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Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells

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ABSTRACT

CSF-1 is broadly expressed and regulates macrophage and osteoclast development. The action and expression of IL-34, a novel CSF-1R ligand, were investigated in the mouse. As expected, huIL-34 stimulated macrophage proliferation via the huCSF-1R, equivalently to huCSF-1, but was much less active at stimulating mouse macrophage proliferation than huCSF-1. Like muCSF-1, muIL-34 and a muIL-34 isoform lacking Q81 stimulated mouse macrophage proliferation, CSF-1R tyrosine phosphorylation, and signaling and synergized with other cytokines to generate macrophages and osteoclasts from cultured progenitors. However, they respectively possessed twofold and fivefold lower affinities for the CSF-1R and correspondingly, lower activities than muCSF-1. Furthermore, muIL-34, when transgenically expressed in a CSF-1-dependent manner in vivo, rescued the bone, osteoclast, tissue macrophage, and fertility defects of *Csf1*$^{op/op}$ mice, suggesting similar regulation of CSF-1R-expressing cells by IL-34 and CSF-1. Whole-mount *IL34* in situ hybridization and CSF-1 reporter expression revealed that *IL34* mRNA was strongly expressed in the embryonic brain at E11.5, prior to the expression of *Csf1* mRNA. QRT-PCR revealed that compared with *Csf1* mRNA, *IL34* mRNA levels were lower in pregnant uterus and in cultured osteoblasts, higher in most regions of the brain and heart, and not compensatorily increased in *Csf1*$^{op/op}$ mouse tissues. Thus, the different spatiotemporal expression of IL-34 and CSF-1 allows for complementary activation of the CSF-1R in developing and adult tissues. *J. Leukoc. Biol.* 88: 495–505; 2010.

Introduction

CSF-1 is the primary regulator of the survival, proliferation, and differentiation of mononuclear phagocytes [1–5] and plays a central role in the regulation of osteoclastogenesis [6–11]. CSF-1 also regulates the development of Paneth cells [12], Langerhans cells [13], lamina propria dendritic cells [14], and microglia [15, 16]. All of the effects of CSF-1 are mediated by the high-affinity CSF-1R, a protein tyrosine kinase encoded by the c-*fms* proto-oncogene [17]. As *Csf1r*$^{-/-}$ mice possess virtually all of the reported defects of *Csf1*$^{op/op}$ mice, all of the effects of CSF-1 appear to be mediated by the CSF-1R [18]. However, *Csf1r*$^{-/-}$ mice display a more severe osteopetrosis, reduced survival, and fewer tissue macrophages—and these differences are more pronounced on single-strain backgrounds [12, 18] (E. R. Stanley and S. Nandi, unpublished observations)—strongly suggesting the existence of another ligand. This was confirmed recently by the discovery of the novel cytokine, IL-34, which binds specifically to the CSF-1R [19].

huIL-34 is a dimeric glycoprotein resembling the dimeric CSF-1 glycoprotein, and like *Csf1* mRNA, *IL34* mRNA is broadly expressed in adult human tissues, including heart, brain, lung, liver, kidney, spleen, thymus, testis, ovary, small intestine, prostate, and colon [19]. Mimicking huCSF-1, purified huIL-34 binds CD14^{+} monocytes specifically, promotes the survival/proliferation of human peripheral blood monocytes, and stimulates macrophage colony formation by human bone marrow cells. Furthermore, the soluble huCSF-1R extracellular domain blocks the binding of IL-34 to human mono-

Abbreviations: AP=alkaline phosphatase, CFU-M=CFU-macrophage, *Csf1*$^{op/op}$=CS F-1-deficient, *osteopetrotic*, *Csf1r*$^{-/-}$=CSF-1R-deficient, DIG=digoxigenin, E=Embryonic Day, HPP-CFC=high proliferative potential colony-forming cell, hu=human, M$^{-/-}$=MacCsf1r$^{-/-}$, mu=mouse, Q81=glutamine 81, QRT-PCR=quantitative RT-PCR, RANKL=receptor activator for NF-κB ligand, SCF=stem cell factor, SG=secreted glycoprotein isoform of CSF-1, SP=secreted proteoglycan isoform of CSF-1, Tg=transgenic, TgZ=*Csf-1-lacZ* reporter transgene, TRAP=tartrate-resistant acid phosphatase, X-gal=4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside

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cytic cells and abolishes IL-34-stimulated monocyte survival and proliferation. When immobilized, soluble huCSF-1R binds huIL-34 with an affinity even greater than its binding affinity for huCSF-1. Binding of biotinylated huIL-34 to monocytic THP-1 cells was also inhibited specifically by IL-34 or CSF-1, as well as by an antibody to the huCSF-1R [19]. Moreover, huIL-34-stimulated monocyte viability was blocked by anti-IL-34 antibodies but not by anti-CSF-1 antibodies, and the huCSF-1 stimulated monocyte viability inhibited by anti-CSF-1 but not by anti-IL-34 antibodies, indicating that the effects of huIL-34 on viability were truly independent of CSF-1 [19]. Despite their congruency in action, huIL-34 and huCSF-1 share no DNA sequence similarity, although huIL-34 secondary structure predictions suggest the existence of four α-helical bundles, as has been shown for huCSF-1 [20]. Interestingly, a recent comparative sequence and coevolution analysis across all vertebrates suggest that the two ligands interact with distinct regions of the CSF-1R [21].

We here demonstrate that although efficacious in all in vitro assays tested, muIL-34 is less potent than muCSF-1 but more potent than an IL-34 isoform lacking Q81. However, when transgenically expressed in mice in a spatiotemporal manner mimicking CSF-1, muIL-34 can rescue the *CSF-1*$^{op/op}$ phenotype. We show further that there are differences in the spatiotemporal expression patterns of muIL-34 and muCSF-1. Thus, IL-34 and CSF-1 have similar CSF-1R-mediated effects, but as a result of their different expression patterns, they are likely to complement each other in their actions via the CSF-1R.

MATERIALS AND METHODS

Growth factors, cell lines, cell culture, and competitive binding assay

muIL-34 and huIL-34 have +Q81 and –Q81 isoforms (see Results). huIL-34 (–Q81), muIL-34 (+Q81), and muIL-34 (–Q81) were expressed in mammalian cells and purified from the cell culture medium as described previously [19]. Purified muIL-34 (+Q81) was also purchased from R&D Systems (Minneapolis, MN, USA). rhuCSF-1 was a gift from Chiron Corp. (Emeryville, CA, USA). muCSF-1 was purified from mouse L cell conditioned medium as described [22] and purchased from R&D Systems. Purity, with the exception of the purchased preparations, was demonstrated by SDS-PAGE and silver staining. Anti-CSF-1R mAb (AFS98) [23] was a gift from Dr. Shinici Nishikawa (RIKEN Kobe Institute, Japan). The anti-muCSF-1R C-terminal and the antiphospho-Y559 peptide antisera were raised in rabbits and affinity-purified against their corresponding peptides as described [24]. Anti-ERK1/2, anti-phospho-ERK1/2 (pT202/pY204), anti-phospho-tyrosine pY100, anti-CSF-1R-pY807, and anti-CSF-1R-pY723 were purchased from Cell Signaling Technology (Beverly, MA, USA).

