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Mucosal mast cells and nematode infection: strain-specific differences in mast cell precursor frequency revisited

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Abstract

Mucosal mast cells (MMC) play an important role in the immune response against selected species of intestinal nematode. The kinetics with which different strains of inbred mice resolve infection with *Trichinella spiralis* correlates with their ability to mount MMC responses in the intestinal mucosa. Homologues of MMC that express and constitutively secrete abundant amounts of the granule chymase, mouse mast cell protease-1 (mMCP-1), can be generated *in vitro* from bone marrow cultures supplemented with interleukins-3 and -9, stem cell factor and transforming growth factor-β_1. Using the enhanced growth characteristics of these MMC homologues, a novel limiting dilution assay for mast cell precursor (MCp) frequency has been developed. The assay is highly specific, in that cultures containing mast cells are identified with mMCP-1 specific antibody, and almost three-fold more sensitive than previously published systems. MCp frequencies were compared in BALB/c and C57/BL10 strains of mice that, respectively, respond rapidly and slowly to infection with *T. spiralis*. MCp frequency (1/378 bone marrow cells) was significantly greater ($P < 0.05$) in BALB/c than C57/BL10 mice (frequency: 1/751). Similarly the rate of growth of MMC homologues and the production of mMCP-1 was significantly ($P < 0.05$) greater in BALB/c than in C57/BL10 bone marrow cultures.

Introduction

The contribution made over the last 35 years by Derek Wakelin and his colleagues to our understanding of host parasite interactions has been very significant (Wakelin, 1978). Their rigorous genetic and immunological analyses of the elements of the adaptive immune response that regulate the protective/permissive responses to helminth infection have set the scene for subsequent molecular studies on mechanisms of resistance and susceptibility to infection and on evasion strategies used by the parasite (Wakelin, 1986). In addition to defining the role of T cells in the protective responses in mice (Wakelin & Wilson, 1979), Wakelin's work established not only that that bone marrow cells contributed significantly to the effector response against nematodes, but that a significant component of the genetic variation in nematode resistance in inbred strains of mice resided in the bone marrow (Wakelin & Donachie, 1981).

Our own studies in the rat had, to some extent, mirrored those carried out by Wakelin in mice and we had shown that sensitized, recirculating T cells, rather than B cells conferred protection (Nawa *et al.*, 1978), that T cells regulated the differentiation of bone marrow-derived mucosal mast cells and that there was substantial genetic variation in mucosal mast cell (MMC) responses (Nawa & Miller, 1979). Mast cell hyperplasia is a feature of almost all helminth infections (Miller, 1996) and recruitment and activation of mast cells is a feature of allergic responses in the airways and gastrointestinal tract (Galli, 1993;

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Metcalfe *et al.*, 1997). Mast cells play a role in the immunological expulsion of some, but not all gastrointestinal nematode parasites (Grencis *et al.*, 1993; Miller, 1996; Artis *et al.*, 2000). Infection with the intestinal nematode *Trichinella spiralis* is prolonged when the MMC response is ablated using anti stem cell factor (SCF) or anti c-kit antibodies (Grencis *et al.*, 1993; Donaldson *et al.*, 1996) and in mutant mice lacking the MMC granule-specific β-chymase, mouse mast cell proteinase-1 (mMCP-1) (Knight *et al.*, 2000).

The kinetics with which different strains of inbred mice resolve infection with *T. spiralis* correlates with their ability to mount a mast cell response at the intestinal mucosa (Tuohy *et al.*, 1990). NIH and BALB/c (high responder) mice support lower worm burdens and resolve infection more rapidly than C57BL/10 (low responder) mice (Tuohy *et al.*, 1990). MMC hyperplasia and both local and systemic increases in the level of mMCP-1, occur earlier and are significantly more pronounced in high responder mice (Tuohy *et al.*, 1990). The genetic differences, which underlie the functional disparity between high and low responder strains of mice, are complex. However, *in vitro* data suggest that both mast cell precursor (MCp) frequency and the rate at which mast cells proliferate in response to cytokines are significant factors (Reed *et al.*, 1988).

