

Constitutive secretion of the granule chymase mouse mast cell protease-1 and the chemokine, CCL2, by mucosal mast cell homologues

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Summary

Background The mucosal mast cell (MMC) granule-specific β -chymase, mouse mast cell protease-1 (mMCP-1), is released systemically into the bloodstream early in nematode infection before parasite-specific IgE responses develop and TGF- β_1 induces constitutive release of mMCP-1 by homologues of MMC *in vitro*. Intraepithelial MMC may also express the chemokine CCL2 (monocyte chemotactic protein-1) during nematode infection but the expression of this chemokine by MMC homologues has not been investigated.

Objective To investigate the expression and to compare the mechanisms of constitutive release of the chymase, mMCP-1, and the chemokine, CCL2.

Methods MMC homologues were generated by culturing bone marrow cells in the presence of TGF- β_1 , IL-3, IL-9 and stem cell factor (SCF). The intracellular distribution of mMCP-1 and CCL2 was examined by confocal microscopy. The involvement of the Golgi complex and of protein synthesis in the constitutive release of mMCP-1 and CCL2 was investigated using the Golgi-disrupting agent brefeldin A and cycloheximide to block protein synthesis. Secreted analytes were quantified by ELISA.

Results mMCP-1 colocalized with Golgi matrix protein 130 but was most abundant in the granules, whereas CCL2 was not found in the granules but appeared to be located uniquely in the Golgi complex. Extracellular release of mMCP-1 was significantly inhibited ($\approx 40\%$) by cycloheximide and by the Golgi-disrupting agent brefeldin A, indicating both continuous protein synthesis and transportation via the Golgi complex are required for optimal mMCP-1 secretion. A similar but more marked inhibitory effect with both compounds was demonstrated on the constitutive secretion of CCL2.

Conclusion The culture conditions that promote mMCP-1 expression and release by MMC homologues also promote the expression and release of CCL2. Constitutive release involves *de novo* protein synthesis and requires a functional Golgi complex, suggesting that similar mechanisms of extracellular secretion operate for both mediators.

Keywords β -chymase, brefeldin A, CCL2, cycloheximide, IgE, MCP-1

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Introduction

Mucosal mast cells (MMC) in rodents are morphologically, biochemically and functionally distinct from connective tissue mast cells (CTMC) [1, 2]. One of key differences between MMC and CTMC is in their expression of neutral granule serine proteases [3–5]. In the mouse and rat, expression of the highly homologous β -chymases mouse mast cell protease-1 (mMCP-1) and rat mast cell protease-2 (rMCP-2) is predominantly restricted to MMC [2, 3, 6–9]. Recent evidence suggests that the

expression of sheep mast cell protease-1 (sMCP-1) is, similarly, restricted to MMC in the sheep [10].

Both mMCP-1 and rMCP-2 are secreted constitutively into the peripheral blood stream in the apparent absence of exogenous stimuli [3, 11–13]. Systemic levels of mMCP-1 and rMCP-2 increase substantially during intestinal nematode infection [3, 13, 14]. In the mouse, peripheral blood concentrations of mMCP-1 increase significantly within 4 to 7 days of nematode establishment in the gut [13]. This rapid increase in systemic mMCP-1 release precedes the onset of a detectable parasite-specific IgE response, suggesting that MMC can secrete mMCP-1 through mechanisms that are distinct from IgE-regulated exocytosis [3]. We have recently shown that mouse bone marrow derived mast cells (mBMMC) grown in the presence of transforming growth factor-beta $_1$ (TGF- β_1) are highly analogous to MMC in that they express the MMC-specific protease, mMCP-1 [15, 16]. In the current study, we address

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the mechanism of the mMCP-1 secretory process in mBMMC homologues of MMC and compare it with the mechanism involved in the constitutive secretion of the chemokine monocyte chemoattractant protein-1 (CCL2). Transcription of CCL2 is up-regulated in the intestinal mucosa during *Nippostrongylus brasiliensis* infection [17] and its expression by mast cells appears to be regulated by SCF [18–20]. We wished to determine whether both mediators are constitutively and concomitantly released through a common process or whether they are released extracellularly by unrelated mechanisms.

Materials and methods

Generation of MMC-like mBMMC

BMC, prepared from 10- to 12-week-old male BALB/c as described previously [15, 16], were washed twice in DMEM (Life Technologies, Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (FCS; Serotec, Kidlington, UK), 100 U/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL fungizone, 2 mM L-glutamine and 1 mM sodium pyruvate (DMEM/FCS). Nucleated cells were counted in an Improved Neubauer counting chamber (Fisher Scientific, Loughborough, UK) after dilution in white blood cell counting fluid (Sigma, Poole, UK). Viability was assessed using 0.2% nigrosin (Sigma) exclusion. BMC cultures were set-up at 1×10^6 viable cells/mL in TGF- β_1 /IL-3/IL-9/SCF (DMEM/FCS supplemented with 1 ng/mL recombinant human TGF- β_1 (rhTGF- β_1 ; Sigma), 1 ng/mL recombinant mouse IL-3 (rm IL-3; R & D Systems, Abingdon, UK), 5 ng/mL recombinant mouse IL-9 (rmIL-9; R & D Systems), and 50 ng/mL rrSCF (Amgen, Thousand Oaks, CA, USA)) [15, 16]. Cultures were maintained in a humidified 5% CO₂ incubator and fed with fresh TGF- β_1 /IL-3/IL-9/SCF every 2 to 3 days for 14 days. Cytosmeas were prepared on day 14 of culture and the percentage of mBMMC and integrin α_E^{+ve} /mMCP-1^{+ve} mBMMC determined by Leishman's staining and paired immunofluorescence [16]. mMCP-1 expression was quantified by enzyme-linked immunosorbent assay (ELISA).

Inhibition of de novo synthesis and Golgi directed secretion of mMCP-1 and CCL2

Fourteen-day-old mBMMC were washed three times in DMEM/FCS, resuspended at 5×10^5 cells/mL in fresh TGF- β_1 /IL-3/IL-9/SCF and seeded into 24-well tissue culture plates in 1 mL aliquots. Brefeldin-A (BFA; Sigma) was dissolved in absolute methanol to yield a final concentration of 5 mg/mL and stored for up to 1 month at -20°C before use; 5 mg/mL solutions of cycloheximide (CHX; Sigma) were made up in absolute methanol immediately before use. In some experiments, a commercial preparation of BFA (GolgiPlugTM; Becton Dickinson, Oxford, UK) was used as directed by the manufacturer to induce Golgi complex disassembly. BFA, CHX and vehicle control (absolute methanol alone) were diluted appropriately in TGF- β_1 /IL-3/IL-9/SCF and filter sterilized before use. Cultures ($n = 4$ to 6) were harvested 24 to 48 h later. Cells were counted in an Improved Neubauer counting chamber after dilution in 0.2% nigrosin to assess viability. The extracellular release of lactate dehydrogenase (LDH) was also quantified as a measure of cell death (Cytotoxicity Detection Kit, LDH; Roche Diagnostics, Lewes, UK) as directed by the manufacturer.