M$^{-/-}$ cells [24] were retrovirally transduced with a MSCVpac retrovirus [25] containing the full-length huCSF1R cDNA (Five Prime Therapeutics, Inc., San Francisco, CA, USA) and cells selected in 5 μg/ml puromycin to create a cloned M$^{-/-}$.huCSF1R macrophage cell line stably expressing wild-type levels of the huCSF-1R, as described previously [24]. muBAC1.2F5 [26] and M$^{-/-}$.huCSF1R macrophages were maintained by culture with 36 ng/ml huCSF-1, and proliferation dose-response assays were carried out by culturing the cells in triplicate in α-MEM (Gibco, Gaithersburg, MD, USA), supplemented with 10% FCS (Gibco) at different concentrations of CSF-1 or IL-34 for 6 days in 96-well plates (huIL-34) or 7 days in 48-well plates (muIL-34, with a medium change at Day 5) and monitoring cell number using a FLUO optima reader (BMG Labtechnologies, Inc., Durham, NC, USA), as described previously [24]. huCSF-1 stimulation of M$^{-/-}$.huCSF1R macrophages or muCSF-1 stimulation of BAC1.2F5 macrophages, immunoprecipitation, SDS-PAGE, and Western blotting for phosphotyrosine, ERK1/2, and phospho-ERK1/2 was carried out as reported previously [24, 27].

HPP-CFC and CFU-M assays were carried out using bone marrow cells from 6-week-old C57BL/6J mice as described previously [27]. HPP-CFC cultures contained SCF (50 ng/ml), IL-6 (20 ng/ml), and IL-3 [20 ng/ml; Stem Cell Technology (Vancouver, BC, Canada)] with 20 ng/ml or 100 ng/ml muCSF-1 or muIL-34 (–Q81 or +Q81) or neither muCSF-1 or muIL-34. CFU-M cultures contained 20 ng/ml or 100 ng/ml muCSF-1 or IL-34 (+Q81 or –Q81). Osteoclast-like cells were obtained by incubating C57BL/6J bone marrow cells in 96-well tissue-culture plates (10^5 cells/well) in α-MEM containing 10% FCS and differentiated in the presence of 100 ng/ml soluble rRANKL (R&D Systems) and 20 ng/ml, 100 ng/ml, or 250 ng/ml muIL-34 (+Q81 or –Q81), or muCSF-1, in triplicate [28] with a medium change at Day 3. Cells were fixed at Day 4 using 4% paraformaldehyde in PBS, pH 7.4 (20 min, 37°C), and stained for TRAP [29]. Images were acquired with a Kodak DC290 digital camera.

For the competitive binding assays [30], duplicate serial dilutions of muIL-34 (+Q81 or –Q81) or muCSF-1 or assay buffer (0.2% BSA, 0.02% NaN_3, 25 mM HEPES in α-MEM medium, pH 7.35) were mixed with ^{125}I-labeled, purified L-cell muCSF-1 (10^6 cpm/ng protein) and CSF-1R-expressing mouse J774.2 macrophages in assay buffer and incubated for 1 h at 4°C. The free and cell-associated ^{125}I was separated by zonal centrifugation and the cell-associated radioactivity determined by γ-counter [30]. For estimation of the on rates, cells were incubated with each unlabeled ligand for the indicated times, the free ligand was removed by rapid zonal centrifugation and washing, and the unoccupied receptors determined by a further incubation with ^{125}I-labeled muCSF-1 for 30 min at 4°C. Ligand binding was determined by subtracting the unoccupied receptor binding at each time-point from the total binding observed in the absence of unlabeled ligand. On-rate constants were determined as described previously [31]. A similar approach was used to estimate off rates that were measured at 20°C.

Mouse generation, maintenance, genotyping, X-ray analysis, histochemistry, and immunohistochemistry

To assemble the TgN(Csf1-IL-34)Ers construct, a fragment containing a full-length muIL-34 cDNA (with Q81), including the endogenous poly A signal and an exogenous human growth hormone poly A signal sequence, was used to replace the portion containing the nucleus-targeted β-galactosidase coding region-SV40 poly A in the TgN(FLCsf1)Ers transgene construct [32]. The TgN(Csf1-IL-34)Ers construct was microinjected into the pronuclei of FVB/NJ zygotes to produce Tg mice. Mice transmitting IL-34 transgenes were mated to FVB/NJ strain *Csf1*$^{op/+}$ mice [33], yielding *Csf1*$^{op/+}$; *Tg*/+ mice that were intercrossed to generate *Csf1*$^{op/op}$; *Tg*/+ mice. For embryo, uterus, and placental samples, mice were mated overnight and females checked for vaginal plugs the next morning. Noon on the day of plug discovery was considered E0.5. Genotyping, X-radiography, and immunohistochemistry (F4/80 and TRAP) were carried out as described previously [32–34]. The transgene in *TgN(Csf-IL-34)Ers* mice was detected by amplifying a 300-bp PCR product using the primers: forward, 5′-GGTAGCTAGGGAGAGGAAG-3′; reverse, 5′-GCACAGCAATCCTGTAGTTGATGG-3′.

RNA isolation and QRT-PCR

Embryonic, extra-embryonic, and individual tissues from 8- to 60-day-old FVB/NJ mice were dissected, frozen immediately in liquid nitrogen, and stored at –80°C. Total RNA was prepared from the frozen tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA synthesis was carried out using SuperScript III RT (Invitrogen). Absence of RT in RT reactions and non-Tg samples was used as the negative controls. QRT-PCR was carried out as described previously [35]. Determinations were made in triplicate. QPCR primers were *Csf1*: forward, 5′-AGTATTGCCAAGGAGGTGTCAG-3′, reverse, 5′-ATCTGGCATGAAGTCTCCATTT-3′; *IL34*: forward, 5′-CTTTGGGAAACGAGAATTTGGAGA-3′, reverse, 5′-GCAATCCTGTAGTTGATGGGGAAG-3′; *Csf1r*: forward, 5′-GCAGTACCACCATCCACTTGTA-3′,

reverse, 5′-GTGAGACACTGTCCTTCAGTGC-3′; *Csf1-IL34* Tg: forward, 5′-CGCGGGGCGCTGCCCTTCTTC-3′, reverse, 5′-TCTCGTTTCCCAAAGCCACGTCAAG-3′; *β-actin*: forward, 5′-CAGGCATTGCTGACAGGAT-3′, reverse, 5′-GTGCTAGGAGCCAGAGCAGT-3′.

To normalize the minor variations in the starting amount of RNA, the differences in the efficiency of cDNA synthesis and PCR amplification and the comparisons between endogenous *IL34* and *Csf1-IL34* transgene mRNA levels *β-actin* was used as an internal control in each QRT-PCR reaction. A relative quantification method, $2^{\text{cycle threshold}}$ [36], was used.

Whole-mount in situ hybridization and β-galactosidase staining

E11.5 FVB/NJ embryos were fixed in 4% paraformaldehyde in PBS, pH 7.4 (4°C overnight with rotation), and stored in methanol at –20°C [37, 38]. Embryos were rehydrated, permeabilized using RNase-free PBS containing 0.1% Tween 20 in the presence of 4.5 μg/ml proteinase K (20°C, 20 min), postfixed in RNase-free PBS containing 4% paraformaldehyde and 0.2% glutaraldehyde (4°C, 15 min), and incubated with hybridization buffer containing 50% formamide, 2× SSC, pH 7, 5 mM EDTA, 1 mg/ml yeast Torula RNA, 0.1% Tween 20, 0.1% CHAPS, and 100 μg/ml heparin (65°C, 3 h). Full-length, single-stranded sense and antisense *muIL34* probes were synthesized using DIG-RNA labeling mix (Roche, Mannheim, Germany) and purified using Sephadex G-50 spin columns (Roche). Embryos were incubated with hybridization buffer in the presence of 200 ng/ml probe (70°C overnight), washed, and incubated further with anti-DIG-AP antibody (Roche; 1:10,000; 4°C overnight). BM Purple, the AP substrate (Roche), was used for color development.

For localization of β-galactosidase in the CSF-1 reporter, Tg [*TgN(Csf1-Z)Ers*] mouse embryos were fixed and stained by incubating with X-gal, as described [32].

