

Mouse bone marrow-derived IL-3-dependent mast cells and autonomous sublines produce IL-6

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SUMMARY

This study deals with the question of whether mouse bone marrow-derived mast cells are able to produce interleukin-6 (IL-6) *in vitro*. For this purpose, a panel of primary mast cell clones from limiting-dilution microcultures, of permanent IL-3-dependent mast cell lines and autonomous malignant sublines, was screened. All of these lines were found to produce growth factor activity for IL-6-dependent mouse hybridoma cells (7TD1), which could be completely neutralized by the monoclonal anti-IL-6-antibody 6B4. Transcriptional activity of the IL-6 gene was demonstrated in both IL-3-dependent mast cells and autonomous sublines using a mouse IL-6-specific cDNA probe.

INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic and multifunctional regulatory glycoprotein acting within a complex cytokine network (Wong & Clark, 1988). It stimulates proliferation, differentiation and/or functional activities in a variety of different cell types, including B cells (Vink *et al.*, 1988), T cells (Garman *et al.*, 1987; Takai *et al.*, 1988), myeloma-plasmacytomas (Vink *et al.*, 1988; Nordan & Potter, 1986; Van Damme *et al.*, 1987a; Kawano *et al.*, 1988) and hybridomas (Van Snick *et al.*, 1986), hepatocytes (Andus *et al.*, 1987; Gauldie *et al.*, 1987), haemopoietic stem cells (Ikebuchi *et al.*, 1987), fibroblasts (Kohase *et al.*, 1986) and phaeochromocytoma cells (Satoh *et al.*, 1988). Up to now fibroblasts (Content *et al.*, 1985), monocytes/macrophages (Aarden *et al.*, 1987; Nordan, Pumphrey & Rudikoff, 1987), endothelial cells (Astaldi *et al.*, 1981), T cells (Vink *et al.*, 1988; Van Snick *et al.*, 1986) and several transformed cell lines (Hirano *et al.*, 1986) have been identified as *in vitro* producers of IL-6. The expression of IL-6 may be enhanced in response to other cytokines such as IL-1 α or β (Content *et al.*, 1985; Van Damme *et al.*, 1987a), tumour necrosis factor α (Van Damme *et al.*, 1987b; Kohase *et al.*, 1986), or platelet-derived growth factor (Kohase *et al.*, 1986), as well as in response to double-stranded RNA (Van Damme *et al.*, 1987a). Increased production of IL-6 has been further shown in cultures of monocytes (Gauldie *et al.*, 1987) or fibroblasts (Helfgott *et al.*, 1987) stimulated with bacterial endotoxin. Recently stromal fibro-

blasts were demonstrated to secrete IL-6 also in response to acute viral infection (Sehgal *et al.*, 1988). On the basis of these findings it is not surprising that IL-6 has emerged as a major component in the host immune response to bacterial infections and tissue damage (Nijsten *et al.*, 1987). Increased levels of IL-6 have been demonstrated in serum of patients with rheumatoid arthritis and systemic lupus erythematosus, in synovial fluid of patients with rheumatoid arthritis and in cerebrospinal fluid of patients with multiple sclerosis or viral meningitis and encephalitis (Aarden *et al.*, 1987; Matsuda, Hirano & Kishimoto, 1988; Frei *et al.*, 1988).

In some of these patho-physiological situations local accumulation and/or activation of mast cells has long been known to occur at the sites of inflammation (Lewis & Austen, 1981). Mast cell activation has been observed in both immediate (Ishizaka & Ishizaka, 1975) and delayed hypersensitivity reactions (Askenase & Van Loveren, 1983), and mast cell hyperplasia occurs in rheumatoid arthritis (Bromley, Fisher & Wolley, 1984) and enteritis following helminthic infections (Miller & Jarrett, 1971; Befus & Bienenstock, 1979). In the latter case IL-3-dependent local proliferation of mucosal mast cells in the gut of infected animals has been specifically implicated in the expulsion of parasites (Guy-Grand *et al.*, 1984). This mechanism is severely impaired in mast cell deficient w/w^v mice (Kojima, Kitamura & Takatsu, 1980; Ha, Reed, & Crowle, 1983).