Culture supernatants and cells were separated by centrifugation at 700 g. Cell pellets were washed in three changes of PBS/1% bovine serum albumin (BSA; Sigma). The amount of mMCP-1 present in culture supernatants and cell pellets was quantified by ELISA.

ELISA quantification of mMCP-1 and CCL2

The concentration of mMCP-1 in culture supernatants and cell pellets was measured by ELISA as described previously [21]. Constitutive secretion of CCL2 was quantified using a commercial ELISA kit as directed by the manufacturer (Becton Dickinson). Mean concentrations were compared using the parametric Student's *t*-test.

Immunofluorescent detection of mMCP-1 in the Golgi complex

Fourteen-day-old mBMMC were washed in PBS, resuspended at 5×10^6 cells/mL in PBS, loaded onto Bio-Rad Adhesion Slides (Bio-Rad Laboratories, Hemel Hempstead, UK) as directed by the manufacturer and fixed in absolute methanol alone for 20 min at 21°C . Non-specific immunoglobulin interactions were blocked by a 1-h incubation, at 21°C in a humidified container, with M.O.M.TM Mouse Ig blocking reagent (Vector Laboratories, Peterborough, UK). After washing with PBS, slides were incubated for 30 min, at -20°C in a humidified container, with 25 µL per reaction field of mouse anti-Golgi matrix protein 130 (GM130) IgG₁ (Monoclonal Clone 35; Becton Dickinson) or normal mouse control IgG₁ (Sigma) diluted to 2 µg/mL in M.O.M.TM diluent (Vector Laboratories). Cells were washed in PBS and incubated for 10 min, at 21°C in a humidified container, with M.O.M.TM biotinylated anti-mouse IgG at the concentration recommended by the manufacturer (M.O.M.TM Immunodetection Kit; Vector Laboratories). Cells were washed in PBS and incubated for 5 min, at 21°C in a humidified container, with 20 µg/mL of streptavidin-Texas Red (Vector Laboratories). Cells were then stained for mMCP-1 (FITC/Alexa Fluor 488) and mounted with Mowiol (CN Biosciences UK, Nottingham, UK) as described previously [16].

Immunofluorescent detection of CCL2

Bio-Rad Adhesion Slide preparations of 14-day-old mBMMC were fixed in absolute methanol for 20 min at -20°C as described above. Slides were blocked for 1 h with 30 µL of staining buffer (PBS/0.5 M NaCl containing 0.5% Tween-80 (Serotec, Kidlington, UK) and 10% normal rabbit serum) at 37°C in a humidified container. Slides were then incubated overnight, at 4°C in a humidified container, with 30 µL per reaction field of polyclonal goat anti-CCL2 (Cambridge Bioscience, Cambridge, UK) or normal goat control IgG (Cambridge Bioscience) diluted to 1 µg/mL in staining buffer. After washing with PBS, slides were incubated with 30 µL per reaction field of Cy3 conjugated rabbit anti-goat IgG monovalent Fab fragments (Strattech Scientific, Luton, UK) at 1 µg/mL in staining buffer for 2 h at 4°C in a humidified container and mounted with Mowiol.

Microscopy and imaging

Bright field images were acquired with a Sony DXC-390P 3CCD colour video camera (Scion Corporation, Frederick, USA) mounted on an Axiovert 100 inverted microscope

equipped with DIC optics (Carl Zeiss, Welwyn Garden City, UK). The RGB video signal from the camera was digitized using Scion Image (Scion Corporation) installed in a G4 Macintosh computer (Apple Computer, Cupertino, USA) fitted with a CG-7 frame grabber (Scion Corporation).

Fluorescent images were acquired using an MRC-600 confocal laser scanning microscope (CLSM; Bio-Rad Laboratories) mounted on an Axiovert 100 inverted microscope equipped with a $\times 63$ Plan-Apochromat[®] 1.4 NA objective lens (Carl Zeiss). FITC and Alexa Fluor 488 were excited with the 488 nm line from a 15 mW Kr/Ar laser (Bio-Rad Laboratories). Texas Red and Cy3 were excited with the 568 nm line from a 15 mW Kr/Ar laser.

Co-localization analysis was performed using LaserSharp 3.2 (Bio-Rad Laboratories). Images were prepared for publication using Object-Image [22]. Object-Image is a public domain software package, based on NIH Image [23], developed by Norbert Vischer (The University of Amsterdam, Amsterdam, the Netherlands) and is available via the Internet at <http://simon.bio.uva.nl/object-image.html>.

Flow cytometric analysis of BFA and CHX induced apoptosis

mBMMC were washed in cold PBS and incubated for 15 min at 21 °C with Alexa Fluor-488 conjugated Annexin-V (5% dilution of stock solution; Molecular Probes, Leiden, the Netherlands) in 10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl₂ (pH 7.4). Samples were then diluted fivefold in 10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl₂ (pH 7.4) supplemented with 10 µg/mL of propidium iodide and stored on ice. Quantitative analysis was performed using a flow cytometer (FACSCalibur; Becton Dickinson, California, USA) with linear amplification for forward/side scatter and logarithmic amplification for Alexa Fluor 488 and propidium iodide fluorescence.

Detection of mMCP-1 mRNA transcripts by RT-PCR

Total RNA was extracted from 5×10^6 cells in 1 mL Tri-Reagent (Sigma) as directed by the manufacturer. Contaminating DNA was removed by incubating 5 µg total RNA with 6 U of DNA-free[™] DNase (Ambion, Austin, USA) as described previously [21]. Quantitative RT-PCR analysis was carried out using an 'IntraSpec[™]' Comparative RT-PCR kit (Ambion) according to the manufacturer's instructions. Briefly, total RNA samples from test cultures (BFA or CHX treated) and from cultures with vehicle alone were reverse-transcribed using unique RT primers, comprising 5' PCR primer binding sites, 3' anchored oligo (dT) domains and an interdomain region of either 10 nucleotides (for vehicle control samples) or 50 nucleotides (for test samples). After removal of unbound nucleotides (NucAway Spin Columns, Ambion), cDNA concentrations were measured spectrophotometrically and normalized by carrying out test amplifications using GAPDH primers provided with the kit. cDNAs from each test sample and vehicle were mixed in a range of ratios, and amplified using an 80-mM PCR primer to the interdomain region (supplied with the kit) and 80 mM of an mMCP-1 gene-specific primer (5'-CAAAGCTTGTCCTCCAGACTAT-3'). Primer specificity was confirmed by failure to amplify any product from TGF- β_1 -induced BMMC from mMCP-1^{-/-} animals [24], which transcribe other MMC-like proteases normally (Knight and

Wright, unpublished observations). Amplifications were carried out for 35 thermocycles (30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C) and PCR products were visualized on ethidium bromide-stained 2% agarose gels. The relative intensities of different sized PCR products from each competing cDNA were used to quantify relative abundance of the mMCP-1 transcript. Image acquisition and analysis were performed using an Image Station 440CF and Kodak 1D software (NEN[®] Life Science Products, Zaventem, Belgium).

