radiotherapeutic cancer treatment is the limitation in dose that can be applied to the tumor because of coirradiation of the normal healthy tissue. Late radiation-induced damage reduces the quality of life of the patient and may even be life threatening. Replacement of the radiation-sterilized stem cells with unirradiated autologous stem cells may restore the tissue function. Here, we assessed the potential of granulocyte colony-stimulating (G-CSF)-mobilized bone marrow-derived cells (BMCs) to regenerate and functionally restore irradiated salivary glands used as a model for normal tissue damage.

Male-eGFP+ bone marrow chimeric female C57BL/6 mice were treated with G-CSF, 10 to 60 days after local salivary gland irradiation. Four months after irradiation, salivary gland morphology and flow rate were assessed.

G-CSF treatment induced homing of large number of labeled BMCs to the submandibular glands after irradiation. These animals showed significant increased gland weight, number of acinar cells, and salivary flow rates. Donor cells expressed surface markers specific for hematopoietic or endothelial/mesenchymal cells. However, salivary gland acinar cells neither express the G-CSF receptor nor contained the GFP/Y-chromosome donor cell label.

The results show that BMCs home to damaged salivary glands after mobilization and induce repair processes, which improve function and morphology. This process does not involve trans-differentiation of BMCs to salivary gland cells. Mobilization of BMCs could become a promising modality to ameliorate radiation-induced complications after radiotherapy.

INTRODUCTION

During radiotherapy, the most important dose-limiting factor is sensitivity of the normal tissue lying in the radiation field. Even with the most optimal radiation schedule, damage will still occur in normal tissues. For radiotherapeutic treatment of head and neck tumors, the salivary glands are one of the tissues at risk. Exposure of the salivary gland to radiation often results in a progressive loss of gland function within the first weeks of radiotherapy 154. The reduction in saliva flow rate and alteration of salivary composition may persist during the rest of the patient’s life, leading to complications, including oral dryness, nocturnal oral discomfort, increased risk for oral infections, dental caries, and difficulties in speech, which severely hamper the quality of life 3,4.

Radiation damage to normal tissue is often due to reduced functioning of the tissue stem cells that can no longer replace differentiated functional cells, resulting in loss of homeostasis. In the salivary gland, early gland dysfunction (0-10 days after irradiation) is evoked by disturbance of intracellular receptor-mediated signaling in differentiated functional acinar cells without significant cell depletion 16. At later time-points (10-60 days after irradiation), progressive loss of acinar cells is observed, which is associated with further decrease in saliva production. The lack of replenishment of these functional cells is thought to be due to the radiation-induced sterilization of endogenous stem cells. Replacement of salivary gland stem cells by stem cell therapy could potentially restore tissue homeostasis after radiation.

At present, there are no reliable methods available to obtain sufficient numbers of well-characterized salivary gland stem cells that could be applicable in such a stem cell therapy approach. Recently, however, bone marrow-derived cells (BMCs) were suggested to serve as a valuable source for the regeneration of damaged tissues 155. Homing and engraftment of BMCs in damaged non-hematopoietic organs such as vascular tissue 73, myocardium 78,156-163, brain 63,65, liver 66-69, kidney 74-76, lung 70,72,154,165 and skin 77 have been observed and were suggested to contribute to the wound healing process. In some tissues like myocardium 157,160-163,166, kidney 79 and liver 96, even improved function has been observed. These studies have provided the proof of principle of damage repair by the application of BMCs. A clinically attractive approach is to use granulocyte colony-stimulating factor (G-CSF) to mobilize bone marrow cells to the circulation. It has been reported that G-CSF is associated with improved cardiac function and survival after myocardial infarction in mice 157,160,166,167.

Many investigators use radiation as conditioning regimen for stem cell transplantation. However, the prevention/repair of therapeutic radiation-induced damage to organs has not been assessed.
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The salivary gland not only provides a unique model, but it is also clinically very relevant to prevent normal tissue toxicity after radiotherapeutic treatment of head and neck cancers. Because these side-effects are mainly caused by damage to the stem cells, a proof of principle in this area may also shed light on the general therapeutic use of stem cells. The architecture of the salivary gland is well known: cells can be readily identified morphologically, and precise function measurements in time have been developed and extensively used in our laboratory for both rats and mice. Thus, the exact time-points of loss of function, the accompanying morphological changes and the incapability of regeneration after stem cell depletion are well known.