In view of these converging actions of IL-6 and mast cells in inflammation, in the present study we investigated whether mast cells might produce IL-6. We have developed a limiting dilution culture system favouring the growth of mast cell progenitors from normal mouse bone marrow. From primary mast cell cultures permanent IL-3-dependent mast cell lines could be established efficiently (Hültner *et al.*, 1989). These, in turn, could be reproducibly converted to autonomous malignant sublines

Abbreviations: BMC, bone marrow cells; CM, conditioned medium; FCS, fetal calf serum; hu, human; IL, interleukin; M, molar; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; mu, murine; OD, optical density; r, recombinant; SCM, pokeweed mitogen-activated spleen cell-conditioned medium.

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(Hültner, Moeller & Dörmer, 1986). Evidence is provided that both IL-3-dependent mast cells and autonomous tumourigenic sublines express IL-6 mRNA and secrete IL-6 *in vitro*.

MATERIALS AND METHODS

Mice

BALB/c or C3H/E1 mice of both sexes were used at an age of 8–16 weeks as bone marrow donors. Mice were bred in our facilities and fed with commercial pellets and water *ad libitum*.

Mast cell cultures

Polyclonal mast cell populations were generated in cultures of BALB/c or C3H/E1 bone marrow cells (BMC) supplemented with pokeweed mitogen-activated spleen cell-conditioned medium (SCM) as described previously (Hültner *et al.*, 1989). Briefly, murine BMC ($1 \times 10^5/\text{ml}$) were grown in RPMI-1640 medium supplemented with 20% FCS, 20% SCM, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin and 10^{-4} M α -thioglycerol. When propagating non-adherent BMC in this culture medium with weekly refeeding, homogeneous populations of mast cells could be obtained within a period of 3–4 weeks. From these primary mast cells permanent IL-3-dependent mast cell lines and agar-cloned derivatives could be derived efficiently. Both primary and IL-3-dependent mast cell lines resembled mucosal type mast cells by functional and phenotypical criteria (Hültner *et al.*, 1989, 1985). At low concentrations of IL-3 (≤ 1 U/ml) autonomous malignant sublines were reproducibly generated (Hültner *et al.* 1986).

Mast cell progenitors from mouse bone marrow were also grown under clonal conditions of a limiting-dilution culture system. Limited numbers of BMC (800–3200/well) were seeded into flat-bottomed 96-well microculture plates (200 μl /well) using the medium as described above. Mast cell precursor frequencies were evaluated on Day 14 of culture according to published methods (Lefkovits & Waldmann, 1979) by reading the number of seeded cells (x) corresponding to 37% negative cultures. In this case $1/x$ represents the precursor frequency.

Cytokines and antibodies

Purification of murine IL-6 (muIL-6), IL-6-dependent hybridoma cells 7TD1 and anti-muIL-6 hybridoma 6B4 have all been described previously (Vink *et al.*, 1988; Van Snick *et al.*, 1986). Recombinant human IL-6 (rhuIL-6) was commercially available from Genzyme (Boston, MA). Murine IL-3 was partially purified from Wehi-3B-conditioned medium (Wehi-3B-CM) (1×10^6 cells/ml; 4–5 days) employing DEAE-cellulose chromatography (Moeller *et al.*, 1989). Murine IL-4 was partially purified from EL-4-CM ($2 \times 10^6/\text{ml}$ EL-4 cells stimulated with 20 ng/ml TPA for 48 hr) using Sepharose chromatography (Moeller *et al.*, 1989). Purified murine IL-3 used as a control in selected experiments was available from Genzyme.

Determination of IL-6 activity

IL-6 was measured employing the MTT-assay (Mosmann, 1983) as described previously (Hültner *et al.*, 1989) using a murine hybridoma cell line (7TD1) which only grows in the presence of IL-6 (Van Snick *et al.*, 1986). In brief, $1 \times 10^4/\text{ml}$ 7TD1 cells stimulated with serial dilutions of the conditioned media to be tested were grown in duplicate microcultures using

RPMI-1640 medium supplemented with 10^{-5} M α -thioglycerol, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 0.1 mM hypoxanthine and 16 μM thymidine (HT, 50x; Gibco, Grand Island, NY) 5 $\mu\text{g}/\text{ml}$ polymyxin B and 10% FCS. After 3 days MTT-formazan produced during the last 4 hr was measured (Hültner *et al.*, 1989). Highly purified native murine IL-6 (Van Snick *et al.*, 1986) as well as rhuIL-6 served as positive controls. IL-6 bioactivity was verified using the monoclonal antibody 6 B4 (Vink *et al.*, 1988).