Primary osteoblast culture

Primary osteoblast cultures were established from newborn wild-type FVB/NJ mouse calvaria [33, 39]. Briefly, calvariae from eight pups were removed aseptically after carefully disposing of the periosteum and endosteum, trimmed and digested further using α-MEM containing 2 mg/ml type II collagenase (37°C, 10 min). Trimmed calvaria were digested further in fresh α-MEM containing 2 mg/ml type II collagenase and 5 μg/ml DNase I (37°C, 2 h). Osteoblast-like cells were separated from the calvariae by filtration through a 70-μM cell strainer, washed in α-MEM, counted, and plated at 2×10^4 cells/cm^2 in six-well tissue-culture plates in α-MEM containing 10% FCS, 15 mM HEPES (Sigma Chemical Co., St. Louis, MO, USA), and 50 μg/ml ascorbate (Sigma Chemical Co.) and cultured for 5 days with change of media every 2 days. Cells were dissociated, replated in triplicate at 1.5×10^4 cells/cm^2 in 24-well tissue-culture plates, and incubated further alone or in the presence of 0.1 or 1.0 μg/ml LPS (Sigma Chemical Co.) or IFN-γ (Stem Cell Technologies, Vancouver, BC, Canada) for 6 and 24 h. Purity of the culture was assessed by AP staining [40].

Statistical analysis

Data were expressed as means ± SD or means ± SEM. Student *t*-test was used to test significance. Differences were considered statistically significant for comparisons of datasets yielding *P* values ≤0.05. Alternatively, as indicated, error bars denote range of duplicates.

RESULTS

huCSF-1 and huIL-34 induce similar proliferation and MAPK activation in huCSF-1R-expressing macrophages

Previous studies have shown that huIL-34 possesses a higher binding affinity than huCSF-1 for the huCSF-1R and that huIL-34 stimulates macrophage colony formation by human bone marrow cells and human monocyte survival/proliferation [19]. We first compared the ability of huIL-34 and huCSF-1 to stimulate macrophage proliferation using the cloned mouse macrophage cell line, M$^{-/-}$.huCSF-1R, in which the huCSF-1R replaces the muCSF-1R. As expected, the huCSF-1 and huIL-34 dose response curves were superimposable (**Fig. 1A**). We therefore compared their ability to stimulate huCSF-1R tyrosine phosphorylation and to activate MAPK in these cells. Both ligands stimulated huCSF-1R tyrosine phosphorylation to a similar degree and with similar kinetics. Both ligands also stimulated ERK1/2 phosphorylation to a similar degree and with similar kinetics (Fig. 1B). We then used the cloned muBAC1.2F5 macrophage line [26] to determine the relative efficacy of huIL-34 and huCSF-1 in stimulating the proliferation of mouse macrophages. Compared with huCSF-1, which was shown previously to be almost as effective as muCSF-1 in stimulating macrophage proliferation [30, 41], the EC_{50} for huIL-34 was 30-fold higher (Fig. 1C). These results show that despite their similar capacity to activate huCSF-1R signaling and huCSF-1R-mediated macrophage proliferation in vitro, huIL-34 exhibits marked cross-species specificity and is much less potent than huCSF-1 in stimulating muCSF-1R-mediated macrophage proliferation.

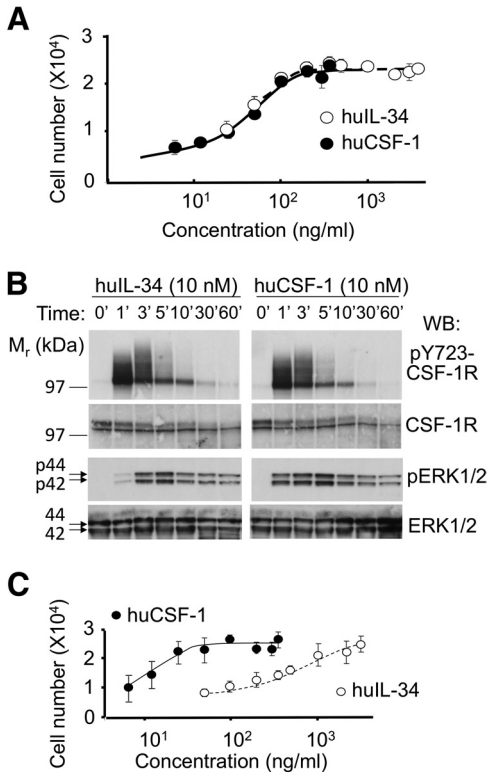


Figure 1. huCSF-1 and huIL-34 stimulated macrophage proliferation and signaling via the CSF-1R. (A) Proliferation dose-response curves of M$^{-/-}$ mouse macrophages expressing the huCSF-1R (M$^{-/-}$.huCSF-1R macrophages; triplicate cultures±SEM). (B) Kinetics of CSF-1R tyrosine 723 phosphorylation (pY723) and ERK1/2 phosphorylation (pERK1/2) of M$^{-/-}$.huCSF-1R macrophages in response to huIL-34 or huCSF-1. WB, Western blot. (C) Proliferation dose-response curves of muBAC1.2F5 mouse macrophages (triplicate cultures±SEM).

Active muIL-34 isoforms regulate CSF-1R signaling and in vitro macrophage and osteoclast proliferation and differentiation but have a lower affinity than muCSF-1 for the muCSF-1R

muIL34 and *huIL34* pre-mRNAs exhibit alternative splicing of a CAG codon that leads to isoforms, with and without a Q81 (in human and mouse; Supplemental Fig. 1A). Previous studies of huIL-34 [19], as well as those described above, used huIL-34 (–Q81). The presence or absence of Q81 is predicted to alter the nearby structure from coiled coil (+Q81) to helix (–Q81; Supplemental Fig. 1B). Therefore, for in vitro studies of growth factor activity, we compared the activities of muIL-34 (+Q81) and muIL-34 (–Q81) with those of muCSF-1. Examination of the dose responses of muCSF-1- and muIL-34-induced BAC1.2F5 macrophage proliferation (**Fig. 2A**) revealed that muIL-34 (+Q81) was significantly less potent than muCSF-1 and that muIL-34 (–Q81) was significantly less potent than muIL-34 (+Q81). We therefore examined the ability of

