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Macrophage lineage phenotypes and osteoclastogenesis—Complexity in the control by GM-CSF and TGF-β

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Abstract

Bone-resorbing osteoclasts (OCs) derive from macrophage lineage precursors under the potential control of many factors. Addition of macrophage-colony stimulating factor (M-CSF or CSF-1) to murine bone marrow cells gives rise to so-called bone marrow-derived macrophages (BMM); this adherent population can then be quantitatively converted into OC lineage cells when receptor activator of NFκB ligand (RANKL) is included. The effect of another CSF, granulocyte macrophage-CSF (GM-CSF), on OC differentiation *in vitro* is quite complex with both enhancing and suppressive actions being described. We report here that GM-CSF can generate a population of adherent macrophage lineage cells from murine bone marrow precursors (GM-BMM) which is also capable of giving rise to OC lineage cells in the presence of M-CSF and RANKL as effectively as BMM. The degree of this differentiation was surprising considering that GM-BMM are often referred to as immature dendritic cells and that, for both BMM and the GM-BMM, GM-CSF suppressed subsequent OC differentiation governed by M-CSF and RANKL. Unlike for BMM, this GM-CSF-mediated suppression for GM-BMM appeared to be independent of c-*fos* expression.

The effects on bone of another cytokine, transforming growth factor-β (TGF-β), are also quite complex although usually found to be stimulatory for OC differentiation. Unexpectedly, we observed that TGF-β1 also potently suppressed M-CSF+RANKL-driven OC differentiation from both BMM and GM-BMM. Using cells from gene-deficient mice, this inhibition of OC differentiation by both GM-CSF and TGF-β1 appeared to be independent of endogenous interferon α/β production.

It appears therefore that the influence of GM-CSF and TGF-β on osteoclastogenesis depends on the presence or otherwise of other stimuli such as RANKL and possibly upon the maturation state of the OC precursors. It is proposed that the findings have particular relevance for the control of bone resorption in pathology, for example, in inflammatory lesions.

Keywords: Cytokines; Inflammation; Transforming growth factor-β; Osteoclast; c-*fos*; Cell number; Interferon; Bone; Cell density

Introduction

An understanding of the complexity of the control of bone remodeling by the osteoclast (OC), particularly in pathology, for example, in inflammatory joint disease [52], requires the development of suitable, purified cell populations to allow the molecular events governing both OC development and activation to be delineated [51]. In normal bone turnover, key communication between OCs and the bone-forming osteoblasts occurs, and key cytokines controlling OC development are macrophage-colony stimulating factor (M-CSF or CSF-1) and receptor activator of NFκB ligand (RANKL) [14,49,65,72]. Bone-resorbing OCs belong to the myelomonocytic lineage and can be generated from monocytes or even macrophage populations [51,66]. Osteoclast progenitors migrate to the

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bone surface where they differentiate and fuse to form multinucleated OCs, but how commitment is established is only partly understood. The bone microenvironment, including the presence of stromal cells (pre-osteoblast precursors) and mature osteoblasts, appears to be essential for OC differentiation [60]. Osteoblasts/stromal cells contribute to osteoclast differentiation by supplying RANKL [14,32,65] and M-CSF (or CSF-1) [57,65]. *In vivo* data from mice with a null mutation of the RANKL gene support these *in vitro* observations [47]. Osteoclastogenesis has been shown to be directly and indirectly regulated by the effects of growth factors, cytokines and hormones acting on OC precursors and osteoblasts, and a number of recent studies have shown an effect of these factors on osteoclastogenesis in pathological states (see [73] for a review).

One of the difficulties faced in understanding osteoclastogenesis at the molecular level is that heterogeneous populations have often been used in culture. These can consist of osteoblasts/stromal cells and OC precursors, the latter from tissues such as bone marrow or spleen; even if the former cells are not present at the start of the culture, the OC precursors usually still represent only a small proportion of the population, and it is not easy to assess the relationship of the number of OCs ultimately generated to the number of the precursors. What is needed therefore are culture systems which are initiated with large numbers of enriched OC precursors [51] and which also monitor the changes in OC lineage cell number during the differentiation process.

Recently, developed cell culture systems have enabled OCs to be generated from precursors *in vitro* in the presence of only M-CSF and RANKL (see, for example, [32,39,42,51]). M-CSF by itself can generate murine macrophage populations *in vitro* in large numbers from bone marrow precursors by proliferation and differentiation; these adherent cells are widely termed bone marrow-derived macrophages (BMM) [51]. Recently, we and others have shown that BMM can be rapidly and quantitatively converted into OCs [51,64]. This culture system has the advantage that BMM can be produced in large numbers, and are highly purified, making them suitable for analysis of the signal transduction pathways governing OC differentiation.

In addition to developmental changes giving rise to subpopulations, macrophage lineage cells respond to a number of stimuli in various ways, thereby generating heterogeneity in function; recently, on the basis of these various responses, macrophages have been classified into different phenotypes (see [36] for a review). M-CSF-treated macrophage populations have been considered by some to have an "anti-inflammatory" phenotype [67]. Another CSF, granulocyte macrophage-CSF (GM-CSF), was first defined by its ability to generate granulocyte and macrophage colonies from precursor cells *in vitro* [2]. However, significant actions for GM-CSF would appear to be on the more mature cells themselves, for example, as part of its action as a proinflammatory cytokine [18,67]. It can prime/activate monocytes/macrophages to produce a number of proinflammatory and host-protective mediators [18], and it is widely used to generate dendritic cells (DCs) or antigen presenting cells *in vitro* [23]. Along with other cytokines, GM-CSF has been considered as generating a "proinflammatory" phenotype in macrophages [67].

Studies examining the effect of GM-CSF on osteoclastogenesis appear to have led to contradictory results. In some studies, GM-CSF has been reported to induce OC-like cell formation [21,31,40,59]. In contrast to these studies showing a stimulatory or facilitating effect of GM-CSF on osteoclastogenesis, other studies examining the effect of GM-CSF in co-cultures of bone marrow cells with osteoblasts [22], and in cultures of murine [42,43] and human [29] precursors containing RANKL and M-CSF, have reported an inhibitory effect on OC formation. The basis for these apparently contradictory results reached by these different approaches is not clear. Given this uncertainty as to the effect of GM-CSF on OC differentiation and the potential significance of its increased production at sites of inflammation where bone is being resorbed [8], we sought to clarify the effect of GM-CSF on osteoclastogenesis using highly enriched OC precursor populations.

Transforming growth factor-β (TGF-β) is another cytokine, present in bone, whose role in bone turnover is quite complex with conflicting *in vivo* and *in vitro* data on whether resorption is enhanced or inhibited [10,37,74]. Once again, there is a requirement for enriched OC progenitor populations if its influence on osteoclastogenesis is to be understood; all studies with murine precursors [6,11,13,26,28,30,50,71], including our own with non-adherent BMM precursors [51], suggest that TGF-β potentiates OC development *in vitro*.

We report here, using a new assay designed to quantitate OC cell number from enriched precursors, that GM-CSF can generate *in vitro* a purified population of adherent macrophage-like cells from bone marrow which surprisingly could give rise to OCs as efficiently as their counterparts generated by M-CSF (i.e., BMM), indicating that they have not committed to the DC lineage. We also found unexpectedly that TGF-β1 suppressed osteoclastogenesis driven by M-CSF + RANKL from both of the above enriched OC precursor populations. Thus, the effects of GM-CSF and TGF-β on OC differentiation depend critically upon the degree of maturation of the precursors and to what else they are exposed to at the time.

Materials and methods

Animals and reagents

C57B1/6 mice (5–10 weeks), purchased from Monash University (Clayton, Australia), were the source of bone marrow cells. GM-CSF gene-deficient (GM-CSF$^{-/-}$) mice, backcrossed onto the C57BL/6 background for 11 generations, were originally provided by the Ludwig Institute for Cancer Research (Parkville, Victoria, Australia) [3]. IFNR1$^{-/-}$ mice, backcrossed on the C57BL/6, were provided by Monash Medical Centre (Monash University) [9]. Mice, female and 8–12 weeks of age, were used in all experiments.