Determination of IL-6 mRNA expression

Total RNA was isolated from exponentially growing cells of the following mast cell lines established in our laboratory: L138.8A (cloned permanent IL-3-dependent cell line cultured in 20% SCM), L138 C auto (autonomous subline growing for 12 weeks in the absence of exogenous growth factors), L138 C 3-10 auto (cloned autonomous subline growing for 6 months in the absence of exogenous growth factors and then expanded either in the absence or presence of SCM), L190D3-B1-Asc (ascites mast cells freshly harvested from the ascites of a mast cell tumour-bearing BALB/c mouse injected i.p. with $1 \times 10^6/\text{mouse}$ tumourigenic L190D3-B1 mast cells 8 weeks before), and L138.8 auto A-T (autonomous subline established *ex vivo* from the malignant mast cell ascites produced in a BALB/c mouse following the injection of tumourigenic L138.8 auto A mast cells). The isolation of cellular RNA essentially followed standard protocols (Chirgwin *et al.*, 1979). Glyoxylated RNA samples (20 μg of total RNA) were transferred to nylon membranes (Amersham, Amersham, Bucks, U.K.) after electrophoresis through 1.2% agarose gels. Northern hybridization was performed without formamide at 65° for at least 30 hr using nick-translated probes labelled with ^{32}P -dCTP (Amersham) to a specific activity of 1×10^8 c.p.m./ μg DNA. The stringency wash was done at 60° for 60 min, using salt conditions of $0.1 \times \text{SSC}$ ($1 \times \text{SSC} = 150$ mM sodium chloride, 15 mM sodium citrate). IL-6 transcripts were detected by the use of a 667 bp ECORI-PSTI fragment of mouse IL-6 cDNA derived from the clone PHP1B5 (Van Snick *et al.*, 1988).

RESULTS

Generation of mouse bone marrow-derived mast cell clones in limiting-dilution microcultures

In pilot studies we found that virtually pure mast cell populations could be grown from BALB/c or C3H BMC in a limited number of microwells under reproducible conditions. BMC were seeded at limiting-dilution densities in replicate wells of microtitre plates and cultured in the presence of an optimum SCM concentration (20% of a pretested, $5 \times$ concentrated batch) as a source of IL-3. Single mast cell clones developed as very uniform mosaic-like monolayers within a culture period of 14–16 days. At this time granulocyte-macrophage clones consisted of mostly degenerating or dead cells. The mast cell type was confirmed morphologically using Alcian Blue-stained centrifuge preparations. Figure 1 shows a representative result obtained with C3H-BMC. When the number of BMC seeded per well was plotted on a semi-logarithmic scale against the fraction of mast cell-negative wells, a linear regression line was repeatedly obtained with an intercept at the ordinate close to 1.0. This finding indicates single hit kinetics, meaning that only

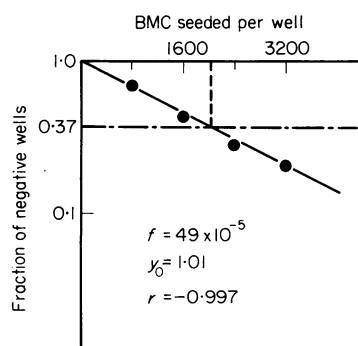


Figure 1. Determination of precursor frequency in C3H bone marrow of mucosal type mast cells by means of limiting-dilution microcultures.

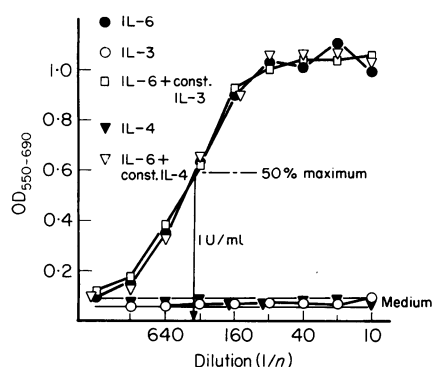


Figure 2. Lack of growth modulation of IL-3 and IL-4 in cultures of IL-6-dependent hybridoma cells (7TD1). Detection system: MTT assay.

one cell type was limiting the number of negative wells. Similar mast cell precursor frequencies as in Fig. 1 were measured when purified IL-3 was used instead of SCM (data not shown).