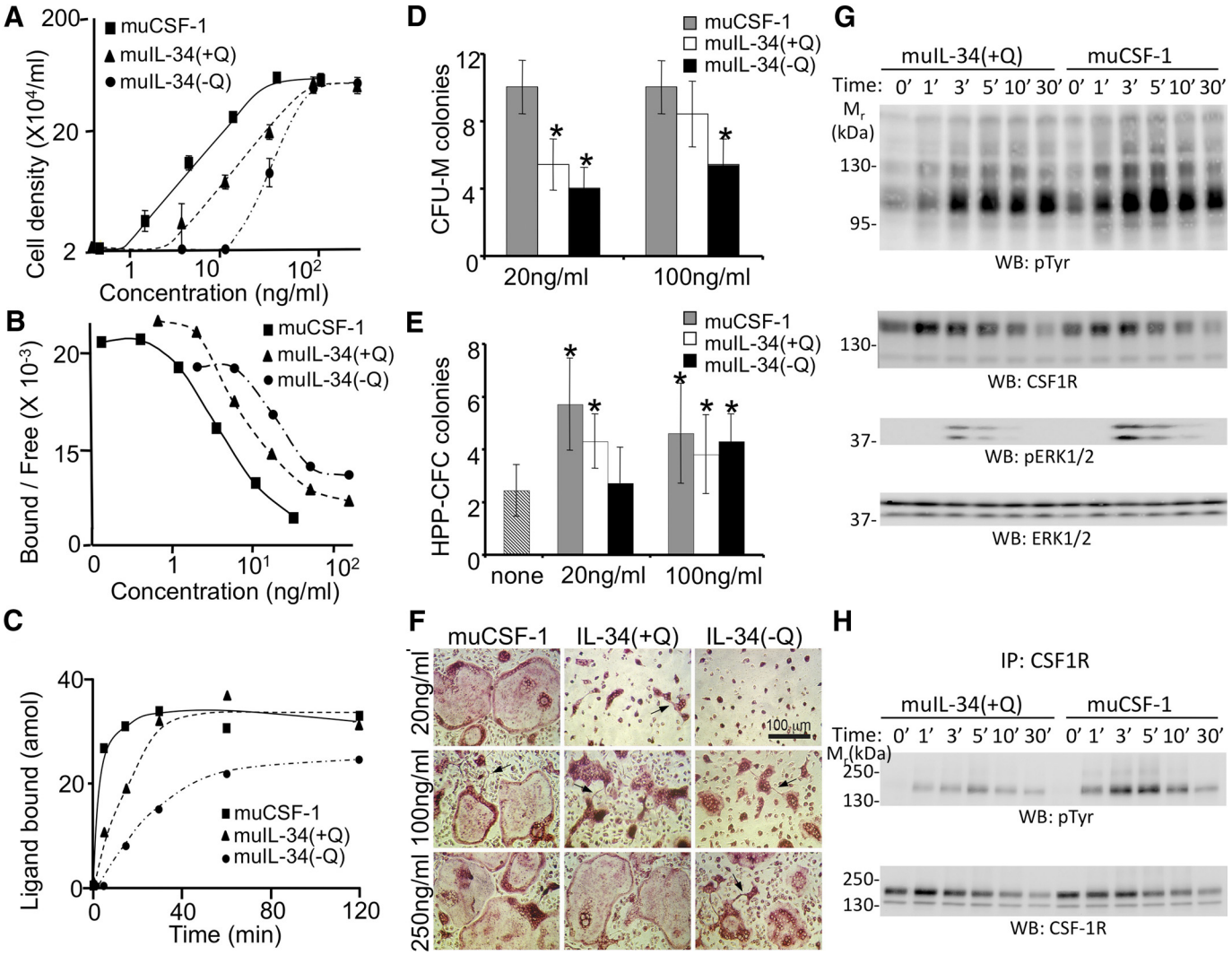


Figure 2. muIL-34 isoforms stimulate mouse macrophage proliferation, macrophage, osteoclast progenitor cell differentiation, and CSF-1R signaling but have a lower affinity for the muCSF-1R than CSF-1. (A) Proliferation dose-response curves of BAC1.2F5 mouse macrophages to muCSF-1 and to two different, purified muIL-34 preparations, differing by the presence (+Q) or absence (–Q) of Q81 (triplicate cultures±SD; EC_{50}=8 ng/ml, 20 ng/ml, and 40 ng/ml, respectively; averages of three experiments). (B) Competition by muCSF-1 and muIL-34 for ^{125}I-muCSF-1 binding to the muCSF-1R on mouse J774.2 macrophages [IC_{50}: muCSF-1=4 ng/ml, IL-34 (+Q)=7 ng/ml, and IL-34 (–Q)=22 ng/ml; averages of two experiments]. (C) Kinetics of association of muCSF-1 (60 ng/ml), muIL-34(+Q; 200 ng/ml), and muIL-34 (–Q; 200 ng/ml) with the muCSF-1R on mouse J774.2 macrophages. (D) Activity of muIL-34 (+Q or –Q) or muCSF-1 alone on macrophage progenitors (CFU-M; ± SD *, significantly different from muCSF-1 alone; $P \leq 0.01$; $n \geq 4$). (E) Thier synergy with IL-3 + IL-6 + SCF on primitive progenitors (HPP-CFC; ± SD *, significantly different from "none"; $P \leq 0.05$; $n \geq 6$). (F) Synergism of muIL-34 isoforms or muCSF-1 with RANKL in osteoclastogenesis from primary mouse bone marrow cells cultured for 4 days. Arrows point to intercellular bridges of fusing cells, indicative of osteoclasts that are not fully differentiated. (G) SDS-PAGE of Nonidet P-40 lysates of BAC1.2F5 macrophages stimulated with 120 ng/ml muIL-34 or muCSF-1 at 37°C, showing the kinetics of muCSF-1R tyrosine phosphorylation (pTyr) and ERK1/2 phosphorylation. (H) SDS-PAGE of CSF-1R immunoprecipitates (IP) of lysates used in G.

the muIL-34 isoforms and muCSF-1 to compete for the binding of ^{125}I-muCSF-1 to the CSF-1R on intact J774.2 macrophages, a system that forms the basis of a CSF-1 radio-receptor assay [30]. Consistent with the rankings of their EC_{50} for mouse macrophage proliferation, muIL-34 (+Q81) possessed an approximate twofold lower ability and muIL-34 (–Q81) an approximate fivefold lower ability to compete for ^{125}I-muCSF-1 binding to the muCSF-1R than muCSF-1 (Fig. 2B). These results indicate that the muIL-34 isoforms have lower affinities for the muCSF-1R than muCSF-1. To determine whether this difference was primarily a result of differences in the on rates or off rates of the ligands, we indirectly determined these for all three ligands, as described in the Materials and Methods. We found that although all ligands showed similar slow rates of dissociation at 20°C (data not shown), the on rate for muCSF-1 at 4°C was greater than the on rate for muIL-34 (+81), which was greater than the on rate for muIL-34 (–Q81; Fig. 2C), in agreement with the differences in their affinities being primarily a result of differences in their association rates. Consistent with the ability of muIL-34 to compete with muCSF-1 for binding to the CSF-1R, proliferation induced by either growth factor was blocked by the CSF-1-neutralizing anti-CSF-1R mAb (AFS-98; data not shown). A similar hierarchy in the actions of the muIL-34 isoforms and muCSF-1 was observed in their stimulation of macrophage colony formation (CFU-M; Fig. 2D) and their synergism with SCF, IL-6, and IL-3 in stimulating colony formation by HPP-CFC (Fig. 2E) and with RANKL to stimulate osteoclastogenesis by mouse bone marrow cells (Fig. 2F). These results demonstrate that muIL-34 is able to stimulate macrophage proliferation and macrophage and osteoclast differentiation from mouse cells in vitro but is less potent than CSF-1. The higher concentrations of the IL-34 isoforms required for these in vitro activities reflect their lower affinities for the muCSF-1R.

To determine whether there were qualitative differences between the muCSF-1 and muIL-34 signal transduction, we compared the ability of muCSF-1 and muIL-34 (+Q81) to stimulate tyrosine phosphorylation and to activate MAPK in BAC1.2F5 macrophages. Both ligands stimulated protein tyrosine phosphorylation and ERK1/2 phosphorylation (Fig. 2G) and muCSF-1R tyrosine phosphorylation (Fig. 2H) with similar kinetics, although the degree of stimulation by muIL-34 was slightly less at the concentration used, probably reflecting the lower affinity of muIL-34 for the CSF-1R. In addition, no qualitative differences between muCSF-1 and muIL-34 stimulation were evident in the phosphorylation of CSF-1R tyrosines Y559, Y807, or Y721, as assessed by Western blotting with appropriate antiphosphotyrosyl peptide antibodies (Supplemental Fig. 2).