In the current study, we assessed the capability of G-CSF-mobilized BMCs to regenerate and functionally restore irradiated salivary glands.

RESULTS

MOBILIZED BMCs HOME TO IRRADIATED SALIVARY GLANDS

To be able to identify bone marrow-derived cells (BMCs) after mobilization, the endogenous bone marrow of wild-type female mice was destroyed by a total body irradiation of 9.5 Gy (salivary glands were shielded) and replaced by male bone marrow isolated from eGFP transgenic mice (Fig. 1). Eight weeks later, mice with peripheral blood chimerism levels > 60% were selected and received an additional irradiation on the salivary glands. At 10, 30 or 60 days after gland irradiation, two doses of G-CSF given 3 days apart were used to induce circulation of BMCs in the blood. A clonogenic assay revealed that this mobilization protocol induced circulating levels of 10,000 granulocyte macrophage colony-forming units and about 1,000 hematopoietic stem cells per mL blood for at least 1 week.

One hundred thirty days after gland irradiation, the number of eGFP+ cells in the salivary glands was established. Figure 2 shows that the irradiated salivary gland in non-G-CSF-treated animals (Fig. 2A) contained few eGFP+ cells. About the same number of eGFP+ cells were found in unirradiated salivary glands of mice after G-CSF treatment (Fig. 2B). The highest level of eGFP+ cells was observed in irradiated glands of mice treated with G-CSF 30 days after irradiation (Fig. 2C-D). Quantification of the % of eGFP+ cells (Fig. 2E) revealed a low level of eGFP in all glands of animals which were not subjected to the combination of G-CSF treatment and salivary gland irradiation (Fig. 2E, left panel). However, a significant 5- to 8-fold increase in eGFP+ cells was observed in irradiated salivary glands of G-CSF-treated animals, irrespective of the time point of mobilization (10, 30 or 60 days after irradiation). For further analysis, these groups were all pooled and treated as one group.

These data show that BMCs home to the salivary gland specifically after irradiation. This effect can be strongly enhanced by mobilizing BMCs by G-CSF.

FIGURE 1. SCHEMATIC REPRESENTATION OF EXPERIMENTAL SETUP. Mice were total body irradiated excluding the salivary glands and transplanted with whole bone marrow of eGFP+ transgenic mice (middle left clockwise). Hereafter, the salivary glands were locally irradiated. At different time points, G-CSF was given, and several parameters were determined 90 to 130 days after irradiation.

FIGURE 2. HOMING OF BMCs TO IRRADIATED SALIVARY GLANDS. Confocal Laser Scanning Microscopy slides of submandibular gland 130 days after (sham)-irradiation. Similar amounts of eGFP+ cells can be seen in sham-irradiated, sham-mobilized (-/-, E), irradiated, sham-treated (+/-) (A,E), and mobilized, sham-irradiated mice (+/-) (B,E). In irradiated mobilized mice, a significant higher number of eGFP+ cells were detected (C-E), DAPI nuclear staining (blue) and eGFP expression (green). Original magnification, 200x (A-C) and 630x (D). eGFP+/DAPI+ cells were enumerated in 200 counted cells and standardized to a 100% detection rate of a eGFP+ normal salivary gland. Columns, mean. Bars, SEM. *, P < 0.05.
G-CSF INDUCED CHANGES IN MORPHOLOGY OF IRRADIATED SALIVARY GLAND

Macromorphological examination of glands, extirpated 130 days after radiation, revealed a 60% reduction in gland weight (Fig. 3B). In addition, the color of the gland was changed after radiation (Fig. 3A). When mice were treated with G-CSF after irradiation, gland weights were significantly increased and gland color was normalized. Although no significance difference in endothelial area was found between the irradiated/G-CSF-treated group and the non-G-CSF-treated or non-irradiated mice, morphological differences were observed. Blood vessels appeared somewhat larger after irradiation and G-CSF treatment, possibly resulting in an improved blood flow, observed as an improved gland color.