Lack of growth modulation by IL-3 or IL-4 in 7TD1 hybridoma cell cultures

As a prerequisite for testing the production of IL-6 in mast cell cultures in the presence of mast cell growth factors, we had to ensure that our preparations of IL-3 and IL-4 were free of IL-6 activity and devoid of IL-6 modulating activity. Figure 2 shows that serial dilutions of IL-3 partially purified from Wehi-3B-cM or IL-4 partially purified from EL-4-cM were indeed free of detectable IL-6 activity. Moreover, the addition of IL-3 or IL-4 (10 U/ml each) to cultures of 7TD1 cells stimulated with serial dilutions of purified IL-6 did not modify the dose-response curve obtained with IL-6 alone (Fig. 2).

Production of IL-6 in mast cell cultures

In vitro production of growth factor activity for IL-6-dependent mouse hybridoma cells (7TD1) was consistently observed in murine bone marrow-derived primary mast cells, in permanent IL-3-dependent mast cell lines and in autonomous malignant sublines (Table 1). The 7TD1-stimulating activity produced by

Table 1. IL-3-dependent and autonomous murine mast cells produce growth factor activity for IL-6-dependent hybridoma cells (7TD1)

Designation*	Origin	Time of <i>in vitro</i> proliferation	IL-3 dependence	Production of growth factor activity for 7TD1 cells
1 MW-1	BALB/c	4 weeks	+	+
2 MW-2	C3H/E1	4 weeks	+	+
3 MW-3	C3H/E1	4 weeks	+	+
4 MW-4	BALB/c	4 weeks	+	+
5 MW-5	BALB/c	4 weeks	+	+
6 LD17-1	BALB/c	4 weeks	+	+
7 LD17-2	BALB/c	4 weeks	+	+
8 LD17-3	BALB/c	4 weeks	+	+
9 LD17-4	BALB/c	4 weeks	+	+
10 LD17-5	BALB/c	4 weeks	+	+
11 L138A	BALB/c	1 year	+	++
12 L138.8A	BALB/c	2 years	+	++
13 L138.8 auto A	L138.8A	2 years	—	++
14 L138C auto	L138A	12 weeks	—	++
15 L138C3-10 auto	L138C auto	6 months	—	++
16 32Dcl.23	C3H/HeJ	> 2 years	+	+++
17 EL-4	C57Bl/6N	> 2 years	—	—
18 HL-60	Human	> 2 years	—	—

* 1–10, clones of primary bone marrow-derived mucosal type mast cells (MMC); 11–12, permanent IL-3-dependent MMC sublines; 13–15, permanent autonomous malignant MMC sublines; 16, early haemopoietic cell line; 17, thymoma cell line; 18, promyelocytic leukaemia cell line (FAB M2).

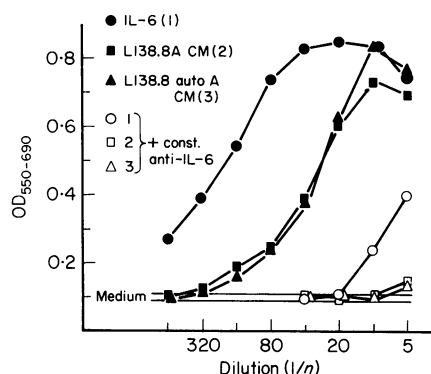


Figure 3. Conditioned medium (CM) from IL-3-dependent mast cells (L138.8A) or autonomous mast cells (L138.8 auto A) contains 7TD1-stimulating activity which is neutralized by the monoclonal anti-IL-6-antibody 6B4 (10 µg/ml). Detection system: MTT assay.

factor-dependent mast cells (L138.8A) or an autonomous subline (L138.8 auto A) was completely neutralized by the monoclonal anti-muIL-6-antibody 6B4 (Fig. 3).

In pilot studies performed with both IL-3-dependent mast cells and autonomous sublines, we observed a near linear relationship between the number of cells cultured per ml (range tested: 10^5 – 10^6 cells per ml) and the amount of IL-6 measured in the culture media conditioned for 24 hr (data not shown). IL-6

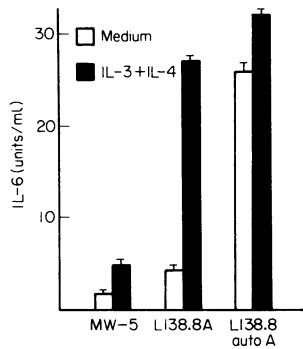


Figure 4. Production of IL-6 in the absence or presence of IL-3 and IL-4 (10 U/ml each) in cultures of primary mast cells (MW-5), permanent IL-3-dependent mast cells (L138.8A) and autonomous malignant mast cells (L138.8 auto A). Replicate cultures of 2×10^5 /ml mast cells were allowed to condition media (24-well plate; 0.5 ml/well) for 24 hr. Serially diluted supernatants were tested individually for IL-6 activity employing the MTT-assay with 7TD1 cells. Values shown are $\bar{x} \pm \text{SD}$ ($n = 4$).