Expression of IL-34 in a CSF-1-specific manner rescues the major defects of CSF-1$^{-/-}$ mice

Evidence suggests that all of the effects of CSF-1 are mediated via the CSF-1R [18]. In vitro, the effects of IL-34 resemble those of CSF-1 in several assays (Figs. 1 and 2). If IL-34 has similar effects on CSF-1R-expressing target cells in vivo, it should rescue the *Csf1*$^{op/op}$ phenotype when expressed in the same spatiotemporal pattern as CSF-1. To address this hypothesis, we created a Tg construct, in which the *Csf1* promoter and first intron, previously shown to drive normal tissue-specific and developmental expression of CSF-1 [32], were used as drivers for full-length muIL-34 cDNA, encoding the +Q81 isoform (**Fig. 3A**), and used this construct to generate several independent Tg lines (**Table 1**). These transgenes were then introduced onto the *Csf1*$^{op/op}$ background. Only two of eight transgenes exhibiting germline transmission conferred significant rescue. The Tg40 transgene elicited a rescue comparable with the rescue of the *Csf1*$^{op/op}$ phenotype, which was observed previously in a strong rescue with the precursor of SGP using the same driver [42]. *Csf1*$^{op/op}$; *Tg40/+* mice exhibited normal male and female fertility. In addition, the growth rate (Fig. 3B), tooth eruption (Fig. 3C), F4/80+ bone marrow

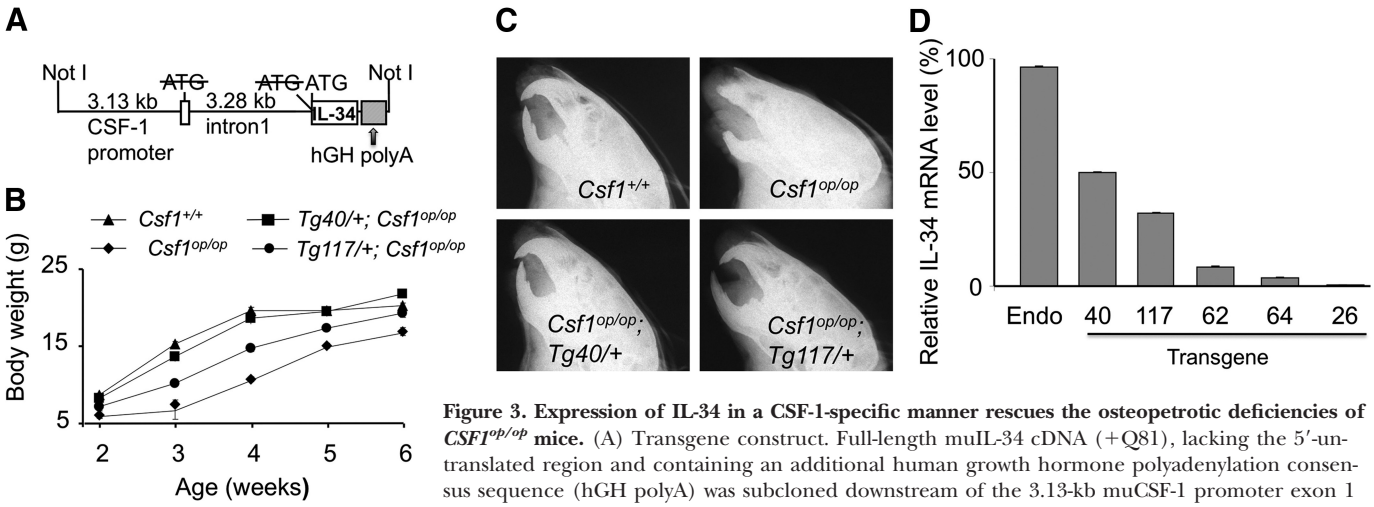


Figure 3. Expression of IL-34 in a CSF-1-specific manner rescues the osteopetrotic deficiencies of *CSF1*$^{op/op}$ mice. (A) Transgene construct. Full-length muIL-34 cDNA (+Q81), lacking the 5′-untranslated region and containing an additional human growth hormone polyadenylation consensus sequence (hGH polyA) was subcloned downstream of the 3.13-kb muCSF-1 promoter exon 1 and the 3.28-kb intron 1. (B) Growth curves of the female mice ($n \geq 3$ mice at each time-point; mean±SEM). (C) X-Radiograms showing wild-type incisor tooth eruption in *CSF1*$^{op/op}$ mice expressing Tg40 or Tg117 transgenes. (D) muIL-34 transgene mRNA expression relative to the level of endogenous IL-34 mRNA (Endo) in 2-month-old Tg spleens, determined by QRT-PCR (triplicate assays±SEM).

TABLE 1. Summary of *TgCsf1-1-IL-34* Mouse Lines

Founders	Germline transmission	Transgene mRNA level	Correction of gross phenotype of $Csf1^{op/op}$ mice					
						Macrophage numbers		
			Tooth eruption	Osteopetrosis	Growth rate	Femur	Kidney	Liver
26	T	—	NC	NC	NC	N/A	N/A	N/A
31	T	N/A	NC	NC	NC	N/A	N/A	N/A
39	T	N/A	N/A	NC	NC	N/A	N/A	N/A
40	T	+++	C	C	C	C	C	C
41	NT	N/A	N/A	N/A	N/A	N/A	N/A	N/A
53	T	N/A	NC	NC	NC	N/A	N/A	N/A
62	T	+	NC	NC	NC	N/A	N/A	N/A
64	T	+	NC	NC	NC	N/A	N/A	N/A
117	T	++	C	PC	PC	PC	PC	PC

T, Transmissible; NT, not transmissible; N/A, not done or not applicable; C, corrected; PC, partially corrected; NC, no correction.

(**Fig. 4C**), dermal (Fig. 4D), and kidney (Fig. 4E) macrophage deficiencies, together with the osteopetrosis (Fig. 4, A and B) and TRAP deficiences (Fig. 4H), were all corrected in $Csf1^{op/op}$; *Tg40/+* mice. The macrophage densities in kidney, which are low in the $Csf1^{op/op}$ mice and extremely sensitive to circulating CSF-1 [4], were quantitated in $Csf1^{op/op}$ (48±3), $Csf1^{op/op}$; *Tg40/+* (213±9), and $Csf1^{+/+}$ (224±4) mice (means±SD; F4/80+ cells/mm^2; two 2-week-old mice/group; ≥20 fields) and shown not to differ significantly between $Csf1^{op/op}$; *Tg40/+* and $Csf1^{+/+}$ mice (P>0.05 Student's t-test). In contrast, although tooth eruption was also corrected in $Csf1^{op/op}$; *Tg117/+* mice (Fig. 3C), their growth rate (Fig. 3B) and osteopetrosis (Fig. 4, F and G) were corrected only partially, in a manner resembling the rescue by the majority of the SG precursor transgenes [42]. To determine whether correction by the IL-34 transgenes was related to their level of expression, we determined the relative levels of transgene mRNA in total splenic RNA by QRT-PCR. The transgenes with the highest levels of expression were Tg40 and Tg117, and consistent with its more effective rescue, the expression of *Tg40* mRNA was ~50% higher than *Tg117* mRNA (Fig. 3D). The levels of expression of the three transgenes that failed to rescue were at least 3.5 times lower than the expression of Tg117. Importantly, the level of expression of *Tg40* mRNA was 50% of the level of expression of endogenous, splenic *IL34* mRNA. These experiments demonstrate that when IL-34 is expressed at high enough levels in the spatiotemporal pattern of CSF-1, it is able to rescue $CSF\text{-}1^{op/op}$ defects in a manner that mimics the action of SG.