Mast cell precursors are derived from bone marrow (Kitamura *et al.*, 1979) and frequencies have been calculated for bone marrow, peripheral blood and mesenteric nodes from parasitized adult mice (Ashman *et al.*, 1991; Kasugai *et al.*, 1995; Lantz & Huff, 1995; Tegoshi *et al.*, 1997; Gurish *et al.*, 2001). MCp frequency in the intestinal mucosa is increased early after nematode infection whereas the numbers in bone marrow remain unchanged (Dillon & MacDonald, 1986; Tegoshi *et al.*, 1997). There was little evidence of MCp proliferation in the bone marrow or circulation, with the main site of proliferation and differentiation occurring in the jejunum (Kasugai *et al.*, 1995; Tegoshi *et al.*, 1997). Therefore it is MCp, not mast cells, which leave the bone marrow and enter the circulation to reach the gut mucosa where they differentiate into MMC.

In vivo analysis of MMC hyperplasia during nematode infection in the mouse shows that mMCP-1 is expressed very early during MMC differentiation (Wastling *et al.*, 1997). *In vitro*, the expression and constitutive secretion of mMCP-1 by cultured mouse bone marrow derived mast cells (mBMMC) is strictly regulated by the multifunctional cytokine TGF-β_1 (Miller *et al.*, 1999; Wright *et al.*, 2002). Within 4 days of initiating a bone marrow culture in the presence of SCF, IL-3, IL-9 and TGF-β_1, approximately 40% of the cells are mMCP-1^{+ve} mBMMC, and by day 7 of culture $\geq$ 85% of the cells are mMCP-1^{+ve} (Wright *et al.*, 2002). This rapid upregulation of mMCP-1 expression is accompanied by the constitutive release of substantial quantities of mMCP-1 into the extracellular milieu (Miller *et al.*, 1999; Wright *et al.*, 2002). Using a novel limiting dilution assay, based on TGF-β_1 regulated expression of mMCP-1 (Miller *et al.*, 1999; Wright *et al.*, 2002) that detects MMC precursors, we have examined high (BALB/c) and low (C57/BL10) responder strains of mice (Tuohy *et al.*, 1990) for differences in MCp frequency. The data supports the hypothesis that bone marrow MCp frequency correlates with resistance to intestinal nematodes (Reed *et al.*, 1988) and suggests that previous studies have substantially underestimated MCp frequency.

Materials and methods

In vitro *culture of mouse bone marrow derived mast cells*

Single cell suspensions of mononuclear cells were prepared from the femoral bone marrow of age matched male BALB/c and C57/BL10 mice as described previously (Miller *et al.*, 1999; Wright *et al.*, 2002). Cells were washed twice in DMEM (Life Technologies, Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (FCS: Serotec, Kidlington, UK), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2.5 μg ml^{-1} fungizone, 2 mM L-glutamine and 1 mM sodium pyruvate (DMEM/FCS) and counted using an Improved Neubauer counting chamber (Fisher Scientific) after dilution in white blood cell counting fluid (Sigma, Poole, UK). Viability was assessed using 0.2% nigrosin (Sigma) exclusion. Cultures were maintained in 96- or 24-well tissue culture plates at 37°C in a humidified 5% CO_2 atmosphere. mBMMC differentiation was induced by culturing bone marrow mononuclear cells in DMEM/FCS supplemented with various combinations recombinant human TGF-β_1 (1 ng ml^{-1}: Sigma), recombinant mouse IL-3 (1 ng ml^{-1}: R&D Systems, Abingdon, UK), recombinant mouse IL-9 (5 ng ml^{-1}: R&D Systems), and recombinant SCF (50 ng ml^{-1}: Peprotech, London, UK) (Miller *et al.*, 1999; Wright *et al.*, 2002). Cultures were harvested on days 4 and 8, cell numbers and viability were measured as described above. Culture supernatants and cells were separated by centrifugation at 730 *g*. mMCP-1^{+ve} mBMMC were enumerated using immunofluorescence (FITC/propidium iodide). Extracellular secretion of mMCP-1 into the culture supernatant was quantified using an enzyme-linked immunosorbent assay (ELISA), as described previously (Knight *et al.*, 2000).