Results

Generation of MMC-like mBMMC

BMC were grown in TGF- β_1 /IL-3/IL-9/SCF for 14 days *ex vivo*. By day 14, cultures were comprised of $\geq 99\%$ mBMMC with typical MMC-like morphology (Figs 1a and c). Cells from these cultures also coexpressed mMCP-1 and integrin α_E (Fig. 1b). ELISA analysis of culture supernatants confirmed that the presence of mMCP-1 in the granules as shown in Figs 1(b, d, g, h) was associated with the extracellular release of 1.0 ± 0.3 µg of mMCP-1 per 10^6 cells over 24 h (data not shown). This is in agreement with previous studies that showed that the expression and extracellular release of mMCP-1 is strictly regulated by TGF- β_1 [15, 16]. In addition to mMCP-1 and integrin α_E , mBMMC also expressed CCL2 (Fig. 1f). In contrast to mMCP-1, CCL2 was not present in the mast cell granules but was restricted to the Golgi complex (Fig. 1f).

Immunodetection of mMCP-1 and CCL2 within the Golgi complex

In order to identify the Golgi complex in mBMMC the presence of the Golgi marker protein GM130 was investigated by immunofluorescence before and after ablating the Golgi using GolgiPlug[™] (Becton Dickinson). GolgiPlug[™] is a proprietary commercial preparation of BFA that induces complete Golgi complex disassembly and is used in this instance to provide an additional negative control for the co-localization analysis.

Fourteen-day-old mBMMC were transferred into fresh TGF- β_1 /IL-3/IL-9/SCF with or without GolgiPlug[™]. After 6 h, mBMMC were fixed and immunolabelled for mMCP-1 and the Golgi marker GM130 (Figs 1d, e, g and h). Confocal sections (1 airy-disk) were acquired through the centre (z-axis) of the Golgi complex. FITC/Alexa Fluor 488 and Texas Red were imaged sequentially using 488 nm and 568 nm excitation, respectively, to avoid bleed-through artifacts. For each culture ($n = 6$), 10 images of approximately 20 cells/field of view were acquired from dual labelled (Fig. 1d), isotype matched single negative controls (Figs 1G and H) and GolgiPlug[™]-treated controls at a resolution of 100.9 nm/pixel (2.18-fold oversampling based on a theoretical optical resolution of 220 nm for Texas Red). Isotype-matched single negative controls were used to determine the level of non-specific background staining present. In the absence of GolgiPlug[™], well-defined GM130 staining is clearly visible in the majority of integrin α_E^{+ve} /mMCP-1^{+ve} mBMMC (Figs 1d, e and g). GolgiPlug[™] treatment was associated with loss of GM130 immunoreactivity (data not shown). mMCP-1 immunoreactivity is most prominent within the mBMMC granules (Figs 1d, g and h) but was also visible proximal to the nuclei (Fig. 1h: arrow). Co-localization