Differential spatiotemporal expression patterns of *IL34* and *Csf1* mRNAs in embryonic and adult tissues

As IL-34 and CSF-1 have similar effects on CSF-1R-expressing target cells in vitro and in vivo, we next examined whether they also have a similar pattern of expression during development. As there is a dramatic increase in the level of expression of *Csf1* mRNA and protein in the uterus during pregnancy [43, 44], and they are also increased in the embryo during embryonic development [44, 45], we initially compared *IL-34* and *Csf1* mRNA expression in embryos, extra-embryonic tissue, and pregnant uterus by QRT-PCR. In contrast to the dramatic increase in uterine *Csf1* mRNA expression between E8.5 and E11.5, *IL34* mRNA levels were low and did not increase (**Fig. 5A**, left panel). In contrast to embryonic *Csf1* mRNA levels, which increased between E11.5 and E13.5, embryonic *IL34* mRNA was expressed at approximately the same level from E8.5 to E17.5 (Fig. 5A, middle panel). *Csf1* and *IL34* expression was similar in the placenta (Fig. 5A, right panel), and *Csf1r* mRNA expression in all tissues was as expected from previous studies [43, 45]. To compare the localized embryonic expression patterns of *IL34* and *Csf1*, embryos were subjected to whole-mount in situ hybridization for *IL34*, and embryos from TgZ/+ mice were stained with X-gal. In contrast to the significant staining of *IL34* mRNA in the telencephalon of E11.5 embryos (Fig. 5B), *Csf1* reporter expression was not apparent, appearing at E13.5 and mostly in other regions of the embryo (Fig. 5C). The expression of *Csf1r* mRNA previously reported in the brains of E12.5 embryos [46] is consistent with this early expression of *IL34*. Additional differences in expression of *IL34* and *Csf1* mRNAs were noted in adult tissues, particularly in brain, heart, and ear (**Fig. 6A**). In particular, *IL34* mRNA was differentially more highly expressed than *Csf1* mRNA in most of the regions of the developing and adult brain examined (Fig. 6B). Osteoblasts are a known source of CSF-1 and are likely to play a role in the hematopoietic stem cell niche [8, 47]. In cultured, primary calvarial osteoblasts (Day 5, ~70% pure by AP staining), *Csf1* mRNA was much more highly expressed than *IL34* mRNA (**Fig. 7**), in agreement with similar data reported for long-term cultures at biogps.gnf.org, where at 5 days of culture (their earliest time-point), IL-34 expression is lowest and CSF-1 expression highest, and their levels change reciprocally with time of culture thereafter. However, a 24-h stimulation with LPS or IFN-γ slightly increased *Csf1* and *IL34* levels (Fig. 7). These studies and similar data at biogps.gnf.org indicate that spatiotemporal ex-

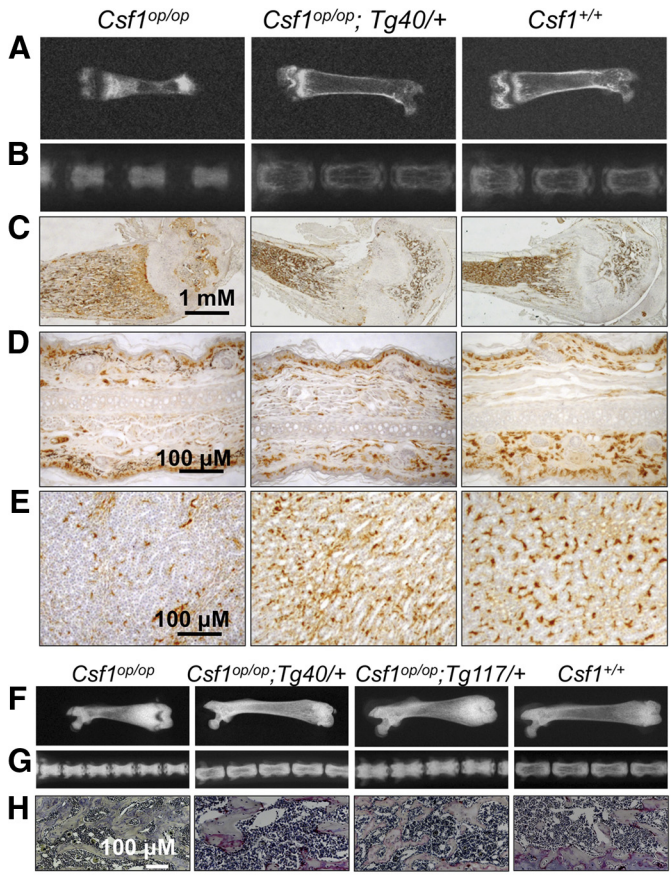


Figure 4. Dose-dependent correction of the *CSF1*$^{op/op}$ deficiencies by Csf1 promoter and first intron-driven IL-34 transgenes. Normal radiopacity in radiograms of the femurs (A) and tails (B) and normal macrophage densities in femoral bone marrow (C), ear (D), and kidney (E) in 2-week-old *Csf1*$^{op/op}$; *Tg40* mice. Partial correction of the osteopetrotic phenotype of femurs (F) and tails (G) and reduced TRAP+ (red) osteoclast staining (H) of 2-month-old *Csf1*$^{op/op}$; *Tg117* mice compared with the absence of obvious osteopetrosis and wild-type levels of osteoclast staining in *Csf1*$^{op/op}$; *Tg40* mice.

pression patterns of CSF-1 and IL-34 differ substantially, suggesting that this difference could lead to complementary regulation through the CSF-1R.

Absence of a compensatory increase in *IL34* mRNA expression in *Csf1*$^{op/op}$ mouse tissues

An important question concerning the *Csf1*$^{op/op}$ mouse is whether in the absence of CSF-1, there is a compensatory increase in the expression of IL-34 that might reduce the difference between the phenotypes of *Csf1*$^{op/op}$ and *Csf1r*$^{-/-}$ mice. At 3 weeks of age, there was no evidence for a significant compensatory increase in the levels of *IL34* mRNA in several tissues of *Csf1*$^{op/op}$ compared with wild-type mice (**Table 2**). However, the level *IL34* mRNA was more elevated in wild-type compared with the *Csf1*$^{op/op}$ ear, suggesting that IL-34 acts downstream of CSF-1, and its expression is positively regulated by CSF-1-mediated signaling via the CSF-1R in CSF-1R-expressing cells or in neighboring cells of the ear.

DISCUSSION

Previous studies showed that huIL-34 stimulated human monocyte survival/proliferation and macrophage colony formation by human bone marrow cells at similar concentrations to those at which huCSF-1 is effective [19]. Consistent with this, we observed that huIL-34 and huCSF-1 were equivalently active in stimulating the proliferation and early signaling in mouse macrophages, in which the muCSF-1R was replaced by the huCSF-1R. In our experiments with muIL-34, we compared the behavior of muCSF-1 with the behavior of two IL-34 isoforms in a variety of assays. These isoforms, derived by alternative splicing of the exon encoding Q81, are highly conserved, having been observed in mouse, rat, chimpanzee, and human. The loss of Q81 is predicted to disrupt the second-most amino terminal of the four α-helices of muIL-34. Both IL-34 isoforms had a higher EC_{50} for macrophage proliferation and a lower affinity for the CSF-1R that was associated with their lower rates of association. In addition, they were less active in stimulating the proliferation and differentiation of macrophage progenitor cells and in osteclastogenesis, and muIL-34 (–Q81) was less active than muIL-34 (+Q81) in all of these assays. Furthermore, although the kinetics of ligand-induced tyrosine and ERK phosphorylation of muCSF-1 and muIL-34 were similar, mulL-34 (+Q81) was less effective, mole for mole, in stimulating CSF-1R signaling. The lower activity of muIL-34 compared with muCSF-1 in the osteoclastogenesis, macrophage progenitor, and macrophage proliferation assays may explain why the macrophage and osteoclast deficiencies of *Csf1*$^{op/op}$ mice, although less than those of *Csf1r*$^{-/-}$ mice, are relatively severe. However, in the absence of information on the relative tissue abundance and circulating levels of IL-34 protein in mouse and human, it is difficult to speculate whether its role in these respects is limited in the mouse. Importantly, at concentrations that compensate for the weaker affinity of muIL-34 for the muCSF-1R, muIL-34 activation of the muCSF-1R results in stimulation of hematopoietic and osteoclast progenitor proliferation and differentiation that is indistinguishable from the stimulation by muCSF-1. These findings, together with the similarity between huIL-34 and huCSF-1 (Fig. 1B) and between muIL-34 and muCSF-1 (Fig. 2, G and H, and Supplemental Fig. 2) in their ligand-induced CSF-1R tyrosine phosphorylation and ERK1/2 activation responses, suggest that once bound by the receptor, CSF-1 and IL-34 similarly activate it to mediate responses.