Limiting dilution analysis of mast cell precursor frequency

Serial dilutions of single cell suspensions from BALB/c and C57/BL10 mice were prepared in DMEM/FCS containing TGF-β_1/IL-3/IL-9/SCF and added in triplicate to 96-well tissue culture plates (4000, 2000, 1000, 500, 250, 125, 62.5 cells per well). After 7 days, dilutions containing one or more mast cell precursors were identified by the presence of granulated mMCP-1^{+ve} mBMMC using immunofluorescence (Cy3). Mast cell precursor frequency was calculated using L-Calc™ version 1.1 (StemCell Technologies, Vancouver, Canada).

Immunofluorescent detection of mMCP-1

Cells were washed three times and resuspended in PBS before being loaded onto Bio-Rad Adhesion Slides (Bio-Rad Laboratories, Hemel Hempstead, UK) as directed by the manufacturer. Bound cells were fixed in modified Bouin's fluid (92.5% saturated aqueous picric acid, 5% concentrated (37%) formaldehyde and 2.5% glacial acetic acid) for 10 min at 21°C, permeabilized for

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10 min at 21°C in absolute methanol and stored in 70% ethanol at 4°C. Non-specific immunoglobulin interactions were blocked by a 2 h incubation with PBS/0.5 M NaCl (High-Salt) containing 0.5% Tween-80 and 10% normal goat serum (NGS) at 21°C in a humidified container. Slides were then incubated overnight at 4°C in an humidified container with rat anti-mMCP-1 IgG_1 (Mab RF6.1) (Scudamore *et al.*, 1997) or normal rat control IgG_1 (Becton Dickinson, Oxford, UK) diluted to 10 $\mu g\,ml^{-1}$ in High-Salt/10%NGS. After washing with PBS, slides were incubated with FITC (2 $\mu g\,ml^{-1}$) or Cy3 (1 $\mu g\,ml^{-1}$) conjugated goat anti-rat monovalent Fab fragments (Stratech Scientific, Luton, UK) in High-Salt/10%NGS for 2 h at 4°C in a humidified container. FITC labelled cells were counterstained for 10 min with 5 $\mu g\,ml^{-1}$ propidium iodide. Slides were mounted with Mowiol mounting media (CN Biosciences UK, Nottingham, UK).

Results

TGF-β_1, IL-9, IL-3 and SCF are required for optimal growth and differentiation of C57/BL10 mucosal mast cell homologues

Bone marrow cells from C57/BL10 mice (n = 4) were cultured in the presence of the same combinations of cytokines that were tested on BALB/c bone marrow cells (Miller *et al.*, 1999). Cell numbers, viability and release of mMCP-1 into the culture supernatant were measured on day 4 (fig. 1). Cytokine combinations containing IL-3 and SCF resulted in optimal cell growth and viability (fig. 1a and b). Growth and viability were poor in cultures maintained in DMEM alone, TGF-β_1 or TGF-β_1/IL-9 (fig. 1a and b). Although growth and viability appeared to be largely dependent on the presence of IL-3 and/or SCF, the combination of TGF-β_1/IL-9/IL-3/SCF resulted in optimal growth and viability (fig. 1a and b) and induced maximal constitutive mMCP-1 release (fig. 1c). mMCP-1 was detected only in the supernatants of cultures supplemented with TGF-β_1 (fig. 1c). Although IL-9 did not induce detectable mMCP-1 production in the absence of TGF-β_1, cultures maintained in TGF-β_1/IL-9/IL-3/SCF secreted over two-fold more mMCP-1 than those maintained in TGF-β_1/IL-3/SCF alone (fig. 1c). These findings are very similar to those described for BALB/c bone marrow cells (Miller *et al.*, 1999).