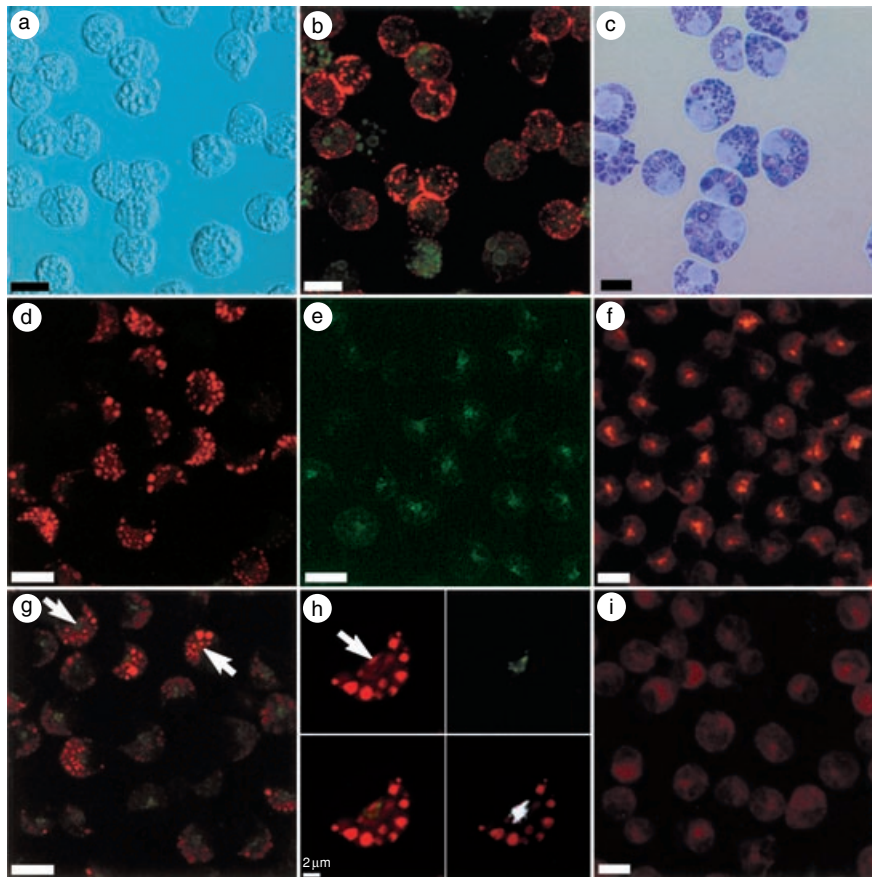


Fig. 1. Adhesion slide preparations of mBMMC grown in the presence of TGF- β_1 /IL-9/IL-3/SCF for 14 days *ex vivo*. For immunofluorescence: integrin α_E and Golgi matrix protein GM130 were labelled indirectly with Texas Red; mBMMC-1 was labelled indirectly with FITC/Alexa Fluor-488; and CCL2 was labelled indirectly with Cy3. (a) Fourteen-day-old mBMMC: DIC image ($\times 500$). (b) mBMMC where integrin α_E is pseudo-coloured red (contrast enhanced) and mBMMC-1 is pseudo-coloured green: extended focus confocal image ($\times 500$; matched with Fig. 1A). (c) A cytosmear of 14-day-old mBMMC stained with Leishmans': bright-field image ($\times 400$). Panels D and E show single negative controls for panels G and H. (d) Isotype matched single negative control for the anti-GM130 monoclonal where mBMMC-1 is pseudo-coloured red: confocal image ($\times 600$). (e) Isotype matched single negative control for the anti-mBMMC-1 monoclonal where GM130 is pseudo-coloured green: confocal image ($\times 600$). (f) CCL2 staining (pseudo-coloured orange) is predominantly restricted to the Golgi complex of mBMMC: confocal image ($\times 400$). Note the absence of CCL2 staining in the mBMMC granules. (g) 14-day-old dual-labelled mBMMC where mBMMC-1 is pseudo-coloured red and GM130 (arrow) is pseudo-coloured green: confocal image ($\times 600$). (h) A high-resolution image of GM130/mBMMC-1 colocalization in the Golgi complex of a 14-day-old mBMMC: confocal image ($\times 1000$). Individual panels show (clockwise): Granular and Golgi (arrow) localized mBMMC-1 staining; Golgi localized GM130 staining; a background corrected RGB image with GM130/mBMMC-1 colocalization highlighted in white (co-localization coefficient = 0.85); and the non-background corrected RGB image. (i) Isotype matched negative control for the anti-CCL2 polyclonal: confocal image ($\times 400$). Scale-bar represents 10 μ m unless stated otherwise. Contrast enhancement was not used on any of the images unless stated otherwise.

analysis of mBMMC-1 and GM130 confirmed the presence of mBMMC-1 within the Golgi complex, with $84.1 \pm 5.4\%$ of GM130-specific immunolabelling also having a mBMMC-1-specific component (Table 1). By contrast, only the Golgi component ($0.8 \pm 0.2\%$) of mBMMC-1 co-localizes with GM130 (Table 1). Co-localization analysis of isotype-matched single negative controls and GolgiPlugTM-treated cultures returned correlation coefficients of 0.000 in all cases, confirming that the level of background correction was sufficient to exclude any non-specific staining artifacts from the analysis (Table 1).

Previous studies in human mast cell lines indicated that CCL2 could be detected using histochemistry [20], but its localization was not entirely clear. However, in mBMMC, the presence of CCL2 was detected in the majority of cells and had a typical crescent shaped perinuclear distribution (Fig. 1f) that was distinct from the granule distribution of mBMMC-1 (Fig. 1d). The distribution was very similar to that shown for GM130 (compare Figs 1e and f) and was distinct from the background staining (Fig. 1i) seen with the controls. The majority of

mBMMC exhibited this pattern of staining and it appears to be predominantly Golgi associated.

Cycloheximide and brefeldin A inhibit the constitutive secretion of mBMMC-1 and CCL2 by mBMMC

To investigate the contribution of the Golgi complex to the constitutive secretion of mBMMC-1 and CCL2, and to determine whether protein synthesis was necessary for secretion to occur, mBMMC were treated with either BFA or CHX. Fourteen-day-old mBMMC-1^{+ve} mBMMC were transferred into TGF- β_1 /IL-3/IL-9/SCF supplemented with CHX (40, 20, 10, 1 and 0.1 ng/mL), BFA (200, 100, 50, 5 and 0.5 ng/mL) or vehicle alone. Quadruplicate cultures were maintained under these conditions for 24 h, after which supernatants and cell pellets were harvested and analysed for mBMMC-1 content by ELISA (Table 2). Cells were counted and nigrosin exclusion and LDH release were used to assess viability (Table 2). CHX did not reduce mBMMC viability at any of the concentrations used in this

Table 1. The CIS-Golgi marker GM130 co-localizes with mMCP-1 in the Golgi complex. Fourteen-day-old integrin $\alpha_E^{+ve}/mMCP-1^{+ve}$ mBMMC were harvested onto adhesion slides and dual-labelled for mMCP-1 and GM130. Control cultures were treated for 6 h with GolgiPlug™ to disrupt the Golgi complex and ablate GM130 immunoreactivity. Single negative controls, in which either the anti-mMCP-1 or anti-GM130 primary antibodies are substituted with isotype-matched control IgG, were used to determine the degree of non-specific background staining present. Co-localization analysis was performed using LaserSharp v3.2. Data are expressed as mean values $\pm$ 1 standard error from the mean ($n = 6$)

	Co-efficient of GM130 Co-localization with mMCP-1	Co-efficient of mMCP-1 Co-localization with GM130
$\alpha_E^{+ve}/mMCP-1^{+ve}$ mBMMC	0.841 $\pm$ 0.022	0.008 $\pm$ 0.002
$\alpha_E^{+ve}/mMCP-1^{+ve}$ mBMMC GolgiPlug™ treated controls	0.000 $\pm$ 0.000	0.000 $\pm$ 0.000
mMCP-1 isotype-matched negative controls	0.000 $\pm$ 0.000	0.000 $\pm$ 0.000
GM130 isotype-matched negative controls	0.000 $\pm$ 0.000	0.000 $\pm$ 0.000

Table 2. Effects of cycloheximide and brefeldin A on mBMMC growth, viability and mMCP-1 synthesis and secretion. Fourteen-day-old integrin $\alpha_E^{+ve}/mMCP-1^{+ve}$ mBMMC were maintained for 24 h in TGF- β_1 /IL-9/IL-3/SCF supplemented with various concentrations of CHX, BFA or vehicle alone. Data are expressed as mean values $\pm$ 1 standard error from the mean ($n = 4$). Differences between CHX/BFA-treated cultures and vehicle control cultures are significant at $P < 0.05$ (*) or $P < 0.01$ (**). A significant reduction in mBMMC viability was observed in cultures maintained in the presence of 200 ng/mL of brefeldin A, ELISA data have therefore been excluded (‡)

Cycloheximide		40 ng/mL	20 ng/mL	10 ng/mL	1 ng/mL	0.1 ng/mL	Vehicle
24 h	Secreted mMCP-1 (ng/10 ⁶ viable cells)	397 $\pm$ 52.8**	546 $\pm$ 30.5**	651.2 $\pm$ 29.9**	823.6 $\pm$ 2	929.3 $\pm$ 89.1	897.1 $\pm$ 46.5
	Intracellular mMCP-1 (ng/10 ⁶ viable cells)	7682.3 $\pm$ 763.8**	11081.3 $\pm$ 428.9*	11476.5 $\pm$ 1525.8	14674 $\pm$ 1794.1	17220 $\pm$ 716	13873.8 $\pm$ 710.6
	Cells/mL ($\times 10^5$)	10.0 $\pm$ 1.7	7.9 $\pm$ 0.5	8.5 $\pm$ 0.2	7.6 $\pm$ 1.0	7.1 $\pm$ 1.2	8.5 $\pm$ 0.3
	Viable (%)	98.7 $\pm$ 0.8	97.6 $\pm$ 0.9	97.7 $\pm$ 1.6	97.5 $\pm$ 1.2	98.4 $\pm$ 1.0	98.24 $\pm$ 1.1
	Cytotoxicity (%)	0.0	0.0	0.0	0.0	0.0	0.0
Brefeldin-A		200 ng/mL	100 ng/mL	50 ng/mL	5 ng/mL	0.5 ng/mL	Vehicle
24 h	Secreted mMCP-1 (ng/10 ⁶ viable cells)	‡	519.8 $\pm$ 17**	696.8 $\pm$ 25.3	763.5 $\pm$ 13.9	828.8 $\pm$ 15.5	784.8 $\pm$ 46.3
	Intracellular mMCP-1 (ng/10 ⁶ viable cells)	‡	10298.4 $\pm$ 714.9	11838 $\pm$ 1452.8	12658.7 $\pm$ 915.6	12736 $\pm$ 1441	10401.4 $\pm$ 1757.8
	Cells/mL ($\times 10^5$)	8.2 $\pm$ 0.6	9.2 $\pm$ 0.6	8.2 $\pm$ 0.6	6.6 $\pm$ 0.4	6.8 $\pm$ 0.8	7.8 $\pm$ 0.6
	Viable (%)	84.8 $\pm$ 3.9*	98.6 $\pm$ 0.8	96.3 $\pm$ 1.6	97.4 $\pm$ 1.7	97.0 $\pm$ 0.3	98.0 $\pm$ 1.2
	Cytotoxicity (%)	2.57 $\pm$ 0.3*	0.0	0.0	0.0	0.0	0.0