Histological analysis revealed that 130 days after irradiation, the number of acinar cells with remaining ducts was substantially reduced compared to normal gland tissue (Fig. 3C, a), and inflammatory aggregations were prevalent, consistent with previous studies 51,174 (Fig. 3C, d). Periodic Acid Schiff (Fig. 3C, b versus e) and Alcian Blue (Fig. 3C, c versus f) staining for mucins and mucopolysaccharides, respectively, showed the reduced presence of acinar cells. When present, these cells were often enlarged and disorganized and surrounded by ductal cells and fibrotic tissue. In animals that were irradiated and subsequently mobilized, however, the reduction in acinar cell number was less pronounced and the glands overall showed improved morphology (Fig. 3C, g, h, and i). Detailed quantitative analysis (Fig. 4A) confirmed a significantly higher acinar cell number in the pooled G-CSF-treated group.

These results imply that G-CSF treatment after irradiation improves micromorphology and macromorphology of the salivary glands, which coincides with homing of BMCs into the glands.

G-CSF ADMINISTRATION INCREASES SALIVARY GLAND FUNCTION

To examine whether the improved morphology after G-CSF treatment is associated with restored function, total salivary flow rates were measured. As expected, irradiation with a single dose of 15 Gy results in a severe drop of stimulated saliva secretion (3.7 ± 2.3 µL/15 minutes, Fig. 4B) when compared to control non-irradiated animals (194.2 ± 10.4 µL/15 minutes). Secretion increased significantly after gland irradiation and G-CSF treatment (21.1 ± 6.2 µL/15 minutes, P < 0.05), when compared with irradiated non G-CSF-treated animals. Only two irradiated, but otherwise untreated mice had a saliva yield of > 10 µL/15 minutes. In contrast, 17 of the 28 mice of the G-CSF treated group responded with a level higher than 10 µL/15 minutes of saliva secretion. These results indicate that G-CSF treatment increases the function of irradiated salivary glands of most animals.

FIGURE 3. IMPROVED MORPHOLOGY AFTER GLAND IRRADIATION AND MOBILIZATION OF BMCs. Salivary glands were photographed (A) and weighted (B) at 130 days after irradiation. An improved gland color and a significant increase in gland weight were observed after G-CSF treatment compared to irradiated gland. The salivary gland morphology (C) was visualized with a H&E (a, d, and g), PAS (b, e, and h) and Alcian Blue staining (c, f, and i), indicating tissue structure, the presence of mucin and mucopolysaccharides containing acini, respectively. Loss of acinar cells (*) with remaining duct cells (∆) appeared after irradiation (d-f) compared to a normal salivary gland (a-c). Irradiated G-CSF-mobilized mice revealed more acinar groups which were normal sized, as visualized by pictures g, h, and i. Ruler, mm. Mean ± SEM. *, P < 0.05. Original magnification, 200x.
ENDURING ACINAR CELLS ARE NEARLY ALL NOT DERIVED FROM THE BONE MARROW

Next, we investigated whether the increased number of acinar cells responsible for the morphological and functional improvement of the irradiated salivary glands after G-CSF treatment were derived from BMCs. We hardly observed any acinar cells expressing the eGFP protein. However, even not all acini of eGFP transgenic mice expressed the eGFP protein to a detectable level (data not shown). It may be that the newly formed cells, although BMC-derived, do not express the eGFP protein. Therefore, Y-chromosome in situ hybridization was also used to detect male cells originating from the donor bone marrow. As control, positive staining (Y+) was revealed in the majority of cells of the male salivary glands (Fig. 5A), whereas no positive staining was observed in glands of female mice (Fig. 5A, inset). In agreement with the eGFP data shown earlier, irradiated/sham-treated mice showed few Y+ cells in open spaces or in close vicinity of duct and acinar cells (Fig. 5B).

As expected, more Y+ cells were observed in the irradiated/G-CSF-treated mice, occurring either as clusters of infiltrating cells in open spaced nodules and arranged around central veins or as single cell connected to duct or acinar cell (Fig. 5C). However, only very few were detectable as potential acinar or ductal cell (Fig. 5D). Anti-eGFP staining confirmed these results showing rare if any overlap with mucopolysaccharide containing acini (Fig. 6A,B).