Growth and differentiation of mBMMC from BALB/c and C57/BL10 mice

Bone marrow cells from age matched male BALB/c (n = 5) and C57/BL10 mice (n = 5) were cultured in the presence of TGF-β_1/IL-9/IL-3/SCF. Cell numbers, viability, percentage mMCP-1^{+ve} mBMMC and mMCP-1 secretion were measured on days 4 and 8 (fig. 2). BALB/c and C57/BL10 cultures yielded similar numbers of viable cells on day 4 (fig. 2a). However, cells from BALB/c cultures expanded more rapidly than their C57/BL10 counterparts between day 4 and 8 (fig. 2a). Whilst this could have been due to enhanced proliferation, cell viability was significantly lower for C57/BL10 cells between day 4 and 8 (fig. 2b). BALB/c cultures also contained significantly more mMCP-1^{+ve} mBMMC on days 4 and 8 than their C57/BL10 counterparts and

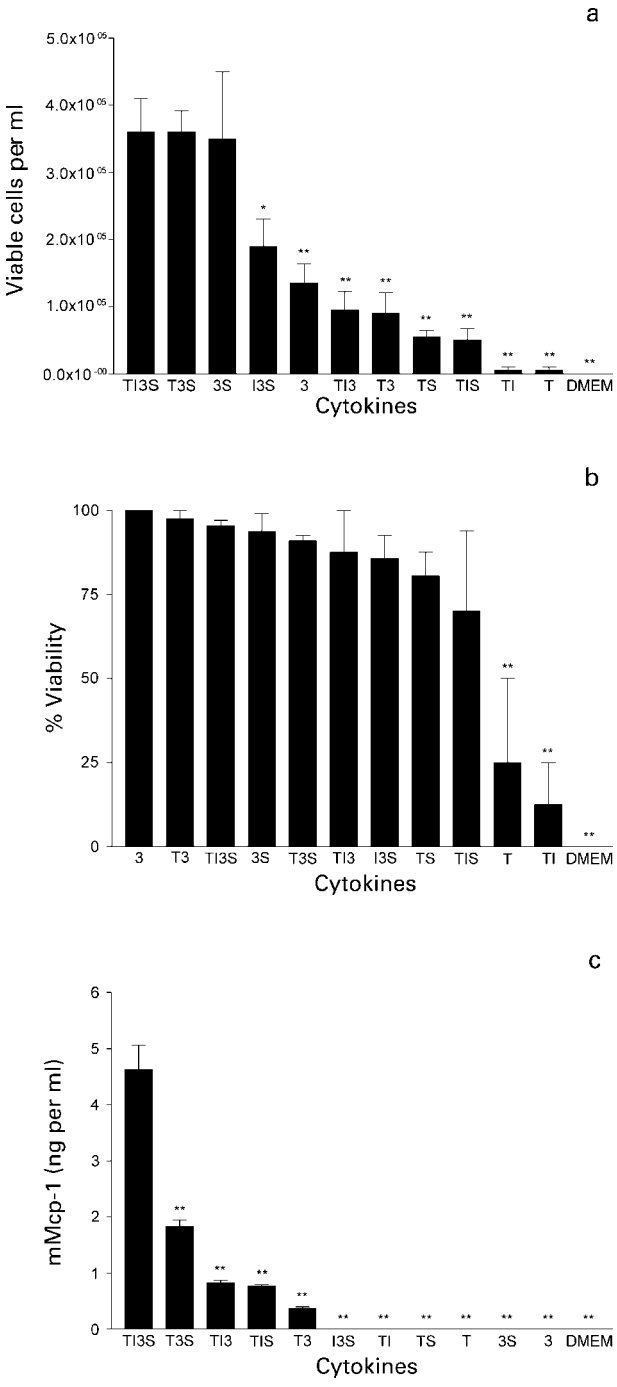

Fig. 1. Effect of different cytokines on the growth and viability of C57/BL10 mBMMC and the extracellular release of mMCP-1. Bone marrow from C57/BL10 mice (n = 4) was cultured for 4 days *ex vivo* in the presence of varying combinations of 1 ng ml^{-1} TGF-β_1 (T), 1 ng ml^{-1} IL-3 (3), 5 ng ml^{-1} IL-9 (I) and 50 ng ml^{-1} SCF (S). Numbers of cells (a) and viability (b) were measured using an Improved Neubauer counting chamber and nigrosin exclusion. (c) ELISA analysis of constitutive mMCP-1 secretion. Data are expressed as mean values ± 1 standard error of the mean. Data differ significantly (unpaired two-tailed Student t-test) from that of cultures maintained in the presence of TI3S at $P < 0.05$ (*) and $P < 0.01$ (**).