experiment. However, mBMMC viability was significantly reduced in cultures maintained in the presence of 200 ng/mL of BFA for ≥ 24 h ($P < 0.05$).

CHX inhibited mMCP-1 synthesis and secretion in a dose-dependent manner (Table 2). mBMMC exposed to ≥ 10 ng/mL of CHX for 24 h secreted significantly less mMCP-1 than mBMMC maintained in vehicle alone ($P < 0.01$). CHX had a similar, but less significant ($P < 0.05$), effect on intracellular levels of mMCP-1 in mBMMC from cultures exposed to ≥ 20 ng/mL over the same time-course (Table 2). BFA inhibited mMCP-1 secretion in a dose-dependent manner but did not reduce intracellular mMCP-1 (Table 2). Although a dose-dependent effect was evident in mBMMC cultures maintained in BFA for 24 h, the reduction in extracellular mMCP-1 levels observed only reached significance in cultures treated with 100 ng/mL ($P < 0.01$).

mBMMC also constitutively secreted significant quantities of the chemokine, CCL2 (Fig. 2). As in the case of mMCP-1, both CHX and BFA significantly reduced the amount of CCL2 in the supernatants of mBMMC cultures in a dose-dependent manner but did not completely block its secretion (Fig. 2c). At the highest concentrations of CHX (120 and 240 ng/mL) and BFA

(120 ng/mL) used in this experiment, the constitutive secretion of CCL2 was inhibited to a significantly greater degree than that of mMCP-1 ($P < 0.01$). Reductions observed in the secretion of mMCP-1 and CCL2 did not differ significantly ($P > 0.05$) when cultures were maintained in the presence of ≤ 60 ng/mL of CHX or 30 to 60 ng/mL of BFA.

The anti-secretory effects of brefeldin A and cycloheximide are not due to non-specific cytotoxicity

To ensure that cell death did not contribute to any observed reduction in synthesis or secretion of mMCP-1, 14-day-old mMCP-1⁺ve mBMMC were transferred into TGF- β_1 /IL-3/IL-9/SCF supplemented with vehicle control or BFA/CHX (at final concentrations of 15, 30, 60, 120, 240, 480 or 960 ng/mL). Cultures ($n = 6$) were maintained under these conditions for 48 h. After 24 h, a proportion of the cells from each culture were harvested and counted; nigrosin exclusion was used to assess viability. Constitutive secretion of mMCP-1 and CCL2 was measured by ELISA (Figs 2b and c). After a further 24 h, the remaining cells were stained with Annexin-V and propidium iodide. The percentages of viable, apoptotic and dead mBMMC were determined using flow cytometry (Figs 2a and 3).

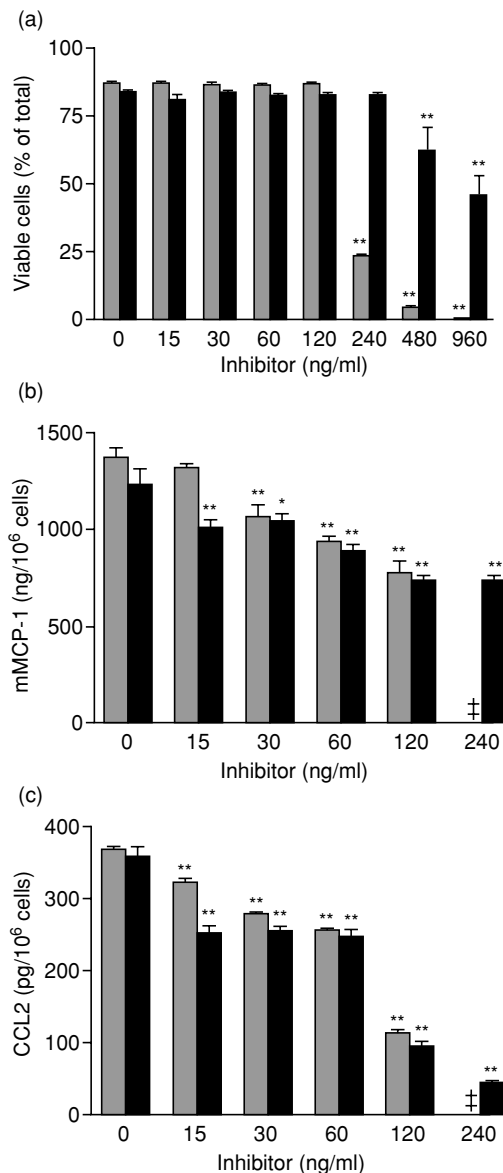


Fig. 2. Brefeldin A and cycloheximide inhibit mMCP-1 and monocyte chemoattractant protein-1 secretion by integrin $\alpha_E^{+ve}/mMCP-1^{+ve}$ mBMMC. Fourteen-day-old integrin $\alpha_E^{+ve}/mMCP-1^{+ve}$ mBMMC were maintained for 48 h in TGF- β_1 /IL-9/IL-3/SCF supplemented with various concentrations of BFA/vehicle (■), CHX/vehicle (■). Cultures were sampled at 24 h: numbers and viability of mBMMC were quantified after dilution in 0.2% nigrosin; and the amount of mMCP-1 present in culture supernatants was quantified by ELISA. (a) To rule out the possibility that any reduction in mMCP-1 or MCP-1 secretion observed at 24 h was due to non-specific cytotoxicity: cultures were maintained for a further 24 h in the presence of BFA/CHX; after which apoptotic, necrotic and viable mBMMC were quantified by flow cytometry (See Fig. 3). Bars represent the percentage (mean value $\pm$ 1 standard error of the mean; $n=6$) of viable non-apoptotic mBMMC present in cultures after 48 h exposure to BFA/CHX. (b) Bars represent mean concentrations of secreted mMCP-1 (ng/10⁶ viable cells; $n=6$) $\pm$ 1 standard error of the mean. (c) Bars represent mean concentrations of secreted monocyte chemoattractant protein-1 (CCL2 pg/10⁶ viable cells; $n=6$) $\pm$ 1 standard error of the mean. Data from cultures in which a significant reduction in viability ($P < 0.05$) was observed at 24 or 48 h have been excluded (‡). Differences between BFA/CHX-treated cultures and their respective vehicle control cultures are significant at $P < 0.01$ (**) and $P < 0.05$ (*).