The following recombinant cytokines were used: recombinant human RANKL (PeproTech), murine GM-CSF (specific activity 3×10^7 U/mg; Amgen), human M-CSF (specific activity 1×10^7 U/mg; Chiron), murine IL-4 (PeproTech) and human TGF-β1 (R&D Systems USA). Commercial monoclonal antibodies (mAbs) against the following antigens were used: CD11b (Mac-1 α chain; M1/70-APC, BD Biosciences), F4/80 (CI: A3-1-biotinylated, Caltag Laboratories), CD11c (HL3-PE, BD Biosciences), CD80 (B7-1-PE, BD Biosciences) and CD86 (B7-2-FITC, BD Biosciences). The mAb derived from the hybridoma to MHC class II (M5/114.15.2) was obtained from the American

Type Culture Collection. The mAbs against the following antigens were gifts: Ly6C (ER-MP20) and ER-MP58—these are used as markers of murine macrophage development [34] (Dr. P.J.M. Leenen, Erasmus University, Rotterdam, The Netherlands); c-Fms (M-CSF receptor; ASF-98) [58] (Dr. S-I. Nishikawa, Kyoto University, Japan). APC-conjugated donkey anti-rat IgG (H+L, $F(ab')_2$ fragment (Jackson Immuno Research Lab Inc), FITC-conjugated anti-rat IgG (Chemicon) and streptavidin-PE (BD Biosciences) were used as secondary antibodies.

Preparation of BMM and GM-CSF-derived macrophage populations

Adherent BMM were prepared as before [51], and GM-CSF-dependent bone marrow-derived macrophages (GM-BMM) were generated by a similar protocol. Briefly, bone marrow cells were flushed from long bones of mice in RPMI 1640 medium (Gibco), centrifuged, and the cell pellet resuspended (10^6 cells/ml) in RPMI containing 10% heat-inactivated fetal bovine serum (FBS; CSL Biosciences, Parkville, Australia) in either 5000 U/ml M-CSF (for BMM) or 1000 U/ml GM-CSF (for GM-BMM). The medium was also supplemented with penicillin (100 U/ml), 100 μg/ml streptomycin sulfate (Gibco) and 2 mM L-glutamine (Gibco). After 3 days, non-adherent cells were harvested and 3×10^6 cells were seeded in 10 ml of the same medium in non-treated, 100 mm dishes (Iwaki, Japan) for another 4 days. The adherent cells were harvested and used for OC differentiation. This time point is referred to as day 0. For the generation of immature DC (iDC), bone marrow cells were cultured at 10^6 cells/ml in medium containing 500 U/ml of GM-CSF and 1 ng/ml of IL-4 in tissue culture flasks (BD Biosciences). For all cell types, on days 3 and 5, 80% of the medium was replaced by the cytokine-containing medium. On day 7, cells in suspension, and those dislodged by vigorous pipetting, were discarded, leaving only adherent cells.

Flow cytometry

Cell staining for flow cytometric analysis, including isotype controls and Fc receptor blocking, was carried out as described previously [7]. Briefly, cells were incubated with the primary antibody (unlabeled or biotin-labeled) followed by an appropriate secondary antibody and directly conjugated antibodies (for more than one-color staining). A typical forward and side scatter gate was set to exclude dead cells and aggregates; a total of 10^4 events in the gate were collected and analyzed using a FACSort (BD Biosciences).

OC differentiation in vitro

The adherent BMM and GM-BMM were harvested by gentle scraping with a rubber policeman and cultured in three ways to generate OC lineage cells. Firstly, using Terasaki culture dishes (Nunc), 1500 cells were seeded per well in 5 μl of RPMI/10% FBS and incubated at 37°C for 1 h. The medium was gently removed, and a total of 20 μl of RPMI/FBS containing cytokine(s) was added to the wells. Cells were cultured up to 9 days with one careful change of medium every 3 days. OC differentiation was determined by TRAP staining following fixation in formaldehyde and acetone/alcohol [51]. Because cells in commonly used culture wells typically form markedly non-uniform distributions (particularly in large culture wells), the total number in a well cannot be estimated from the numbers found in samples of the well area. However, the entire base of the well of the Terasaki plate can be included in a single microscope view (at 100× magnification). Total cell numbers and of the proportion of the total mononuclear and multinuclear cells that are TRAP positive were estimated in an area of 1.3 mm^2.

Secondly, using 6-cm tissue culture dishes (BD Bioscience), cells were plated at a density of 3×10^6 cells per dish in 5 ml of medium (RPMI/FBS) containing cytokine(s) and incubated for either 48 h or 6 days with one change of medium at day 3. OC differentiation was investigated by osteoclastic gene expression.

Thirdly, using 96 well tissue culture dishes, cells were plated at 3×10^4 cells per well, containing cortical bone slices, in 0.1 ml RPMI/FBS containing M-CSF (5000 U/ml) and RANKL (50 ng/ml). Cells were incubated for 21 days, with complete change of medium and mediators twice per week. OC formation was investigated by detection of resorption pits in the bone.

RNA extraction and quantitative reverse transcription polymerase chain reaction analyses

Total RNA was isolated from BMM and GM-BMM at day 0 and also from cultured cells, according to the manufacturer's instructions using the RNAeasy kit (Qiagen).

cDNAs were synthesized from 1 μg total RNA by reverse transcription. RNA was diluted in distilled water (total volume 12 μl) followed by addition of 1 μl of random hexamer p(dN6) (500 ng/ml; Promega, Madison, WI, USA) and incubated for 10 min at 70°C. cDNA was then synthesized in a total volume of 20 μl, containing 1 μl of dNTP mixture at 10 mM (Pharmacia), 1 μl of 10 mM dithiothreitol (Gibco-BRL), 1 μl of reverse transcriptase (Superscript III RNase; Gibco-BRL) and 4 μl of the recommended buffer (Gibco-BRL). The reaction mixtures were incubated at 37°C for 50 min followed by 10 min of heat inactivation at 70°C. Pre-Developed TaqMan Assay Reagents (Applied Biosystems, Scoresby, Victoria, Australia) were used for sequence detection of CTR, Cath K, RANK, c-*fos*, CD11c, CD86, and CD80. Quantitative polymerase chain reaction (Q-PCR) analyses were used to quantify transcripts with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Scoresby, Victoria, Australia).

Pit formation assay

Cells were removed from bone slices by brief sonication and lysing in 1% of Triton-X 100 for 30 min. Hematoxylin was applied to the resorbed surface of each slice, and the residual stain was removed by wiping against absorbent paper. Resorption was detected by transmission light microscopy [21].

Statistical analysis

Data are represented as means±standard error (SE). Significant differences were determined using the two-tailed Student's *t*-test; a p value<0.05 was considered as significant.

Results

Generation of highly enriched osteoclast-lineage precursors by GM-CSF

As mentioned, BMM are a convenient precursor system to study OC differentiation [51,64]. In the first part of the current study, we determined whether the progeny of GM-CSF-treated murine bone marrow cells, following proliferation and differentiation *in vitro*, were also able to be differentiated efficiently along the OC lineage. Bone marrow cells were therefore cultured in GM-CSF for 7 days and the adherent cells collected. Some properties of these cells were initially assessed and a comparison made with BMM. The morphology of the two adherent populations differed, with the GM-CSF-derived cells displaying a rounded morphology in culture, and the BMM appearing elongated and spindle-shaped [64]. When the GM-CSF-derived cells and BMM were treated with either CSF for a further 6 days, GM-CSF still tended to produce a more rounded morphology and M-CSF an elongated one; when the CSFs were added together, the two types of morphology were apparent for both starting populations.

Flow cytometry was used to characterize the two populations by surface marker expression. As summarized in Table 1, both populations were positive for Mac-1, F4/80 and c-Fms, consistent with their belonging to the macrophage lineage—we have previously demonstrated that c-Fms (M-CSFR or CSF-1R) cell surface expression is a useful marker for defining macrophage

Table 1
Surface marker expression in GM-BMM and BMM

Antigen	GM-BMM	BMM	iDC
Myeloid/monocytic			
CD11b (Mac-1)	94±3		
F4/80	82±4	83±5	87±6
c-Fms (M-CSFR)	75±3	81±4	49±8[b]
Maturation			
ER-MP58	94±3	95±2	87±3
Ly6C (ER-MP20)	38±8	34±6	33±6
Immune/accessory			
CD11c	47±2[a]	3±1	63±5[c]
MHC class II	32±7	29±5	27±4
CD80	88±2	86±3	85±2
CD86	6±1	5±1	10±2

GM-CSF-derived bone marrow cells (GM-BMM), BMM and iDC were generated *in vitro* (Materials and methods). The percentage of GM-BMM, BMM and iDC positive for various cell surface markers was determined by flow cytometry (Materials and methods). Data are expressed as mean percentages (±SE) from three independent experiments.