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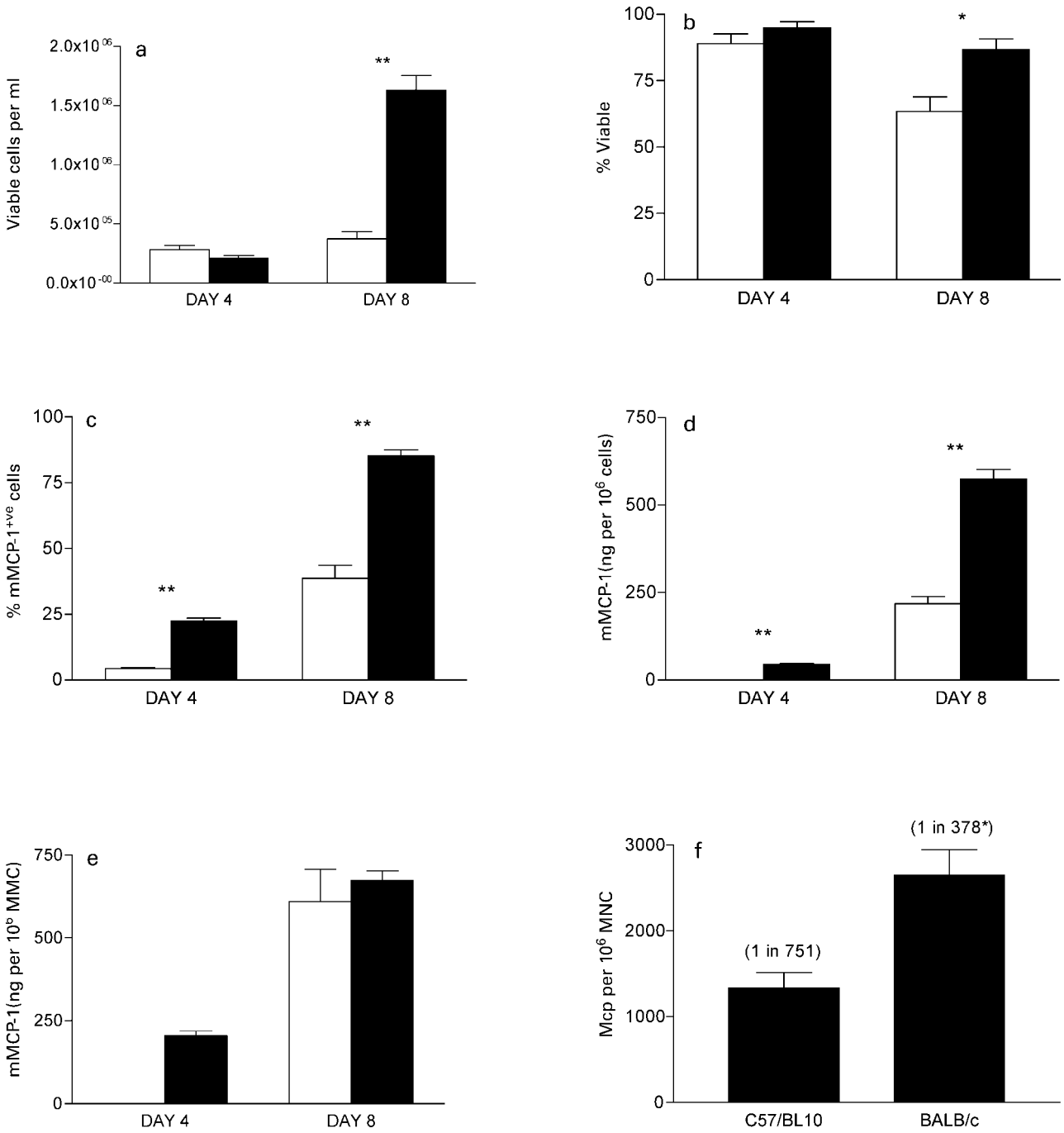