Since many eGFP+ cells were found to reside in close vicinity with duct and acinar cells but not being acinar cells, an immunohistochemical staining (Cy3, red) for a-smooth-muscle-actin (a-SMA) was performed. Figure 6C shows colocalization, although no overlap of the two signals. Both signals were also found as myofibroblasts (Fig. 6D) known to be involved in the tissues reaction to irradiation. So, it is still possible that BMCs are involved in the formation of myoepithelial cells surrounding ducts and acini. Although a significant part of the interstitial eGFP+ cells were CD45+ (Fig. 6E), most were unrelated to hematopoietic cells. CD31 staining revealed that these cells at least in part are likely to be mesenchymal cells derived from the bone marrow (Fig. 6F). These results indicates that at best a very limited number of cells potentially transdifferentiate from BMCs to salivary gland cells. The majority of the eGFP+ seem to be hematopoietic or mesenchymal cells.
To elucidate whether the morphological and functional improvements were due to a direct protective or stimulatory effect of the cytokine G-CSF, we examined the expression of the G-CSF receptor (G-CSFR) in the mouse salivary glands using reverse transcription-PCR, real-time quantitative PCR, and immunohistochemistry. Reverse transcription-PCR revealed very low, barely detectable amount of \( g-csf \) expression in the salivary glands (Fig. 7A, lane 3).

However, measured with a more sensitive method real-time quantitative PCR, some \( g-csf \) expression was detectable although much lower than the expression in bone marrow (data not shown). Furthermore, irradiation of the salivary glands with 15 Gy, did not increase the expression of \( g-csf \) measured at different time points after the irradiation (data not shown). To indicate which cells in the salivary glands expressed the G-CSFR, immunohistochemistry was used. Figure 7B indicates that the expression of the G-CSFR is solely detectable in endothelium and myoepithelial cells surrounding ducts and acini. This is confirmed with double labeling for G-CSFR and a-smooth muscle actin (Fig. 7C,D). At the time of G-CSF treatment (10, 30, or 60 days after irradiation), no changes in G-CSF receptor expression were observed.

These results indicate that it is unlikely that G-CSF directly stimulates the morphological (i.e. formation of new acinar cells) and functional improvements.
In summary, a significant higher number of acinar cells associated with improved salivary function were observed when BMCs were mobilized with G-CSF after irradiation. Most, if not all, newly formed cells, however, were not derived from BMCs.

**DISCUSSION**

Coiirradiation-induced hyposalivation after cancer therapy for head and neck cancers remains a major clinical problem. The depletion of resident viable stem cells after treatment has directed efforts to a search for alternative stem cell sources. Although bone marrow cells have been suggested to take part in the regeneration of damaged organs after irradiation, no study has yet shown improved organ function after radiation with clinically relevant doses. In the current study, we have assessed the ability of G-CSF to stimulate salivary gland regeneration by promoting mobilization of bone marrow cells to the irradiated tissue. The presence of eGFP and male Y chromosome-positive cells in irradiated glands was clearly enhanced by G-CSF treatment. The accompanying morphological and functional improvements, however, are unlikely to be related to bone marrow-derived stem cell transdifferentiation.

The present study provides the first evidence that mobilization of bone marrow cells after salivary gland irradiation has beneficial effects in preventing/reducing radiation-induced damage.

Yet, many researchers have observed that bone marrow cells transplanted in lethally irradiated subjects were detectable in tissues, such as heart, lung, liver and skin. Most of these studies used radiation doses (≤ 10 Gy) that are necessary to allow formation of a chimeric hematopoietic system. Although these doses induce damage in numerous tissues, they are well below the ED50s as described for organ dysfunction in relation to curative radiotherapy. This is of importance as the number of bone marrow cells that home to the irradiated organ is likely to be dependent on the extent of damage inflicted.

In our experimental set-up, we used a dose that inflicts damage to a similar level as what is observed in clinical practice. We observed a high number of BMCs homing to the salivary glands as well as morphological and functional improvements. Potentially, G-CSF could directly protect salivary gland stem cells and stimulate surviving stem cells to divide, resulting in the abundance of acinar cells. It has been shown that G-CSF is able to directly bind to G-CSF receptors (G-CSFR) present on cardiomyocytes to promote their survival after myocardial infarction. Also, Schneider et al. revealed that G-CSF directly protects against programmed cell death in neurons after ischemia. We did detect the presence of G-CSF receptors on myoepithelial and endothelial cells.

These cells are probably responsible for the very low expression of the G-CSF receptor in the gland as determined by real time quantitative-PCR. However, we were not able to detect the expression of G-CSFR on salivary gland acini or ducts, which are suggested to contain the salivary gland stem cell and thus normally will not be able to respond to G-CSF.