Significant cell death was detected using nigrosin exclusion in mBMMC cultures exposed to > 240 ng/mL of CHX or > 120 ng/mL of BFA (data not shown). However, both

compounds significantly reduced constitutive mMCP-1 and CCL2 secretion in a dose-dependent manner at concentrations below the threshold required to induce cell death (Fig. 2). Furthermore, there was no evidence for an increase in BFA- or CHX-induced apoptosis or cell death at these concentrations after 48 h of continual exposure (Figs 2a and 3). The Annexin-V and propidium iodide staining characteristics of mBMMC exposed to 120 ng/mL of BFA (Fig. 3b) or 240 ng/mL of CHX (Fig. 3e) for 48 h were indistinguishable from vehicle control-treated cultures (Figs 3a and d). By contrast, mBMMC treated with 240 ng/mL of BFA showed a marked increase in Annexin V binding, indicative of increased numbers of apoptotic cells (Fig. 3c). mBMMC exposed to 480 ng/mL of CHX exhibited a less substantial, but significant ($P < 0.05$ when compared with control cultures; Fig. 2a), increase in Annexin-V and propidium iodide staining (Fig. 3f).

Indirect effects of cycloheximide and brefeldin A on mMCP-1 gene transcription

Additional experiments were performed in order to determine whether CHX or BFA could influence mMCP-1 gene transcription indirectly through generalized reduced protein synthesis or post-translational modification of proteins associated with the transcription process (Fig. 4). In these studies, 14-day-old integrin $\alpha_E^{+ve}/mMCP-1^{+ve}$ mBMMC were transferred into TGF- β_1 /IL-3/IL-9/SCF supplemented with 40 ng/mL of CHX, 100 ng/mL of BFA or vehicle alone and maintained for a further 48 h. Cultures were harvested at 24 and 48 h and analysed by ELISA and comparative RT-PCR.

Neither compound had a detectable effect on mBMMC viability at the concentrations used in these experiments (data not shown). Treatment of mBMMC cultures with CHX or BFA resulted in a significant reduction in mMCP-1 secretion over the first 24 h of exposure ($P < 0.01$; Fig. 4a). This effect was less marked but still significant when cultures were exposed to either compound for 48 h ($P < 0.01$ and $P < 0.05$ for BFA and CHX treated cultures, respectively; Fig. 4a). Intracellular levels of mMCP-1 were also significantly reduced in mBMMC exposed to 40 ng/mL of CHX for 24 h ($P < 0.01$; data not shown). Comparative RT-PCR analysis, showed that there was a significant reduction in mMCP-1 transcript abundance ($34 \pm 9\%$ relative to vehicle control cultures; $P < 0.05$) when mBMMC were maintained in CHX for 48 h (Figs 4c and d). By contrast, there was no evidence for any reduction in mMCP-1 transcript expression in mBMMC exposed to BFA for 48 h (Figs 4c and d).

Discussion

A substantial body of evidence exists to support the hypothesis that MMC can secrete granule chymases through mechanisms that are distinct from IgE-mediated exocytosis [3, 9, 11–14]. Previous work by this group has identified a central role for the multifunctional cytokine, TGF- β_1 , in both the expression and IgE-independent release of mMCP-1 by mBMMC [15, 16].

The main focus of this study was on mMCP-1, because little or nothing is known of the mechanisms of constitutive release of a mediator that is packaged in the Golgi complex and subsequently stored in the granules. The confocal studies described here clearly show the expected co-localization of mMCP-1 and