Fig. 2. Mouse strain dependent variation in mBMMC growth and differentiation. Bone marrow cells from age matched male BALB/c (n = 5 : filled bars) and C57/BL10 mice (n = 5 : clear bars) were cultured in the presence of TGF-β_1/IL-9/IL-3/SCF. Cell numbers (a), viability (b), percentage mMCP-1^{+ve} mBMMC (c) and constitutive mMCP-1 secretion (d and e) were measured on days 4 and 8. (f) Limiting dilution analysis of MCp precursor frequency in age matched male BALB/c (n = 3) and C57/BL10 mice (n = 3). Data are expressed as mean values ± 1 standard error of the mean. BALB/c and C57/BL10 derived data differ significantly (unpaired two-tailed Student t-test) at $P < 0.05$ (*) and $P < 0.01$ (**).

this was associated with enhanced extracellular secretion of mMCP-1 (fig. 2c and d). However, correcting the mMCP-1 ELISA data for mMCP-1^{+ve} mBMMC numbers suggests that mBMMC from BALB/c and C57/BL10 mBMMC secrete comparable amounts of mMCP-1 by Day 8 (fig. 2e). mMCP-1 levels in the supernatants of day 4 C57/BL10 cultures fell below the detection limit of the mMCP-1 ELISA (fig. 2d).

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MCp frequency in BALB/c and C57/BL10 mice

The novel limiting dilution assay that detected mMCP-1^{+ve} mast cells was employed to measure MCp frequency in BALB/c and C57/BL10 bone marrow. Serial dilutions of bone marrow cells from BALB/c (n = 3) and C57/BL10 (n = 3) mice were prepared in DMEM/FCS supplemented with TGF-β_1/IL-9/IL-3/SCF. By day 7, cultures that originally contained one or more MCp were easily identifiable by the presence of granulated mMCP-1^{+ve} mBMMC. MCp frequencies calculated for BALB/c mice (1/378) were significantly higher than their C57/BL10 (1/751) counterparts (fig. 2f).

Discussion

Previous studies in this laboratory have produced compelling evidence of a central role for TGF-β_1 in the differentiation of MMC (Miller *et al.*, 1999; Wright *et al.*, 2002). mBMMC expression of the MMC-specific β-chymase, mMCP-1, is strictly regulated by TGF-β_1 (Miller *et al.*, 1999). Furthermore, with $\geq$ 85% of cells expressing abundant mMCP-1 and the putative MMC integrin $\alpha_E\beta_7$ by day 7 of culture in the presence of TGF-β_1/IL-9/IL-3/SCF (Wright *et al.*, 2002), the kinetics of TGF-β_1 mediated mMCP-1^{+ve} mBMMC differentiation are remarkably similar to that of MMC hyperplasia during intestinal nematode infection *in vivo* (Wastling *et al.*, 1997). By contrast, $\leq$ 14% of mBMMC grown in the presence of IL-9/IL-3/SCF alone express mMCP-1 (Wright *et al.*, 2002) and even this limited expression can be ablated by anti-TGF-β_1 antibody (Miller *et al.*, 1999).

The same combination of cytokines, TGF-β_1/IL-9/IL-3/SCF, that promotes optimal growth and differentiation of mMCP-1^{+ve} mBMMC from BALB/c bone marrow (Miller *et al.*, 1999) also mediates optimal growth and differentiation of mBMMC in C57/BL10 bone marrow cultures (fig. 1). However, bone marrow cultures from BALB/c yield significantly more mMCP-1^{+ve} mBMMC, when maintained in TGF-β_1/IL-9/IL-3/SCF, than their C57/BL10 counter parts (fig. 2c). This increase in the proportion of mMCP-1^{+ve} mBMMC in BALB/c cultures is associated with improved cell growth and viability (figs. 2a and b). mMCP-1 secretion is also significantly higher in BALB/c mBMMC cultures when expressed relative to total viable cell numbers (fig. 2d). When supernatant concentrations of mMCP-1 are expressed relative to mMCP-1^{+ve} mBMMC numbers, by contrast, there appears to be little of no difference in the amount of mMCP-1 produced by BALB/c and C57/BL10 derived mBMMC (fig. 2e). Previous studies using IL-3, alone, to induce mBMMC differentiation support a role for strain specific differences in both MCp frequency and IL-3 mediated mast cell proliferation in accounting for differential susceptibility to intestinal nematode infection (Reed *et al.*, 1988). MCp represent a relatively small population within the mononuclear cell fraction of bone marrow (fig. 2f) and it seems likely that most of the differences observed between BALB/c and C57/BL10 derived cultures (fig. 2) may be accounted for by a lower frequency of MCp in C57/BL10 mice (fig. 2f). Longer-term culture of pure populations of mature mBMMC will confirm whether this hypothesis is correct.