Possibly, irradiation induces up-regulation of the G-CSF receptor as has been shown to occur on neurons in the brain after ischemia. Irradiation, however, did not enhance the expression of G-CSF receptors in the salivary glands. Furthermore, our treatment was performed rather long after irradiation damage was inflicted, and, if anything, we observed a rather enhanced beneficial effect of G-CSF after delayed application (10 versus 30 days after irradiation). This is in contrast to the observed reduction in beneficial effect on cardiac function by delayed start of G-CSF receptor stimulation. Taken these findings together, it seems unlikely that G-CSF directly acts on acinar cells in our experimental setup.

The question remains what is responsible for the improvement of salivary glands function after irradiation and G-CSF treatment. In view of the very low (if any) numbers of salivary gland cells found to be potentially derived from the bone marrow (mostly myoepithelial and fibroblasts), it seems that “trans-differentiation” of bone marrow cells to acinar salivary gland cells is not observed.

However, still many bone marrow-derived cells resided in the salivary gland long after irradiation and mobilization. Most of these cells were interstitial and CD45-, indicating that they lost epitopes characteristics of hematopoietic cells. This is in agreement with results described in a study using an ischemia/reperfusion model of kidney damage. Moreover, like us, they also showed the presence of bone marrow-derived myoﬁbroblasts cells in the interstitium, which is also known to occur after a radiation insult.

The finding that some of the interstitial GFP+ cells were also CD31+ indicates that these cells are of mesenchymal cell origin. Thus, apparently, G-CSF also induces the mobilization of these cells from the bone marrow in the irradiated salivary glands. Indeed, it is known that next to hematopoietic stem cells, G-CSF also induces the mobilization of a number of mesenchymal progenitor cells, which may even home to salivary glands in healthy mice. Therefore, one can postulate that mesenchymal cells are involved in the amelioration of radiation-induced damage to the salivary gland.
Because the percentage of donor-derived bone marrow cells trans-differentiated in salivary gland cells is at the best very low, we must assume that G-CSF-induced mobilization and homing of hematopoietic and/or mesenchymal cells somehow stimulates the recovery of the salivary gland cells. The most likely hypothesis is that this occurs through cytokine secretion and subsequent induction of proliferation of the endogenous tissues progenitor/stem cell. A study with long-term follow-up will need to be performed to reveal whether the effects observed are transient or long lasting.

In summary, our data indicate that G-CSF treatment is effective in ameliorating radiation-induced salivary gland dysfunction after radiotherapeutic treatment of head and neck cancer, likely via homing of bone marrow-derived cells, which subsequently secrete stimulatory or protective factors. This study indicates a clinical applicable protocol for the use of bone marrow-derived cell mobilization to ameliorate radiation-induced damage. In order to be able to exploit this treatment most effectively, the molecular mechanism behind the observed protection and duration needs to be further explored.

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MATERIALS AND METHODS

ANIMALS

Female C57BL/6 mice, 6 to 8 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME) and used in a sex-mismatched transplantation setting. Enhanced GFP male C57BL/6-TgN (ActbEGFP) mice, were bred in the animal facility of the University Medical Center Groningen and used as donor mice for GFP+ whole bone marrow. All mice were kept under clean conventional conditions and fed ad libitum with food pellets (RMH-8, Hope Farms B.V., Woerden, The Netherlands) and acidified tap water (pH = 2.8). All experiments were approved by the animal Ethical Committee on animal testing of the University of Groningen.

TRANSPLANTATION PROTOCOL

Mice were splenectomized under halothane/O2-anesthesia and allowed to recover for at least two weeks. Splenectomized female acceptor mice were given 9.5 Gy total body irradiation of X-rays (Philips CMG 41 X, 200 kV, 10 mA, 5 Gy/minute) shielding the salivary glands (12 x 50 x 3 mm lead plate) and were transplanted with 3 x 106 male bone marrow cells obtained from femurs from GFP+ transgenic mice by orbital injection. Bone marrow cells were obtained by flushing the femoral content with Iscove’s Modified DMEM (IMDM, GibcoBRL, Paisley, Scotland). Chimerism in peripheral blood was documented 8 weeks later by analysis on a FACS Calibur flow cytometer (Becton Dickinson, Erembodegem, Germany). Only mice with > 60% chimerism were used for the experiments.