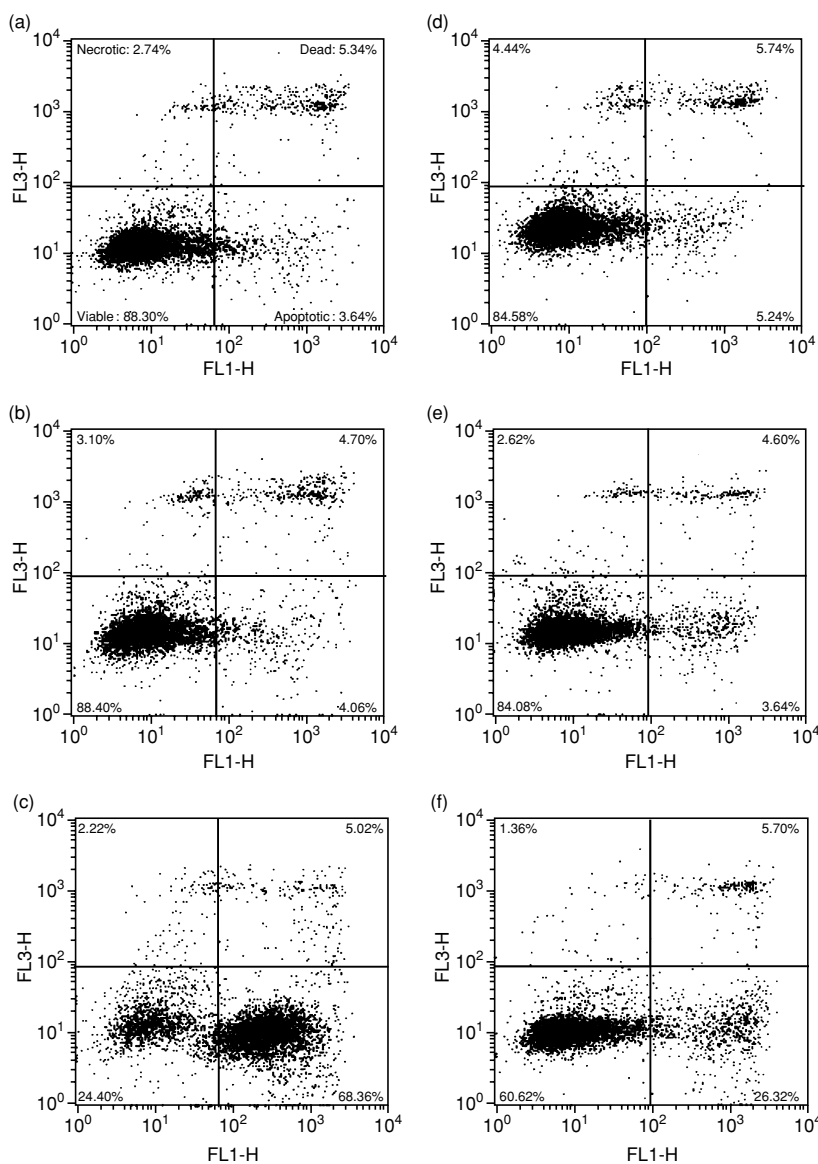


Fig. 3. The effects of brefeldin A and cycloheximide on integrin $\alpha_E^{+ve}/mMCP-1^{+ve}$ mBMMC viability. Fourteen-day-old integrin $\alpha_E^{+ve}/mMCP-1^{+ve}$ mBMMC were maintained for 48 h in TGF- β_1 /IL-9/IL-3/SCF supplemented with various concentrations of BFA, CHX or vehicle alone. Cells were stained with Alexa Fluor-488 conjugated Annexin-V and propidium iodide. Quantitative analysis was performed using a FACSCalibur flow cytometer with linear amplification for forward/side scatter and logarithmic amplification for Alexa Fluor 488 (FL1-H: x-axis) and propidium iodide (FL3-H: y-axis) fluorescence. Representative scatter plots are shown for cultures treated with: (a) vehicle control; (b) 120 ng/mL of BFA (no significant reduction in viability); (c) 240 ng/mL of BFA (significant reduction in viability); (d) vehicle control; (e) 240 ng/mL of CHX (no significant reduction in viability); and (f) 480 ng/mL of CHX (significant reduction in viability).

the Golgi matrix protein GM130 within the Golgi complex (Figs 1g and h and Table 1). Interestingly, CCL2 also localizes to the Golgi complex but is apparently not stored in the granules (Fig. 1f). This appears to be the first clear description of this localization in mast cells.

CCL2 was constitutively released by mBMMC and has been shown to be constitutively released in other mast cell culture systems as a consequence of stimulation with SCF [18–20]. It is likely therefore that the constitutive release of CCL2 by MMC homologues was due to the presence of SCF in the cultures. Although the stimuli for synthesis and release of this chemokine and of mMCP-1 are different, the pathways for extracellular release may be similar for both proteins. It is possible, for example, that mMCP-1 leaches non-specifically from mBMMC granules as a form of spontaneous degranulation. Alternatively, constitutive release of mMCP-1 could result from the Golgi-directed packaging directly to the cell exterior via secretory vesicles, as occurs with many exported proteins.

We therefore compared the effects of Brefeldin A, which disrupts the Golgi complex, on the constitutive release of these two distinct inflammatory mediators with very different patterns of storage. We also used CHX to determine whether constitutive secretion of mMCP-1 is dependent on continuous protein synthesis, as opposed to the release of pre-formed mMCP-1 via spontaneous degranulation (Table 2). CHX inhibits protein synthesis in eukaryotic cells by preventing tRNA from binding correctly to ribosomes [25]. By contrast, BFA inhibits constitutive protein secretion via the Golgi complex but does not affect protein synthesis [26, 27]. Both compounds significantly reduced constitutive secretion of mMCP-1 and CCL2 in a dose-dependent manner (Table 2 and Fig. 2). In the case of CHX, this effect was accompanied by a significant decrease in intracellular mMCP-1 (Table 2). Although neither compound blocked the extracellular release of mMCP-1 release completely, the 40 to 50% reductions in secretion observed supports the involvement of both *de novo* protein synthesis