[a] $p<0.001$ compared to CD11c surface expression on BMM.

[b] $p<0.02$ compared to M-CSFR (c-Fms) on GM-BMM.

[c] $p<0.04$ compared to CD11c surface expression on GM-BMM.

lineage cells [7]. Both populations were also positive for ER-MP58 (Table 1), a marker found on monocytes but not normally on tissue macrophages, and a similar percentage of cells from each population was positive for the more immature myeloid cell marker Ly6C [34]. Detection of both Ly6C and ER-MP58 suggests that both populations are not fully differentiated consistent with their high proliferative capacity; Ly6C expression is lost as precursor cells develop into BMM [34], data consistent with that in Table 1 where it can be seen that most of our BMM were Ly6C negative. However, both populations were negative for CD31 (PECAM-1, ER-MP12), a very early immature myeloid marker [34] (data not shown). Like BMM, the GM-BMM were capable of rapidly phagocytosing 1 μm diameter latex beads (data not shown). Our BMM are prepared in a similar fashion to osteoclast progenitors that have been extensively characterized [64] and appear to be phenotypically similar.

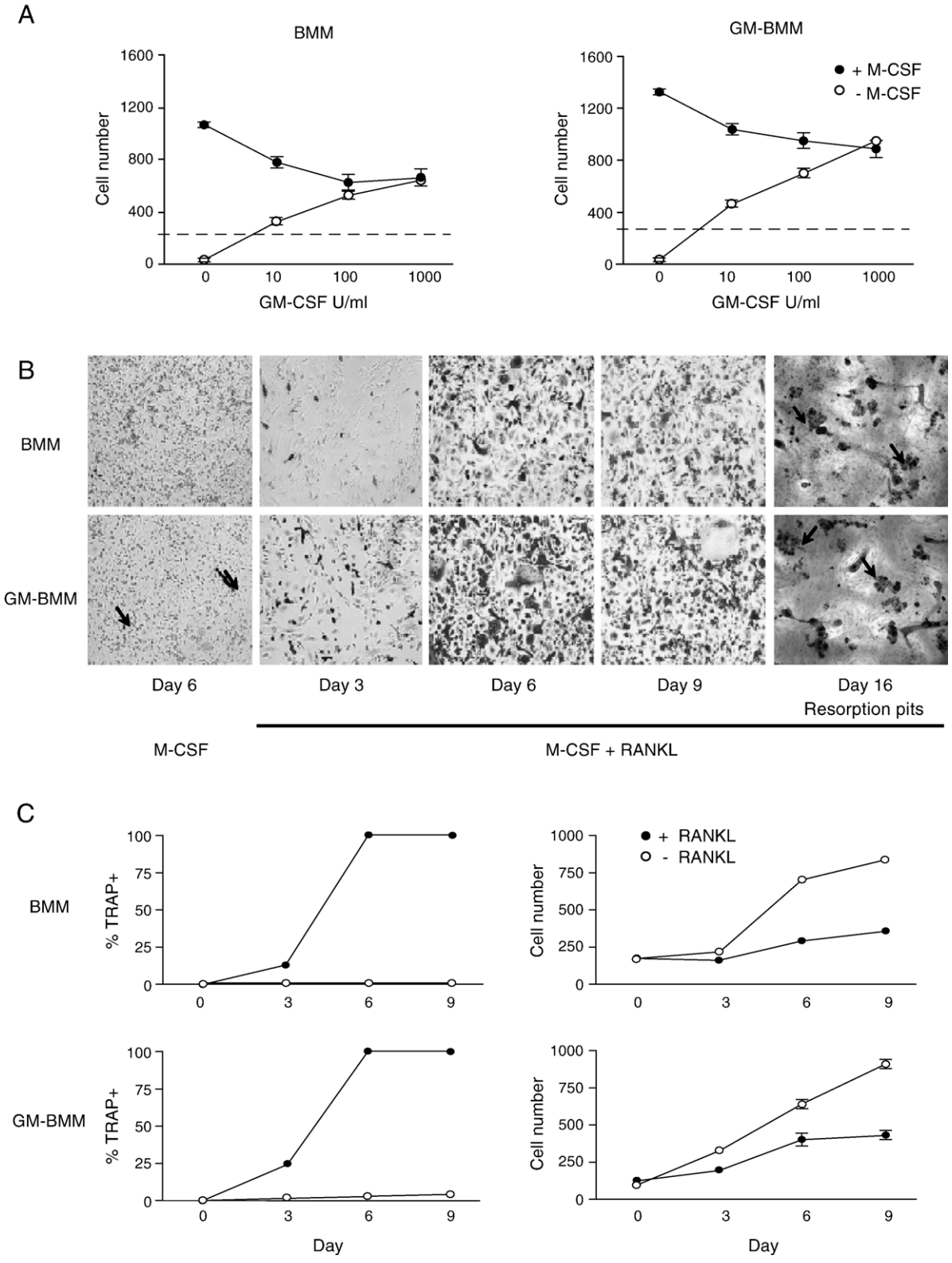
GM-CSF, in conjunction with cytokines such as IL-4 or TNFα, is often used to generate DC lineage populations [23,35,38]. CD11c is frequently used as a surface marker in murine systems to identify DC lineage cells, although it can be found on certain macrophage populations such as alveolar macrophages [46]. In contrast to the markers above, significantly more of the GM-CSF-derived cells expressed CD11c as compared with BMM, which were essentially negative for this marker (47±2% vs. 3±1%; $p<0.001$, Table 1). Following this observation, a further comparison was made with bone marrow cells grown in GM-CSF and IL-4 and referred to as immature DC (iDC) for the purposes of this study. A significantly greater percentage of these iDC expressed CD11c compared to their counterparts derived in the absence of IL-4 (63±5% vs. 47±2%; $p<0.04$, Table 1), while significantly less iDC were positive for c-Fms (M-CSFR) (49±8% vs. 75±3%; $p<0.02$, Table 1). The percentage of iDC positive for MHC class II, CD80 and CD86 was no different from the other two populations in question (Table 1). Seeing that the GM-CSF-derived cells are adherent and phagocytic and that, apart from CD11c, their surface marker expression is similar to that for BMM, for convenience, we shall refer to them as GM-BMM. Other comparisons, such as their vastly different abilities to produce cytokines upon stimulation and to stimulate allogeneic T cells, have been made elsewhere (in preparation).

In vitro studies on osteoclastogenesis usually express the degree of differentiation at the time of assay as the number of multinucleated cells per unit area or as the percentage of cells positive for a marker, usually TRAP. These readouts are not usually related to the number of cells present at the start of the culture. Since cellular differentiation is commonly accompanied by a reduction in proliferation rate, we considered it desirable to establish an osteoclastogenesis assay which could also monitor cell number changes in response to different stimuli. This assay is described in the Materials and methods section and involves the addition of low numbers of cells into the small wells of a Terasaki plate in order to make it easy to count total cell numbers in a large number of replicate cultures. The data obtained below using this assay are presented as the total number of OC lineage cells, i.e., mononuclear and multinuclear cells, although their respective numbers could have been reported. Using this assay, we first monitored the proliferative effects of GM-CSF and M-CSF on both GM-BMM and BMM. GM-BMM had a higher proliferative response to different GM-CSF concentrations than BMM ($p<0.05$) (Fig. 1A). When compared to BMM, they had a similar proliferative response to M-CSF in this assay—the data for an optimal concentration (5000 U/ml) are provided in Fig. 1A. The effect of different GM-CSF concentrations on the proliferative response to this optimal M-CSF concentration was also examined. It can be seen for both cell populations that GM-CSF suppressed the proliferative response to the optimal M-CSF concentration in a dose-dependent manner, the result

Fig. 1. Proliferation and osteoclastogenesis in BMM and GM-BMM. (A) BMM and GM-BMM were cultured in increasing concentrations of GM-CSF either in the absence (○–○) or presence (●–●) of M-CSF (5000 U/ml) in Terasaki wells and the cell number measured after 6 days (Materials and methods). For both cell types at the concentrations shown, GM-CSF suppressed the proliferative response to M-CSF (5000 U/ml) ($p<0.05$). Data are expressed as mean±SE from triplicate cultures and are from a representative experiment which was repeated three times. The total cell number at the start of the experiment (day 0) is indicated by the dashed horizontal line. (B) BMM and GM-BMM were treated with M-CSF (5000 U/ml) for 6 days and with M-CSF+RANKL (50 ng/ml) for 3, 6 and 9 days (100× magnification). Cells were stained for TRAP expression. Arrow indicates TRAP positive cells in GM-BMM in the presence of M-CSF alone. BMM and GM-BMM were also cultured in the presence of M-CSF and RANKL (50 ng/ml) on bone for 16 days (Materials and methods). The dentine bone slices were stained with hematoxylin (100× magnification). Arrow indicates pits on bone surface. (C) BMM and GM-BMM were cultured in M-CSF (5000 U/ml) in the absence (○–○) or presence (●–●) of RANKL (50 ng/ml) in Terasaki wells for 3, 6 and 9 days. The proportion (%) of TRAP positive cells and cell number (mononuclear and multinuclear) were counted (Materials and methods). A multinuclear cell was counted as a single cell. Values are mean±SE from triplicate cultures and are from a representative experiment which was repeated twice.

with BMM confirming previous observations with a different assay [19].