IRRADIATION AND MOBILIZATION PROTOCOL

One month post-transplantation, the salivary glands of the chimeric mice were locally irradiated with a single dose of 15 Gy (as described above), shielding the rest of the body with 3-mm lead. This dose is known to induce sufficient damage without compromising the general health of the animals. At days 10, 30, or 60 after gland irradiation, bone marrow cells were mobilized by s.c. injections of 2 x 25 µg PEG-rHu-G-CSF (Amgen, Inc., Thousand Oaks, CA) given 3 days apart.

COLONY-FORMING CELL ASSAYS

Progenitor cells were assayed as described earlier. Briefly, cells were plated out in alpha-medium (StemCell Technologies, Inc., Vancouver, Britisch Columbia, Canada) containing 0.8% methylcellulose (Fuka, Bachs S.G., Switzerland), 30% fetal calf serum (Life Technologies), and 10^{-4} mol/L 2-mercaptopethanol (Merck, Darmstadt, Germany) at concentrations varying from 10^4 to 5 x 10^5 nucleated cells/mL. Colony growth is stimulated by granulocyte macrophage colony-stimulating factor and stem cell factor. Cultures were plated in 35-mm polystyrene culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) and grown at 37°C in a 5% CO2 humidified atmosphere. Colonies (> 50 cells) were scored after 7 days of culture.

The cobblestone area-forming cell (CAFC) assay allows the assessment of primitive hematopoietic stem cells. The CAFC assay was performed by establishing confluent FBMD-1 cell cultures in 96-well plates (Costar, Corning, NY). The confluent cell cultures were overlaid with mobilized peripheral blood cells in a limiting dilution set-up. Eight dilutions 2-fold apart were used with 10 replicate wells per dilution. The cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 20% horse serum (Life Technologies) at 33°C in a 10% CO2 humidified atmosphere with a half-volume medium change every week.
The percentage of wells with at least one phase-dark hematopoietic clone of at least five cells beneath the stromal layer was determined 4 to 5 weeks after initiating the culture. Cobblestone area frequencies were calculated using Poisson statistics.

**SALIVA COLLECTION**

Before and 90 days after irradiation, whole saliva flow rate was determined. The animals were placed in a restraining device \(^{172}\) after pilocarpine injection (2 mg/kg, s.c.). Saliva was collected for 15 minutes and determined gravimetrically, assuming a density of 1 g/mL for saliva.

**IMMUNOHISTOCHEMISTRY**

At 130 days after irradiation of the salivary glands, animals were sacrificed for immunohistochemistry. The submandibular glands were extirpated and incubated for 29 hours at 4°C in 4% buffered formaldehyde. Following dehydration, the tissue was embedded in paraffin. Five-micrometer sections were analyzed following dehydration, the tissue was embedded in paraffin. Five-micrometer sections were analyzed for the presence of eGFP using a Confocal Scanning Laser Microscopy (CSLM) (Leica TCS SP2). Counterstaining was done with 4',6-diaminophenylindole (DAPI). Overnight staining with anti-eGFP antibody (Chemicon, Temecula, CA; MAB3658; 1/500) was visualized with light microscopy by diamobenzidine (DAB) chromogen. Avidin-biotin-horseradish peroxidase complex (Vector Elite Avidin-Biotin Complex Kit) and anti-mouse rabbit F(ab')2 biotine (Dako, Carpinteria, CA; E0413; 1/300) were used for a 0.01 mol/L, citrate antigen retrieval, following dewaxing and blocking the endogenous peroxidase with 0.3% H2O2 for 30 minutes. Anti-G-CSF receptor (G-CSFR; Santa Cruz Biotechnology, Santa Cruz, CA; clone H-176; 1/100) overnight labeling was done using 0.01 mol/L, citrate antigen retrieval and anti-rabbit F(ab')2 biotine (Dako; E0431; 1/300). In addition, anti-a-smooth muscle actin (Sigma, St. Louis, MO; clone 1A4; 1/100) staining was done using anti-mouse rabbit F(ab')2 biotine for 1 hour at room temperature each. Visualization was made using streptavidine-Cy3 (Sigma; S6402) for 30 minutes. Counterstaining was performed using DAPI. Overnight CD31 (BD Pharmingen, San Diego, CA; 550274; 1/100) labeling was done following 0.05% trypsin pretreatment at 37°C. Visualization was made by using anti-rat biotine biotine (Dako; E0431; 1/300). In addition, anti-a-smooth muscle actin (Sigma, St. Louis, MO; clone 1A4; 1/100) staining was done using anti-mouse rabbit F(ab')2 biotine for 1 hour at room temperature each. Nuclear staining was performed using DAPI. Overnight CD31 (BD Pharmingen, San Diego, CA; 550274; 1/100) labeling was done following 0.05% trypsin pretreatment at 37°C. Visualization was made by using anti-rat biotine biotine (Dako; E0431; 1/300) and streptavidine-Cy3. In addition, anti-CD45 (RDI-MCD45-F11; 1/100) labeling required the anti-rat biotine and streptavidine-Cy3 in combination with a double labeling for anti-eGFP using avidine-FITC (Sigma; A2050). Double labeling of G-CSFR and anti-smooth muscle-actin was done using avidine-FITC and streptavidine-Cy3, respectively.