In all previously published studies, MCp cultured with IL-3 alone or IL-3/SCF or conditioned media developed into toluidine blue^{+ve} mast cells of unknown phenotype after 7–21 days (Ashman *et al.*, 1991; Kasugai *et al.*, 1995; Lantz & Huff, 1995; Tegoshi *et al.*, 1997; Gurish *et al.*, 2001). In the current study, we used a novel approach to determining MCp frequency that has two major advantages. Firstly, TGF-β_1/IL-9/IL-3/SCF induces faster growth and differentiation of mBMMC than IL-3/SCF or IL-3 alone (fig. 1) (Miller *et al.*, 1999; Wright *et al.*, 2002). Secondly, TGF-β_1 mediates expression of the MMC specific β-chymase, mMCP-1, and cultures containing mBMMC can therefore be identified using MMC specific antibody (Miller *et al.*, 1999; Wright *et al.*, 2002) rather than toluidine blue staining or simple morphology. It is therefore, perhaps, unsurprising that MCp frequencies are several fold higher than previously reported (Ashman *et al.*, 1991; Kasugai *et al.*, 1995; Lantz & Huff, 1995; Tegoshi *et al.*, 1997; Gurish *et al.*, 2001). In the most recent study, Gurish *et al.* (2001) report MCp frequencies of $924/10^6$ mononuclear cells in BALB/c bone marrow, almost three-fold lower than the MCp frequency of $2648/10^6$ mononuclear cells calculated for the same anatomical compartment in the current study. Similarly, MCp frequencies calculated for C57/BL10 bone marrow in the current study are over six-fold higher ($1332/10^6$ mononuclear cells) than reported previously, where conditioned media from concanavalin A stimulated spleen cells was used to induce mast cell differentiation (Reed *et al.*, 1988). Despite this increase in sensitivity, the two-fold disparity in MCp frequency between high responder BALB/c and low responder C57/BL10 mice is similar to that previously described for NIH and C57/BL10 (Reed *et al.*, 1988) and for BALB/c and C57/BL6 (Gurish *et al.*, 2001).

We have recently found that MMC hyperplasia and expression of mMCP-1 during nematode infection require the expression of $\alpha_V\beta_6$ by jejunal epithelium (Knight *et al.*, 2002). One of the functions of this integrin is to bind the latency-activated peptide of TGF-β_1 and to activate latent TGF-β_1 at the cell surface (Munger *et al.*, 1999). Thus, the presence of $\alpha_v\beta_6$ on jejunal epithelium provides a highly focused and selective mechanism for the epithelial cell-to-MMC presentation of activated TGF-β_1 (Knight *et al.*, 2002) Work in this laboratory has also established a significant role for mMCP-1 in the expulsion of *T. spiralis* (Knight *et al.*, 2000). This is consistent with the observation that MMC hyperplasia and both local and systemic increases in the level of mMCP-1, occur earlier and are significantly more pronounced in high responder mouse strains during *T. spiralis* infection (Tuohy *et al.*, 1990). The findings of the current study therefore support the hypothesis that MCp frequency influences resistance to intestinal nematodes (Reed *et al.*, 1988) and may account, at least in part, for differences observed between different inbred mouse strains (Tuohy *et al.*, 1990). Furthermore, it reinforces the much earlier work by Wakelin and colleagues (Wakelin & Donachie, 1981) showing the major contribution of bone marrow in the development of resistance against intestinal nematodes.

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