We and others have previously shown that BMM could be converted quantitatively to TRAP$^+$ cells in the presence of M-CSF and RANKL [51,64]. Perhaps surprisingly (see below), GM-BMM can be similarly converted into TRAP$^+$ cells (Fig. 1B). Between days 3 and 6, all cells in both populations were usually differentiated into mainly mononuclear TRAP positive cells. To assess the functional capability of GM-BMM-derived OC lineage cells, their ability to resorb bone was monitored. GM-BMM and BMM were cultured on bone slices. In both groups of the cells, pits formed in presence of M-CSF and



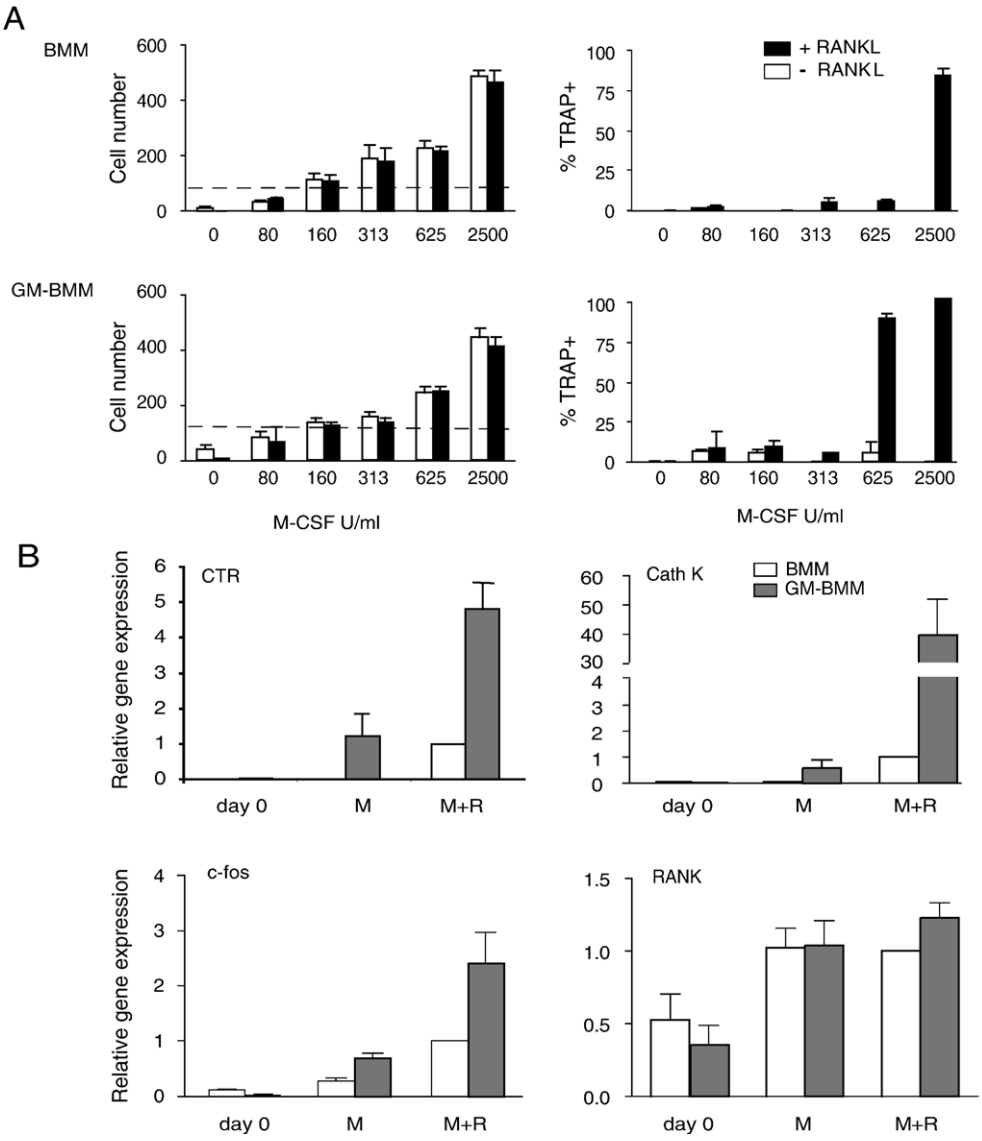


Fig. 2. M-CSF dose response and gene expression for osteoclastogenesis in BMM and GM-BMM. (A) BMM and GM-BMM were cultured in increasing concentrations of M-CSF either in the absence or presence of RANKL (50 ng/ml) in Terasaki wells and the cell number (mononuclear and multinuclear) and the percentage of TRAP positive cells measured after 6 days (Materials and methods). A multinuclear cell was counted as a single cell. Values are mean ± SE from triplicate cultures and are from a representative experiment which was repeated twice. The total cell number at the start of the experiment (day 0) is indicated by the dashed horizontal line (– – –). For both cell populations, the cell number at 160 U/ml M-CSF was not statistically different from the $t=0$ value, in the presence or absence of RANKL, indicating that cell number (survival) was maintained at this concentration (19); for the percentage of TRAP positive cells, in the presence of RANKL, the values at 2500 U/ml M-CSF for BMM and at 625 U/ml M-CSF for GM-BMM were significantly greater than ($p<0.05$) the corresponding values in the absence of RANKL. (B) BMM and GM-BMM were cultured in M-CSF in the absence or presence of RANKL (50 ng/ml) for 6 days; CTR, cathepsin K, c-*fos* and RANK mRNA expressions were measured by quantitative PCR (Materials and methods). Data were normalized to the 18S values and expressed relative to the value for BMM treated with M-CSF and RANKL (arbitrary value of 1) for each gene. Values are means from three independent experiments ± SE. For CTR, CathK and c-*fos* but not RANK, the "M + R" bars were significantly greater ($p<0.05$) than the corresponding "M" bars for both cell types and the "GM-BMM bars" were significantly greater ($p<0.05$) than the corresponding "BMM bars" for either treatment.

RANKL (Fig. 1B). As shown in Fig. 1C, the rate of OC lineage differentiation appeared to be similar for the GM-BMM compared to that for BMM, and there was an increased proportion of larger and multinucleated cells at day 6 and day 9 (Fig. 1B). The selected low starting cell density and number also allowed us to estimate total cell number easily, particularly at the early time points where the cells remained mainly mononuclear. For the particular experiment shown, RANKL reduced over time the total number of cells in both groups as compared to the effect of M-CSF alone (Fig. 1C) although the extent of this reduction varied from experiment to experiment depending upon the degree of multinucleation obtained (see below).

We next used our dual purpose assay to determine, for both BMM and GM-BMM, whether M-CSF was simply a pro-survival factor as suggested for macrophage lineage cells [33], allowing RANKL by itself to promote osteoclast differentiation. To test this, we compared the effect of increasing M-CSF concentrations on osteoclast differentiation and number, the rationale being that low M-CSF concentrations promote BMM

survival and higher concentrations stimulate their proliferation [19]. As expected, low M-CSF concentrations (≤160 U/ml) prevent cell death (Fig. 2A); GM-BMM also showed a similar response. RANKL did not reduce cell number in the experiment shown. In Fig. 2A, it can be observed that for both osteoclast progenitor populations higher M-CSF concentrations were required to induce TRAP expression than for their survival, suggesting that M-CSF is providing more than simply an anti-apoptotic stimulus.