Based on these similar in vitro activities of IL-34 and CSF-1, we hypothesized that expression of IL-34 in the spatiotemporal expression pattern of CSF-1 could correct the *CSF-1*$^{op/op}$ phenotype. One Tg line, *Tg40*, was able to rescue, to a degree comparable with wild-type, and with the rescue achieved by a strong, wild-type level of expression of the precursor of the SG (*TgSGP*) [42]. In addition, one other line, *Tg117*, exhibited only partial rescue in a manner similar to what was observed with most *TgSGP* Tg lines [42]. However, consistent with the lower affinity of muIL-34 for the muCSF-1R compared with muCSF-1, only two of the eight transmitting *TgCsf1-IL-34* lines rescued the *Csf1*$^{op/op}$ defects. By comparison, at least partial rescue was observed by all four *TgSGP* Tg lines and complete

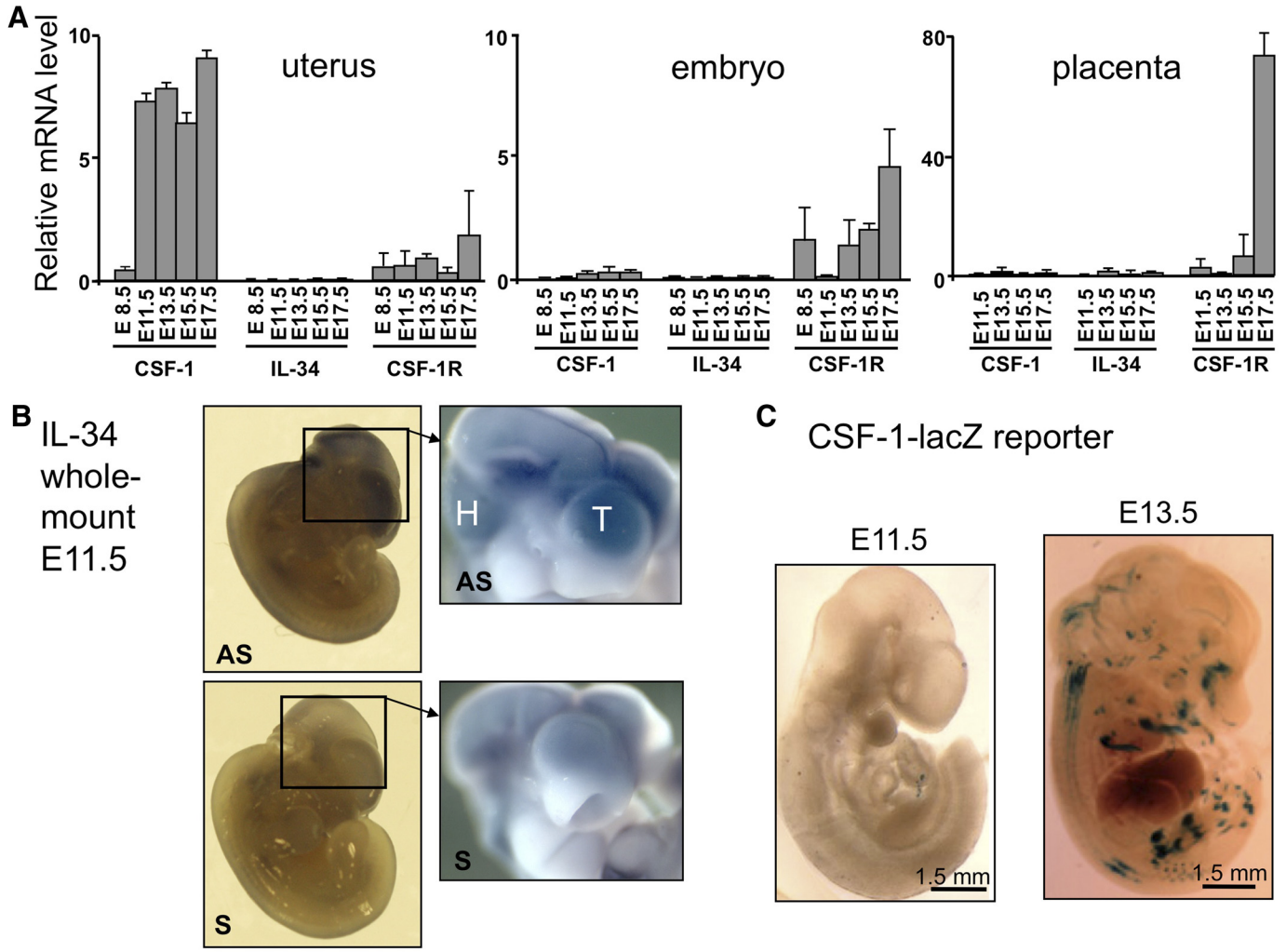


Figure 5. Differential expression of CSF-1, IL-34, and CSF-1R mRNA in embryos and extra-embryonic tissues. (A) QRT-PCR measurement of CSF-1, IL-34, and CSF-1R mRNA expression in E 8.5–E17.5 uteri, embryos, and placentae. Average of duplicates from two FVB/NJ mice; bars indicate range of duplicates. mRNA levels are normalized with respect to β-actin mRNA. (B) Whole-mount in situ hybridization of IL-34 mRNA in E11.5 embryos. AS, Antisense probe; S, sense probe; H, hind brain; T, telencephalon. (C) X-gal staining of E11.5 and E13.5 TgZ embryos.

rescue by six of six transgenes encoding the precursor of the SP (TgSPP) [42]. That rescue by the *TgCsf1-IL-34* Tg lines was a result of their higher level of expression is indicated by the rank order of expression reflecting the degree of rescue (Fig. 3D). It is likely that the rescue observed in the case of the Tg40 line was not a result of massive overexpression but rather, a normal spatiotemporal expression pattern for CSF-1, as the splenic Tg40 expression was only half that of the endogenous splenic IL-34. Thus, these in vivo observations support results of the in vitro studies, which suggest that there are no major differences in the biological response of activation of the CSF-1R by one ligand or the other.