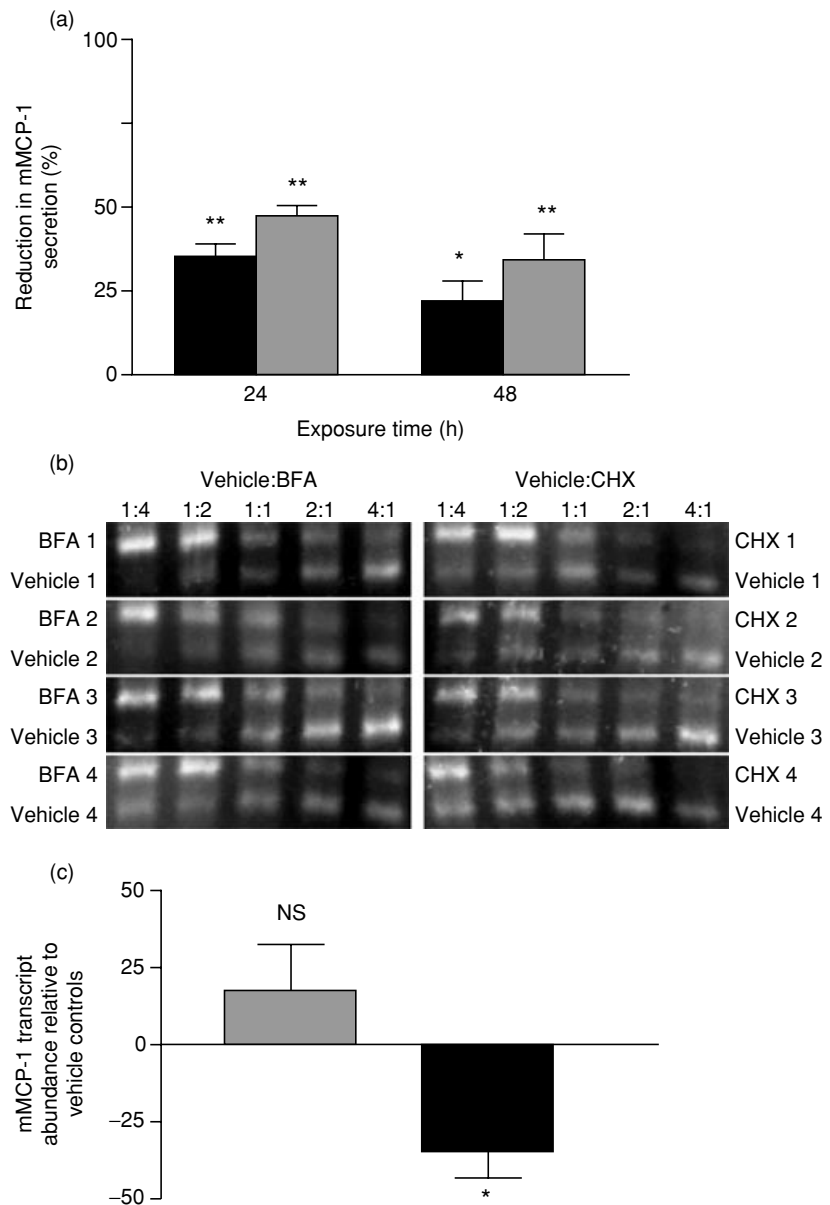


Fig. 4. Effects of cycloheximide and brefeldin A on mMCP-1 gene transcription. Fourteen-day-old integrin α_E^{+ve} /mMCP-1 $^{+ve}$ mBMMC were transferred into TGF- β_1 /IL-9/IL-3/SCF supplemented with 40 ng/mL of CHX ■, 100 ng/mL of BFA ▣, or vehicle alone and maintained for a further 48 h. (a) Histogram representing the percentage reduction (compared with vehicle control cultures) in secretion of mMCP-1 over 24 and 48 h. (b) mMCP-1 transcripts, amplified from competing cDNAs from 48 h CHX-, BFA- and vehicle-treated cultures, using a comparative an 'IntraSpec' Comparative RT-PCR kit (Ambion). RT-PCR products amplified from vehicle control cDNAs are 305 bp, products from CHX/BFA cDNAs are 345 bp. (c) Histogram representing the abundance of mMCP-1 transcripts in BFA/CHX-treated cultures relative to that in mBMMC maintained in vehicle control. Results presented in histogram format are expressed as mean values $\pm$ 1 standard error of the mean ($n = 4$). Differences between CHX- or BFA-treated cultures and vehicle control cultures are significant at $P < 0.05$ (*) or $P < 0.01$ (**).

and Golgi directed transport in the constitutive secretion of mMCP-1.

Extracellular release of mMCP-1 was reduced by $40 \pm 1.7\%$ and $43 \pm 47\%$ in mBMMC cultures treated with 240 ng/mL of CHX and 120 ng/mL of BFA, respectively (Fig. 2b). By contrast, secretion of CCL2 was reduced by $87 \pm 0.3\%$ (CHX) and $69 \pm 1\%$ (BFA) in the same cultures (Fig. 2c). Although CCL2 is constitutively secreted by mature mMCP-1 $^{+ve}$ mBMMC (Fig. 2c) it is not, as mentioned above, stored as a pre-formed mediator within the mast cell granules (Fig. 1f). This may explain why CHX and BFA are approximately 2.2- and 1.6-fold more effective at reducing constitutive secretion of CCL2 (Fig. 2) relative to mMCP-1. It is likely therefore that additional mechanisms may be involved in the constitutive release of MMC mediators that are stored within the mast cell granules. However, it should be noted that the percentage reductions observed in the secretion of mMCP-1 and CCL2 were virtually identical

when cultures were maintained in the presence of ≤ 60 ng/mL of CHX or 30 to 60 ng/mL of BFA. Furthermore, remarkably similar (40 to 50%) maximal inhibition to that reported here (Table 2 and Fig. 2b), in the absence of non-specific inhibition of protein synthesis, of TNF- α secretion has been reported in RBL-2H3 rat mast cells treated with 500 ng/mL of BFA [28].

Xia et al. [29] have previously reported that IL-10-regulated stabilization of mMCP-2 mRNA in mBMMC is abolished by concomitant exposure to ≥ 1 μ g/mL of CHX over 24 h. They hypothesize that this effect results from CHX-mediated inhibition of IL-10-induced *trans*-acting factors responsible for maintaining the half-life of mMCP-2 mRNA [29]. In the current study, CHX has a similar but less absolute effect on mMCP-1 transcript abundance in mBMMC (Fig. 4c, d). It is outside the scope of the current study to investigate the precise mechanisms through which CHX down-regulates mMCP-1 transcript abundance in mBMMC. However, it seems likely that CHX inhibits the synthesis of downstream elements involved in mMCP-1

transcription and/or, as has been described for mMCP-2 [29], in maintaining mMCP-1 transcript stability. Importantly, there was no evidence for any reduction in mMCP-1 transcript expression or protein synthesis in mBMMC maintained in the presence of BFA (Fig. 4).

Our results show that mBMMC are highly susceptible to BFA- and CHX-induced cell death (Figs 2a and 3). In our hands $\geq 1 \mu\text{g/mL}$ of CHX significantly reduced mBMMC viability when maintained in the culture conditions for more than 6 h (data not shown). This contrasts with a previous study, in which a decrease in mBMMC viability was not reported when cultures were exposed to 1 to $20 \mu\text{g/mL}$ of CHX over 24 h [29]. The reason for this discrepancy is not clear but it is conceivable that mBMMC grown in IL-3 or IL-3/IL-10, as used by Xia et al. [29], are less sensitive to CHX than mBMMC grown in TGF- β_1 /IL-9/IL-3/SCF. Significant cell death was also detected when mMCP-1⁺ve mBMMC cultures were exposed to $\geq 200 \text{ ng/mL}$ of BFA for 24 h or longer (Figs 2a and 3). Baumgartner et al. [28] have shown that 0.5 to $10 \mu\text{g/mL}$ of BFA down-regulates IgE-dependent secretion of the cytokine TNF- α by the rat mast cell line, RBL-2H3. In this case [28], cultures were exposed to BFA for much shorter periods of time ($< 3 \text{ h}$) than in the current study. We have found the cytotoxic effects of BFA to be both time and dose dependent (data not shown). Nevertheless, the finding that $\geq 5 \mu\text{g/mL}$ of BFA inhibits TNF- α synthesis [28], suggests that even brief exposure to high concentrations of BFA is sufficient to induce non-Golgi complex specific effects.

It is clearly of paramount importance to delineate the cytotoxic effects of CHX and BFA from their respective inhibitory actions on protein synthesis and Golgi directed secretion. To resolve this issue, we used sufficiently low concentrations of both compounds to ensure that mBMMC viability was not compromised (Fig. 3a). Viability was strictly monitored in each experiment using both nigrosin exclusion and a commercially available LDH release assay. More detailed analysis of mBMMC apoptosis, using the Annexin V-affinity assay [30], confirmed that the concentrations of CHX and BFA required to significantly inhibit constitutive secretion do not affect mBMMC viability (Figs 2a and 3).

This is the first time that the Golgi complex has been directly implicated in the constitutive secretion of a mast cell protease. The existence of such a mechanism *in vivo* would bypass the requirement for antigen mediated IgE/Fc ϵ R1 activation of MMC and account for the constitutive secretion of mMCP-1 observed in normal, uninfected mice [13]. Similarly, up-regulation of constitutive secretion of mMCP-1 by TGF- β_1 is likely to be responsible for the rapid increase in systemic levels of mMCP-1 observed, in the absence of parasite-specific IgE, during primary intestinal nematode infections [3, 13]. It would clearly be more efficient for the process of constitutive release of peptide mediators if there was a final common pathway transporting these molecules to the cell surface. The present study indicates that this may be the case for the concomitant release of mMCP-1 and CCL2 and it would appear that a significant proportion of the extracellular mMCP-1 is derived by a process that by-passes the secretory granules. It is possible that a similar mechanism applies to the differential release of alpha and beta tryptase by human mast cells and the current observations suggest that this process may apply to many different mast cell populations [2].

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