As further support for increased TRAP expression and the morphology changes observed as being indicative of OC differentiation from GM-BMM, we assessed the gene expression of various additional OC lineage markers by Q-PCR. BMM and GM-BMM were cultured for 6 days in M-CSF or M-CSF and RANKL. In BMM, as expected during their osteoclastogenesis, calcitonin receptor (CTR), cathepsin K and c-*fos* mRNA levels increased in the presence of M-CSF and RANKL relative to the effect of M-CSF (Fig. 2B); similar data were obtained for GM-BMM. Interestingly, the mRNA levels at day 6 of each of the indicated OC lineage markers were higher in the GM-BMM treated with M-CSF + RANKL (or with M-CSF) than for BMM treated in the same way. RANK mRNA expression was noted in both GM-BMM and BMM; however, it was not modulated by RANKL in either cell type and there did not seem to be any significant difference between the cells (Fig. 2B).

Effect of GM-CSF on osteoclastogenesis from pre-formed GM-BMM

It would be predicted, based on previous studies [29,42,43] and indeed shown in Fig 3A, that addition of GM-CSF to BMM cultures containing M-CSF and RANKL would suppress the development of osteoclast-lineage cells. We now show that GM-BMM also respond in the same way (Fig. 3A). Fig. 3B shows that the suppressive effect of GM-CSF on TRAP expression in both GM-BMM and BMM is seen only if it is present during the first 3 days of exposure to M-CSF and RANKL; the suppressive effect was not evident when GM-CSF was added 3 days after differentiation to OC was initiated with M-CSF + RANKL. In the same experiment for both cell types GM-CSF reduced the total cell number in the presence of M-CSF ± RANKL (data not shown) (but see Fig. 4 below).

The relatively early (2 days) effect of GM-CSF on the gene expression of other OC markers induced by M-CSF + RANKL is shown for both cell types in Fig. 3C. As for the percentage of $TRAP^+$ data (Fig. 3B), the expression of CTR in GM-BMM and of CTR and c-*fos* in BMM was downregulated; interestingly, GM-CSF did not have any effect on c-*fos* expression in GM-BMM (see Discussion). GM-CSF could not replace M-CSF as a co-stimulus for RANKL in increasing gene expression of any of these markers (data not shown). It can be noted at this relatively early time point that CTR and c-*fos* expression in the presence of M-CSF + RANKL was higher in BMM than in GM-BMM in contrast to what was seen at 6 days above (Fig. 2B); the situation was reversed for CD11c suggesting at this 2-day time point that GM-BMM still expressed more of a DC phenotype even following M-CSF + RANKL addition.

Since GM-CSF can play an important role in the differentiation of DCs *in vitro* [23,35,38] and since RANK is present on DC precursors [69], it is possible that while GM-CSF is suppressing OC differentiation it may be promoting that for DCs. In the presence of M-CSF and RANKL, both GM-BMM and BMM showed increased gene expression for CD11c, CD80 and CD86 in the presence of GM-CSF, indicating enhanced DC differentiation (Fig. 3C).

Interferon α/β (IFN) has been implicated in bone homeostasis via negative control of osteoclastogenesis [63]; one of the strategies in these prior studies was to use mice and their BMM deficient in one of the IFNα/β receptor components, IFNAR1. We checked whether this IFN might be responsible for the suppressive action of GM-CSF shown above for BMM and GM-BMM by studying cells from $IFNAR1^{-/-}$ and $IFNAR2^{-/-}$ mice, the latter being deficient in the other IFNα/β receptor component, IFNAR2. We found that the effect of GM-CSF on the %TRAP + ve cells and cell number was the same for BMM and GM-BMM from wild type mice when compared to cells from $IFNAR1^{-/-}$ (Fig. 4) and $IFNAR2^{-/-}$ mice (data not shown), indicating that endogenous IFNα/β was not involved in the downregulation of osteoclastogenesis induced by GM-CSF for both BMM and GM-BMM.

Effect of TGF-β1 on osteoclastogenesis from BMM and GM-BMM

TGF-β1 can have either stimulatory or inhibitory effects on osteoclastogenesis *in vitro* [6,11,13,24,26,28,37,45,48,50,55,61,71]. The observations of a negative action by this cytokine are usually made in cultures containing both stromal cells and osteoclast precursors [45,48,61] while potentiation of osteoclastogenesis is usually observed when stromal elements are not present [24,50,51,55]. Since both BMM and GM-BMM are highly purified populations of osteoclast precursors, we reasoned that they are suitable to assess the direct actions of TGF-β1. Surprisingly, given the prior literature [24,50,51,55], TGF-β1 strongly suppressed osteoclastogenesis in BMM as judged by %TRAP + ve cells (Figs. 5A and B) and gene expression (CTR, c-*fos*) (Fig. 5C); similar results were found for GM-BMM. For both cell types, it also reduced the total number of cells in the cultures whether or not RANKL was present (Fig. 5B) (in this particular experiment, there was little to no effect of RANKL on total cell number). TGF-β1 also raised gene expression of the DC markers, CD11c and CD86, in BMM but reduced expression of CD11c in GM-BMM (Fig. 5D).

As for the negative effect of GM-CSF on osteoclastogenesis shown above, the effects of TGF-β1 on TRAP expression in the presence of M-CSF and RANKL were no different in BMM and GM-BMM from wild type mice when compared to cells from $IFNAR1^{-/-}$ (Fig. 6A) and $IFNAR2^{-/-}$ mice (data not shown), suggesting that endogenous IFNα/β was also not involved here. The inhibitory effect of TGF-β1 on TRAP expression in both cell types would not seem to be due to endogenous GM-CSF either since cells from $GM\text{-}CSF^{-/-}$ mice demonstrated no differences from their wild type counterparts (Fig. 6B). The above