If IL-34 has a nonredundant role in regulating CSF-1R-expressing cells, the corollary of the above conclusion is that IL-34 and CSF-1 should be expressed differentially in vivo. Our expression studies, although compromised by a lack of reliable antibodies and therefore, limited to mRNA expression, clearly indicate that this is the case. Embryonic *IL34* mRNA expression is low, detectable at E8.5, and does not change with embryonic development, whereas CSF-1 is comparatively higher than *IL34* expression, develops at E13.5, and increases with time. *IL34* expression is detectable in embryonic brain at E11.5 prior to the appearance of *Csf1* reporter expression at E13.5. Although mRNAs for both ligands (and their shared receptor) are broadly expressed in adult tissues, some differential expression is evident. In particular, mimicking the embryonic *IL34* expression pattern, *IL34* is expressed more strongly than CSF-1 in most areas of the postnatal and adult brain examined. This result points to a significant role for IL-34 in the brain, especially as *CSF-1*$^{op/op}$ have only a minor reduction in the number of microglia [15, 16], and our preliminary results (S. Nandi and E. R. Stanley, unpublished) indicate a substantial reduction of microglia in the brains of *Csf1r*$^{-/-}$ compared with

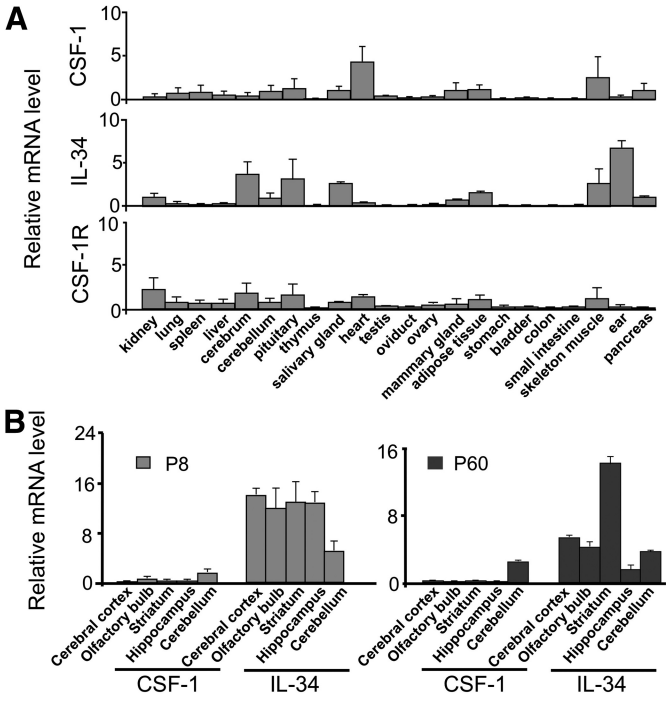


Figure 6. Broad but differential expression pattern of CSF-1, IL-34, and CSF-1R mRNAs in adult tissues. (A) mRNA expression in 2-month-old adult tissues determined by QRT-PCR analysis of tissue RNA. (B) CSF-1 and IL-34 mRNA levels in RNA isolated from the indicated regions in Postnatal Day 8 (P8) and P60 brains. Average of duplicates from two FVB/NJ mice; bars indicate range of duplicates. mRNA levels are normalized with respect to β-actin mRNA.

Csf1r$^{op/op}$ mice. Levels of *IL34* mRNA are also higher in salivary gland and particularly, in the ear, where the development of dermal macrophages and Langerhans cells is regulated by the CSF-1R [4, 13, 18]. A third area of differential expression was revealed in cultured osteoblasts, where levels of *IL34* mRNA are substantially lower than *Csf1* mRNA. This could reflect a less-important role for IL-34 than CSF-1 in osteoclastogenesis and hematopoiesis. This would be consistent with the strong skeletal phenotype of *Csf1*$^{op/op}$ mice, which on an outbred background, almost matches that of the *Csf1r*$^{-/-}$ mice. Thus, although the importance of comparing the expression of CSF-1 and IL-34 proteins cannot be underestimated, these mRNA expression/reporter results point to differences in spatiotemporal expression of IL-34 and CSF-1 and point out tissues in which the significance of the differences in their expression can be pursued.

The differential expression of IL-34 and CSF-1, coupled with the absence of a compensatory increase in the levels of tissue IL-34 mRNA in CSF-1$^{-/-}$ mice, is consistent with CSF-1 and IL-34 having independent roles in regulating CSF-1R-expressing cells and that their actions through the CSF-1R will be largely complementary. However, by comparison with the wild-type ear, we observed lower levels of *IL34* mRNA in the *Csf1*$^{op/op}$ ear. Loss of CSF-1 in the ear has been associated with a significant loss of deep dermal macrophages [4, 18] and of the epidermal Langerhans cells [13, 18]. IL-34 expression in the ear could therefore be under the control of CSF-1-mediated signaling through these cell types, which synthesize IL-34 directly or in a paracrine manner, induce its synthesis by other cell types. Identification of IL-34-expressing cells using IL-34 immunohistochemistry or *IL34* reporter mice could provide valuable information about the nature of the IL-34-expressing cells and their regulation by CSF-1.

Apart from defining a differential expression of CSF-1 and IL-34, we have shown that when expressed as CSF-1, IL-34 exhibits the same kind of biological function as CSF-1. Thus, if the CSF-1R is the only receptor for IL-34, the effects of these ligands on development and function should be complementary. However, should IL-34 also use another receptor, the situation would be more complex. In the former case, the targeted inactivation of the *IL34* gene is expected to resemble aspects of the *Csf1r*$^{-/-}$ phenotype but be less severe. A more severe phenotype of *IL-34*$^{-/-}$ mice would indicate the existence of another IL-34R or alternatively, that the action of embryonic IL-34 on maternally expressed CSF-1Rs is necessary for embryonic survival. Irrespective of the existence of another receptor for IL-34, our studies indicate that the analysis of regulation of tissue development and function by IL-34 will be rewarding.

AUTHORSHIP

S. W.: Performed and conducted the core experiments, collected and analyzed data, participated in manuscript preparation. S. N.: Brain dissection, calvarial osteoblast experiments, competitive binding studies, proliferation assays, lacZ staining, assisted in preparation of manuscript. V. C.: Colony assays, in vitro osteoclast differentiation. Y-G. Y.: Proliferation, signaling, competitive binding studies. W. Y., M. H., L. T. W., and H. L.: Signaling studies. E. R. S.: Research design and supervison, data analysis, colony assays, manuscript preparation.

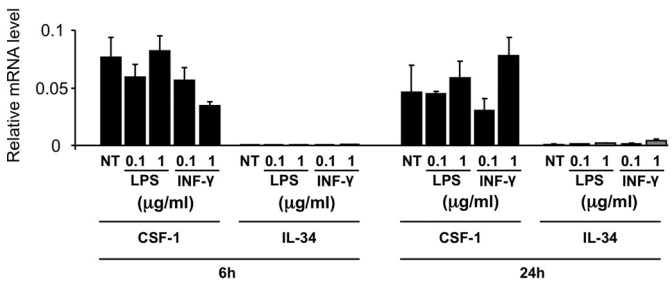


Figure 7. Differential expression of IL-34 and CSF-1 mRNAs in primary osteoblasts and regulation of expression by LPS and IFN-γ. Cultured primary cavarial osteoblasts were incubated with the indicated concentrations of LPS or IFN-γ (INF-γ) for the indicated times at 37°C prior to isolation of RNA for estimation of mRNA by QRT-PCR. mRNA levels are normalized with respect to β-actin mRNA. Means ± SEM; $n = 3$; NT, not treated. All results obtained for muCSF-1 are significantly different from the corresponding results with muIL-34 ($P<0.05$; Student's *t*-test).

TABLE 2. Absence of Compensatory Expression of IL-34 mRNA in *Csf1*$^{op/op}$ Mice

Mouse	Relative tissue IL-34 mRNA level[a]				
	Ear	Skeletal muscle	Spleen	Salivary gland	Liver
Csf1$^{+/+}$	13.88 ± 3.84[b]	0.68 ± 0.01[c]	0.21 ± 0.10[c]	1.44 ± 0.68[c]	0.33 ± 0.21[c]
Csf1$^{op/op}$	2.56 ± 1.87[b]	0.33 ± 0.09[c]	0.52 ± 0.13[c]	2.64 ± 1.90[c]	0.19 ± 0.03[c]

[a]QRT-PCR measurement of tissue mRNA from 3-week-old *Csf1*$^{+/+}$ and *Csf1*$^{op/op}$ mice. [b]Means ± SEM; $n \geq 3$; $P < 0.01$; Student's *t*-test. [c]Average and range of duplicate estimations of RNA from tissues of two FVB/NJ mice.

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