The Y chromosome was stained by fluorescence in situ hybridization (Cambio, Cambridge, UK; 1187-MIB) according to manufacturer’s instructions. The following changes were made: 5 minutes of 1 mol/L sodium thiocyanate and 0.4% pepsin instead of 10 minutes. Nuclear staining was performed with methyl green. Negative controls were included in the protocols. Gland morphology was visualized by routine histologic techniques with hematoxylin-eosin. Acini were detected by Periodic Acid Schiff’s base and Alcian Blue staining by which mucin and mucopolysaccharides are detected, respectively.

**QUANTIFICATION OF eGFP+ CELLS AND ACINI**

Tissue sections were analyzed using the CSLM at a magnification of 630x. The number of eGFP+DAPI+ cells in 200 counted cells was enumerated. The amount of eGFP+ cells in a salivary gland of C57BL/6 eGFP transgenic mice, was set at 100%, as not all salivary gland cells appear to express the eGFP protein. The percentage of surface area occupied by acinar cells was counted by light microscopy (Olympus CX40, Germany) under 400x magnification using 100 squares of 0.25 mm² each. Of each gland 5 squares in three sections (top-middle-bottom) were scored, and subsequently the data were pooled.

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**SALIVARY GLAND REGENERATION BY BMCs**

From mice, the submandibular glands were excised, cut into small pieces, snap frozen in liquid nitrogen, and stored at -80 °C until used. Total RNA was isolated from frozen submandibular gland tissue using TRizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Total RNA was isolated from fresh mouse whole bone marrow using the RNaseasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA was checked on an agarose gel for integrity, and RNA concentration was measured spectrophotometrically. Reverse transcription was performed on 2.5 μg of total RNA using random primers in a final volume of 38 μL (Reverse Transcription System, Promega, Madison, WI) for 10 minutes at 25 °C, followed by 1 hour at 45 °C. Samples were subsequently heated for 5 minutes at 95 °C to terminate the reverse transcription reaction. The cDNA was amplified using mouse g-csf (Csf3r) primers with the following sequences: sense primer, 5'-GTACTCTTGTCACACTGTTG-3' and anti-sense primer, 5'-CAAGATAAACGAGCCCCCA-3' (accession no. NM_007782). The PCR was performed under the following conditions: an initial denaturation at 94°C for 2 minutes followed by a cycle of denaturation at 94 °C for 1 minute, annealing at 58 °C for 1 minute, and extension at 72°C for 1 minute. The samples were subjected to 40 cycles, and the PCR products were analyzed on an ethidium bromide stained agarose gel.

Real-time quantitative PCR was performed on the (irradiated) submandibular gland and bone marrow cDNA samples on a Bio-Rad iCycler iQ Real-Time Detection System. The following primer sequences were used for β-actin; sense primer, 5'-AGACCTCTATGGCCAACGACG-3' and anti-sense primer, 5'-TGGGACGAGCGACGAAATCC-3' (accession no. NM_007393) and for g-csf (Csf3r): sense primer, 5'-ATGGCGCCGCTGTAGTATCTGCTC-3' and anti-sense primer, 5'-CAGCTGTTGATCCGATCTC-3'. Real-time PCR was conducted by amplifying the cDNA with the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). For each set of primers, the PCR-efficiency was determined. Melting curve analysis of amplification products was performed at the end of the PCR reaction to confirm that a single PCR product was detected. For every PCR reaction, β-actin was used as the internal control. Quantification of the samples was calculated from the threshold cycle (CT) by interpolation from the standard curve.

**STATISTICAL ANALYSIS**

The results were analyzed using an unpaired Student’s t tests and the Mann-Whitney test. Statistical significance was defined as \( P < 0.05 \) using SPSS.