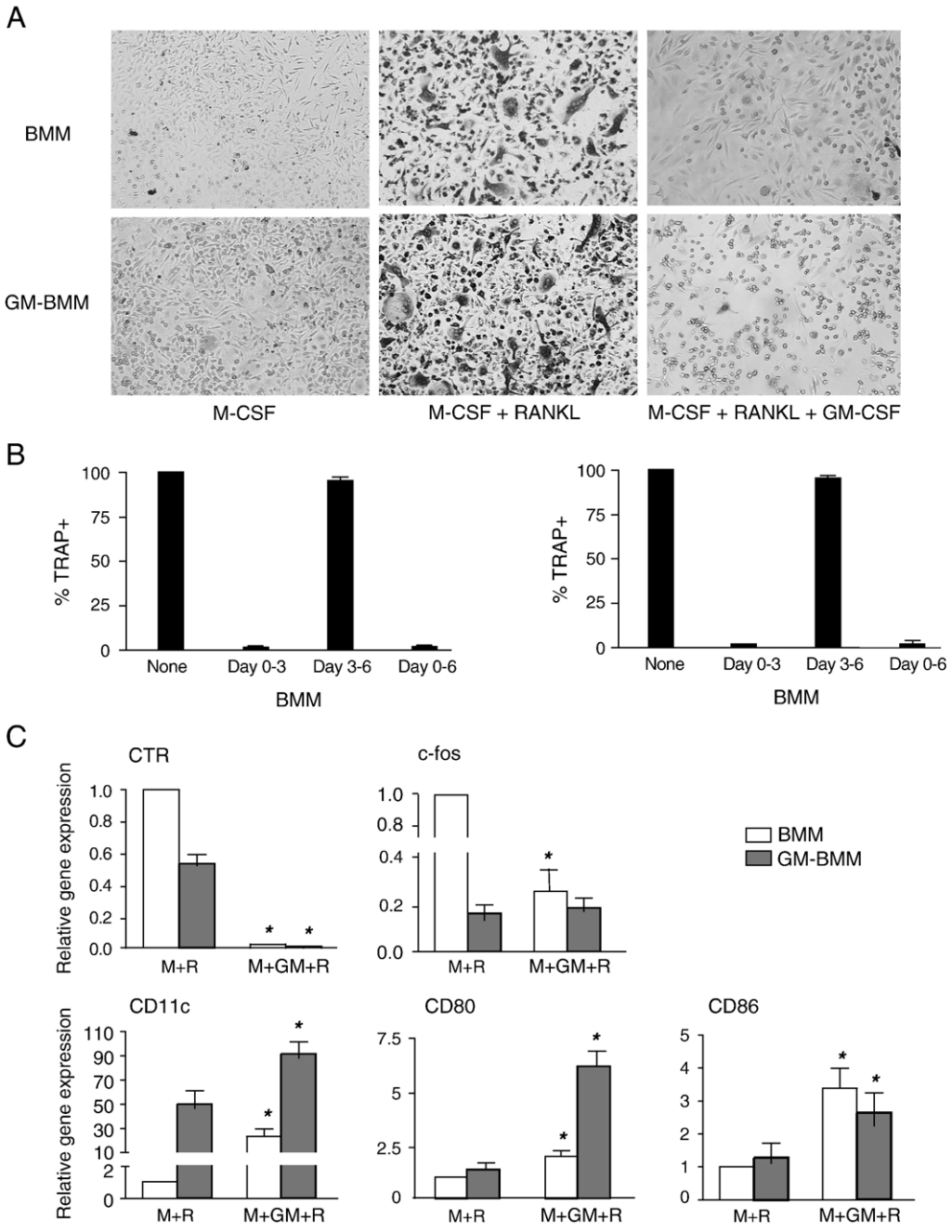


Fig. 3. GM-CSF inhibits osteoclastogenesis from GM-BMM. (A) BMM and GM-BMM were cultured in Terasaki wells in the presence of M-CSF (5000 U/ml) alone and also in the presence of M-CSF and RANKL (50 ng/ml)±GM-CSF (1000 U/ml). After 6 days, the cells were stained for TRAP expression (100× magnification). (B) BMM and GM-BMM were treated with M-CSF (5000 U/ml) and RANKL (50 ng/ml) in Terasaki wells for 6 days, either without (None) or with GM-CSF added for first 3 days (0–3), during the last 3 days (3–6) or for all 6 days of culture (0–6). The percentage of TRAP positive cells was estimated. Values are mean±SE from triplicate cultures and are from a representative experiment which was repeated three times. (C) BMM (white bars) and GM-BMM (gray bars) were cultured for 48 h in M-CSF (5000 U/ml) and RANKL (50 ng/ml) in the absence or presence of GM-CSF (10 ng/ml). CTR, c-*fos*, CD11c, CD80 and CD86 mRNA expressions were measured by Q-PCR (Materials and methods). Data were normalized to 18S expression and expressed relative to the values for BMM treated with M-CSF and RANKL (arbitrary value of 1) for each gene. Values are means of three independent experiments±SE. Bars indicated with the symbol "*" are significantly different from the other bar of the same color (i.e., white or gray) for the one gene ($p<0.05$).

inhibitory effects of TGF-β1 occurred in a dose-dependent manner over a concentration range of 0.01 ng/ml to 10 ng/ml for both cell populations, and potentiation of OC differentiation from BMM and GM-BMM was not seen at any TGF-β1 concentration (data not shown).

Discussion

Macrophage populations are quite heterogeneous and have many diverse functions with both M-CSF and GM-CSF contributing to these properties [36,67]. In mice, based on

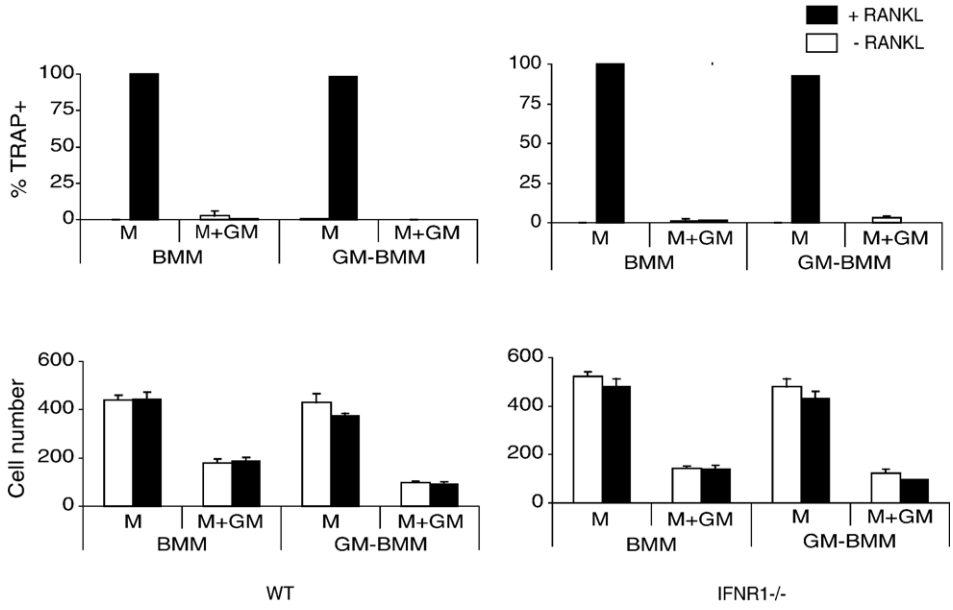


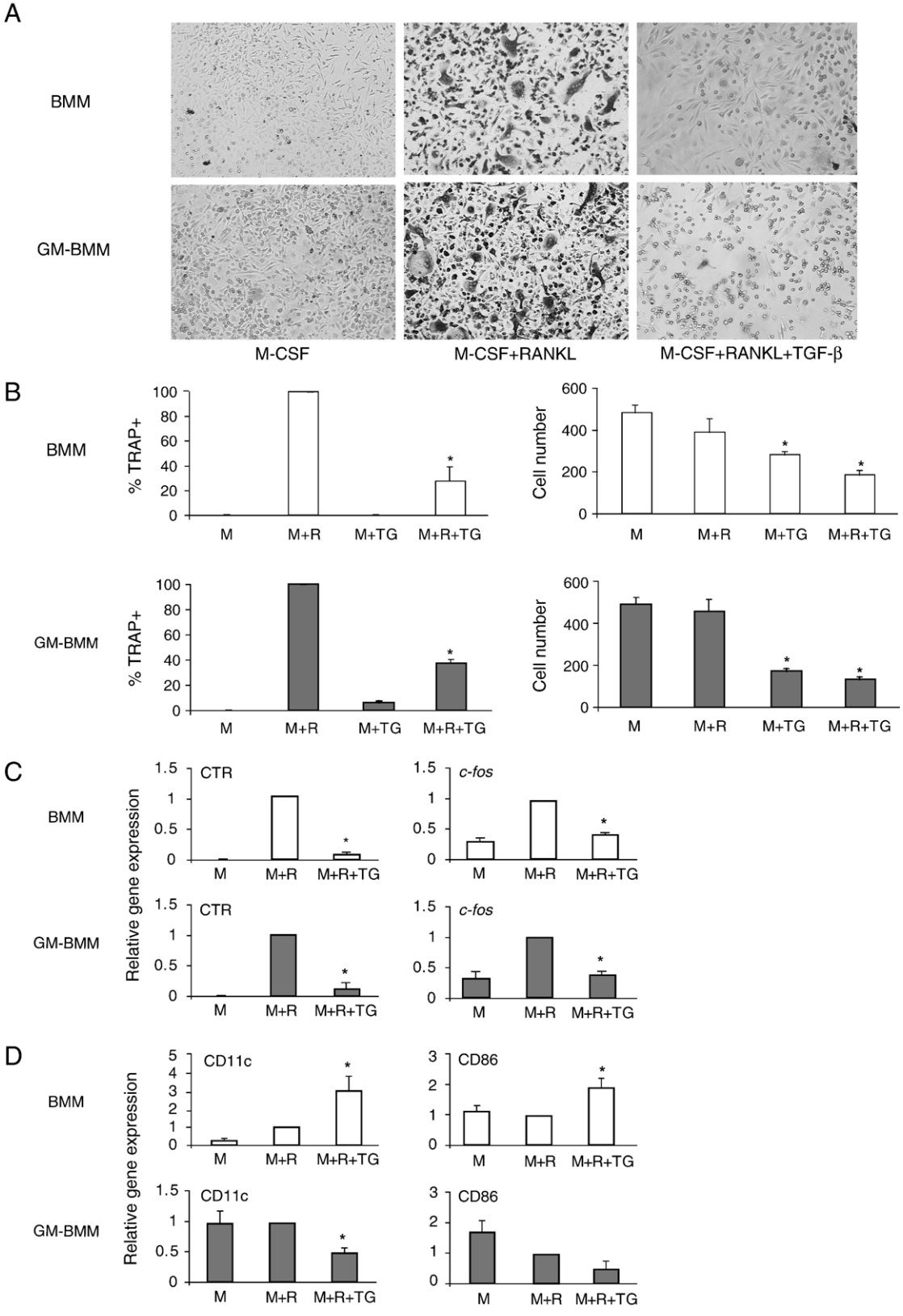
Fig. 4. Lack of involvement of endogenous type I IFN in the inhibitory effect of GM-CSF on osteoclastogenesis, BMM and GM-BMM from wild type (WT) and IFNR1 knockout mice were treated with M-CSF (5000 U/ml), with or without GM-CSF (10 ng/ml), in the absence or presence of RANKL (50 ng/ml) in Terasaki wells. After 6 days, the percentage of TRAP positive cells and the total cell number were estimated. Values are means ± SE from triplicate cultures and are from a representative experiment which was repeated three times. There were no significant differences between the values for wild type and IFNR1 deficient mice.

data from the op/op mouse with its inactive M-CSF protein, M-CSF controls macrophage numbers in a number of tissues [4]; in contrast, the major phenotype of the GM-CSF$^{-/-}$ mouse is alveolar proteinosis due to a defect in alveolar macrophage maturation [56]. Furthermore, GM-CSF but not M-CSF is widely used *in vitro* as a cofactor for DC development. It would be predicted that macrophage lineage cells generated by GM-CSF and M-CSF would exhibit some differences and that M-CSF might be more likely to produce cells capable of differentiating into osteoclasts [42,43,70]. In line with this concept, it has been proposed that GM-CSF and M-CSF drive common progenitors down bifurcating DC and OC differentiation pathways, respectively [42,43]. In the first part of our study, we compared some of the features of GM-BMM and BMM prior to RANKL addition. What we found was that GM-BMM and BMM had some common surface marker expression but they differed in CD11c levels and also in their appearance; GM-BMM have been referred to as immature DCs in some literature [23,35]. As regards their ability to function as OC precursors, we showed for the first time that GM-CSF can generate an adherent cell population which, like BMM, can be completely converted into TRAP^{+} cells. In other words, GM-BMM are not yet committed irrevocably to the DC lineage. These findings would appear to be different to the model proposed [42,43] in which commitment to the DC lineage appears to occur following GM-CSF treatment of osteoclast precursor cells. Our findings suggest that there is more "plasticity" in the GM-CSF-treated cell population than previously depicted [42,43]. As a result of this observation, we are now able to compare the osteoclastogenic potential of two highly purified macrophage lineage populations both of which can easily be produced in high quantities, making the study of the bifurcation along the two (i.e., OC and DC) pathways amenable to molecular analysis.

The ability of GM-CSF shown above to generate large numbers of effective OC precursors is consistent with its ability to restore osteoclastogenesis in op/op mice [44]. Our data are also consistent with the findings that the murine colony forming unit-granulocyte macrophage (CFU-GM) progenitors can form OCs at high efficiency when cultured with M-CSF, RANKL and dexamethasone [40] and that immature human peripheral blood DCs transdifferentiate into functional OCs [53]; they are also in line with the observations in one study that human CFU-GM progenitors have a higher osteoclastogenic potential than CFU-macrophage (CFU-M) progenitors and that short-term GM-CSF treatment potentiates OC differentiation by proliferating human precursors but persistent exposure favors DC formation [21]. However, for reasons unknown, our findings would appear to be different to those of Yamazaki et al. [70] who found that the OC precursors in GM-CSF-induced murine bone marrow colonies developed into OCs less frequently than those found in the corresponding M-CSF-induced colonies. Our cell system has advantages over all of the above previous studies with GM-CSF since we are able to generate large numbers of purified GM-CSF-dependent OC precursors—the prior studies mentioned used colony forming systems or longer term cultures containing a low proportion of OC precursors [21,40,53,70].

By employing the Terasaki plate assay, we were also able to establish culture conditions enabling the absolute numbers of OC lineage cells to be determined, as well as their proportion, regardless of the uneven cellular distribution on the culture surface—this was because the well surface was the entire sample area. When this assay was used above, the total number of OC lineage cells was quantified, i.e., mononuclear and

multinuclear cells, although their respective numbers could have been reported in our study. Because of the potentially high degree of conversion of both BMM and GM-BMM to the OC lineage, they are particularly suitable populations for monitoring both the proportion and number of OC lineage progeny. The low density cultures tended to favor mononuclear OC lineage formation since the degree of multinucleation of OC lineage cells can increase with cell culture density; in other words in the



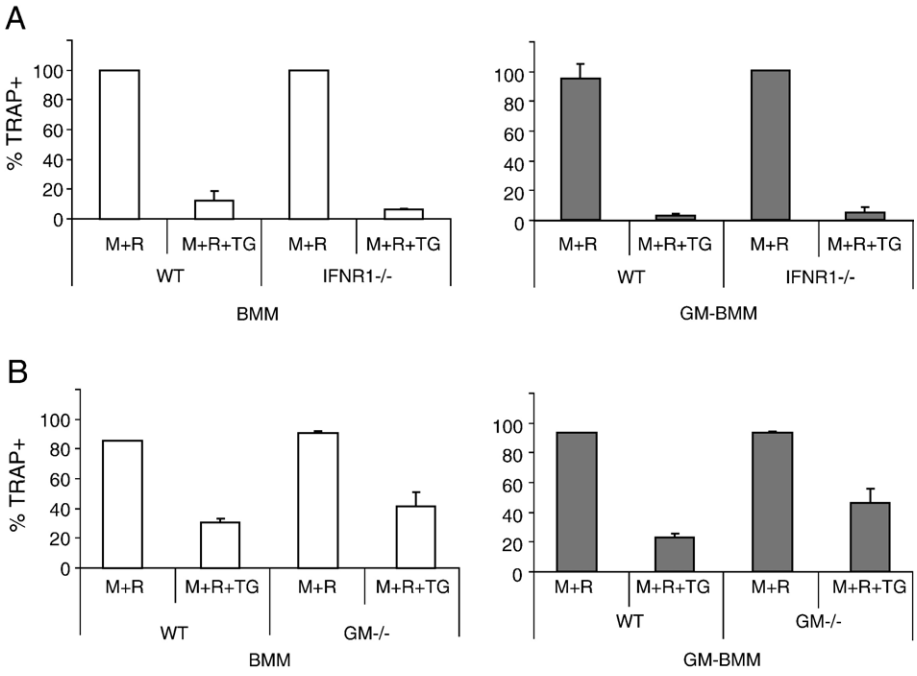


Fig. 6. Lack of involvement of endogenous type I IFN or GM-CSF in the inhibitory effect of TGF-β on osteoclastogenesis. BMM and GM-BMM from IFNR1 (A) or GM-CSF (B) knockout mice, and from wild type (WT) mice, were treated in Terasaki wells with M-CSF (5000 U/ml) and RANKL (50 ng/ml), in the absence or presence of TGF-β (10 ng/ml). After 6 days, the percentage of TRAP positive cells was estimated. Values are mean±SE from triplicate cultures and are from representative experiments which were repeated twice for the IFNAR1$^{-/-}$ cells and three times for the GM-CSF$^{-/-}$ cells. There were no significant differences between the values for wild type and gene-deficient mice.

current study we focused mainly on the OC development program prior to fusion and activation.

From our results above, GM-BMM, as well as BMM as shown also before [51], can be quantitatively driven along the OC lineage. For this "differentiation" phase [21,42] in the presence of RANKL, for both precursor populations, GM-CSF and M-CSF now have in fact competing functions. High concentrations of GM-CSF suppressed both the proliferative actions of M-CSF as well as the OC differentiation which occurs upon the addition of RANKL, the latter observation with BMM agreeing with a previous report [42]. Since, as mentioned above, the density and contact between OC precursors are important factors for multinuclear OC differentiation [41], we would like to suggest that a reduction in the number of OC precursors would appear also to form part of the inhibitory effect of GM-CSF.

Addition of GM-CSF in the first 3 days to cultures of GM-BMM and BMM containing M-CSF+RANKL had almost the same inhibitory effect on OC differentiation as it had when it was applied during the entire 6 days of culture. The presence of GM-CSF during a later stage of differentiation (days 3–6) failed to suppress osteoclastogenesis. Therefore, GM-CSF has an inhibitory effect on OC differentiation only at an early stage of this differentiation, consistent with previous studies with BMM [42]. This result suggests that the cells which had GM-CSF added at days 3–6 following M-CSF+RANKL addition had already committed to differentiate into OCs, and therefore addition of GM-CSF at this stage cannot suppress the OC differentiation induced by M-CSF and RANKL. TRAP has been reported to be expressed in early OC precursors [54]. However, for both GM-BMM and BMM at day 3 following M-CSF+RANKL addition, the vast majority of the cells were still TRAP negative (Figs. 1B and C); therefore, OC commitment would appear to occur well before full expression of TRAP. In contrast to the two macrophage populations which had committed to the OC lineage after 3 days in M-CSF+RANKL, the corresponding cells which had also been treated with GM-CSF for the first 3 days would appear to have committed to another pathway, and the removal of GM-CSF after this stage does not change this fate. This pathway is likely to involve DC differentiation (Fig. 3C) [42]. In other words, even in the presence of cytokines which will promote OC

Fig. 5. TGF-β inhibits osteoclastogenesis from BMM and GM-BMM. BMM and GM-BMM were cultured in Terasaki wells in the presence of M-CSF (5000 U/ml) alone and also in the presence of M-CSF and RANKL (50 ng/ml)±TFG-β (10 ng/ml). After 6 days, the cells were stained for TRAP expression (100× magnification). (B) BMM and GM-BMM were treated with M-CSF (5000 U/ml)±RANKL (50 ng/ml), in the absence or presence of TFG-β (10 ng/ml) in Terasaki wells. After 6 days, the percentage of TRAP positive cells and the total cell number were estimated. Values are mean±SE from triplicate cultures and are from a representative experiment which was repeated three times. Bars indicated with the symbol "*" are significantly different ($p<$s0.01) from the corresponding bar without TGF-β added. (C and D) BMM and GM-BMM were treated with M-CSF (5000 U/ml) alone and also in the presence of M-CSF+RANKL (50) ng/ml ±TFG-β (10 ng/ml). After 6 days CTR, c-*fos* (C), CD11c and CD86 (D) mRNA expressions were measured by Q-PCR (Materials and methods). For each cell type, data were normalized to 18S expression and expressed relative to the values for cells treated with M-CSF and RANKL (arbitrary value of 1) for each gene. Values are means from three independent experiments±SE. Bars indicated with the symbol "*" are significantly ($p<0.05$) different from the corresponding bar without TGF-β added.

differentiation, namely M-CSF+RANKL, GM-CSF dominates and the cells begin to adopt a DC phenotype (Fig. 3C). It is possible that RANKL contributes also to this pathway as well since it can affect DC function [1]. We also observed for GM-BMM, as for BMM, that TNFα could synergize with RANKL, in the presence of M-CSF, to promote osteoclastogenesis (data not shown).

c-Fos is one of the essential transcription factors for osteoclastogenesis [16]. It has been reported that GM-CSF reduces expression of both c-*fos* and *fra-1* (the Fos related protein) in Fms^+ $RANK^-$ OC precursors. By contrast, DC maturation is inhibited when c-*fos* is expressed at an early stage of differentiation, and it has been suggested that the OC/DC lineage determination from OC progenitors by GM-CSF might be via regulation of c-Fos expression [42]. Confirming one of these results, we have shown the downregulation of c-*fos* mRNA in BMM when GM-CSF is added in the presence of M-CSF and RANKL (Fig. 3C). In contrast, during the early stage, inhibition by GM-CSF of the osteoclastogenesis from GM-BMM c-*fos* mRNA levels did not fall (Fig. 3C). Therefore, it is possible that, for at least GM-BMM, inhibition of OC differentiation by GM-CSF is not due to altered c-*fos* expression implying some other mechanism.

As for GM-CSF, the effects of TGF-β on bone homeostasis, including osteoclastogenesis, are quite complex (for a review, see [25])—it has been proposed that there is virtue, as we have done above, in using purified osteoclast precursor populations for the analysis of its effects on osteoclastogenesis [12,27,51] which have been reported to be both stimulatory or inhibitory [25]. In general, even with stromal elements removed, almost all of the studies, including with macrophage populations and our own separate studies with non-adherent BMM precursors [51], indicate that TGF-β promotes OC development [24,30,55] and even to be an essential autocrine factor [26] in one model. A hypothesis has been presented in which the OC is a mononuclear phagocyte directed towards "a debriding" function by TGF-β, activated for this function by RANKL and induced to become specifically osteoclastic by additional signals from bone [6]. However, what we have shown above, for both of our BMM and GM-BMM populations, is that TGF-β, like GM-CSF, can dramatically suppress their differentiation into OC lineage cells, both in terms of function and at the level of cell number. In the absence of RANKL, we found (Fig. 5B) that TGF-β1 also inhibited the M-CSF-dependent proliferation of BMM and GM-BMM, this result being opposite to that reported with the former cell system [5]. For both BMM and GM-BMM, the synergistic effect of TNFα and RANKL on M-CSF-dependent osteoclastogenesis was also suppressed by TGF-β1 (data not shown). In only one prior study has TGF-β been shown to have an inhibitory effect on OC development [27] in which it was found with human monocytes that TGF-β showed an initial positive effect but a negative one with continuous exposure. In this study, the authors concluded that the effects of TGF-β depend most importantly on the species under investigation such that, in contrast to mice in which TGF-β seems to be stimulatory overall, the role in human cells is more complicated, with both stimulatory effects on monocytes and inhibitory effects on preosteoclasts and mature osteoclasts. However, our studies show that even with murine cells TGF-β can have dramatic inhibitory effects on osteoclastogenesis from two types of macrophage populations. Our data are consistent with at least some of the complex *in vivo* findings where TGF-β appears to suppress OC formation (see, for example, [6,25]) thereby providing another mechanism to favor bone formation.

Others have reported [62,63] that more multinucleated $TRAP^+$ cells can be derived *in vitro* from $IFNAR1^{-/-}$ bone marrow cells after several days in M-CSF and RANKL when compared to wild type cells. However, the absolute numbers of precursor cells in the cultures over time were not measured [62,63] and such differences could be contributing to the differences in the number of OCs reported to develop. We have found (data not shown) that BMM and GM-BMM were generated faster from $IFNAR1^{-/-}$ bone marrow in the presence of M-CSF and GM-CSF, respectively, the result with BMM having been published before by us as evidence for the growth inhibitory action of endogenous IFN in their cultures [17,20]. We made sure to quantify the number of starting BMM and GM-BMM (i.e., osteoclast precursor cells) and contend that these findings with $IFNAR^{-/-}$ cells again highlight the desirability where possible of quantifying cell number during OC differentiation. Our studies with cells from $IFNAR1^{-/-}$ mice indicated that the later stage suppression of osteoclastogenesis either by GM-CSF or by TGF-β1 would appear not to be due to endogenous IFN; in this context, the analogous GM-CSF-mediated suppression of human osteoclast formation has been shown recently to be reversed by addition of MCP-1, leading the authors to conclude that the absence of MCP-1 in the cultures treated with GM-CSF and RANKL is a key deficit [29].

Cytokines, including TNFα, GM-CSF and TGF-β, are likely to be contributing to the control of the bone loss by OCs particularly occurring in pathologic conditions, such as arthritis, orthopedic implant loosening, metastatic cancer and periodontal disease [6,15,52,53,68]. Our studies above support the notion that the actions of both GM-CSF and TGF-β on osteoclastogenesis depend critically on the state of maturation of the target OC precursors as well as the nature and timing of action of the other influences (stimuli and inhibitors) to which these precursors are exposed. GM-CSF generates osteoclast progenitors from early precursors in the absence of RANKL but suppresses osteoclastogenesis from the more mature osteoclast progenitors in the presence of M-CSF and RANKL; TGF-β promotes osteoclastogenesis from early precursors in the presence of M-CSF and RANKL but suppresses it from more mature osteoclast progenitors in the presence of the same stimuli. We would like to suggest that both cytokines could promote the generation of preosteoclasts (macrophage-like cells) at a site of resorption but then prevent full OC maturation in the presence of M-CSF and RANKL, perhaps close to the bone surface. Our studies also indicate that macrophage lineage cells can be exposed to GM-CSF for some time without necessarily being irreversibly committed to the DC lineage, i.e., there is more plasticity in this system than perhaps has been considered.

Acknowledgments

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