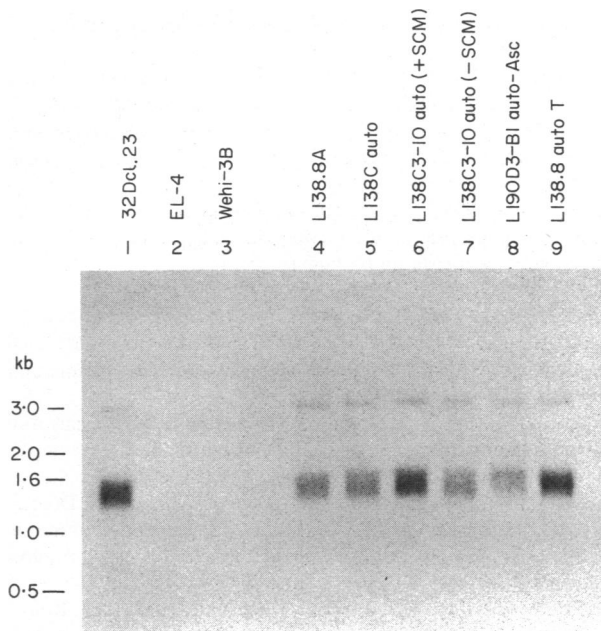


Figure 5. Detection of IL-6 mRNA in IL-3-dependent (lane 4) and autonomous malignant mast cells (lanes 5–9). Positive control: 32Dcl.23 cells (lane 1); negative controls: EL-4 (lane 2) and Wehi-3B cells (lane 3).

levels measured at daily intervals (up to 4 days) in supernatants of replicate cultures of either L138.8A mast cells (2×10^5 /ml) stimulated with IL-3 and IL-4 (10 U/ml each) or L138.8 auto A mast cells (2×10^5 /ml) cultured in the absence of growth factors, continuously increased until Day 3 of culture (data not shown). The capacity of IL-6 production was compared over a period of 24 hr in the presence and absence of IL-3 (Fig. 4). Factor-dependent MW-5 and L138.8A cells (2×10^5 /ml each) showed a significant enhancement in the presence of both cytokines. In contrast, the autonomous subline L138.8 auto A at the same density showed high IL-6 levels in the presence and absence of exogenous growth factors (Fig. 4). However, an additional increase of IL-6 activity was observed in the presence of IL-3 and IL-4 (Fig. 4).

Expression of IL-6 mRNA in IL-3-dependent mast cells and autonomous malignant sublines

Total RNA was isolated for Northern blot analysis from a series of exponentially growing mast cell lines established in our laboratory. The murine IL-3-dependent cell line 32Dcl.23 (Greenberger *et al.*, 1983) served as a positive control. It had been previously identified as an IL-6 high producer cell line (Hültner *et al.*, 1988; L. Hültner, H. Szöts, J. van Snick, J. Moeller, M. Welle and P. Dörmer, manuscript submitted for publication). Wehi-3B and E1-4 cells were taken as negative controls. In 32Dcl.23 as well as in all mast cell lines a major 1.4 kb mRNA species was detected by Northern blot analysis (Fig. 5). A minor RNA species corresponding to about 3.0 kb was found to cross-hybridize with the IL-6 probe used. The nature of this signal was not investigated further. This RNA subspecies appeared only in samples containing the typical 1.4 kb IL-6 transcript. A comparable amount of RNA had been loaded in all lanes as confirmed by reprobing for β -actin (Cleveland *et al.*, 1980) (data not shown).

DISCUSSION

In the present study IL-6-specific transcripts were identified (Fig. 5) and biologically active IL-6 detected (Table 1, Figs 3 and 4) in cultures of mouse bone marrow-derived IL-3-dependent mast cells and autonomous malignant sublines established in our laboratory (Hültner *et al.*, 1989, 1986). IL-3-dependent murine mast cells developing *in vitro* from haemopoietic organs are phenotypically and functionally closely related to mast cells residing *in vivo* in the intestinal mucosa (Jarrett & Haig, 1984). By histo-morphological and functional criteria murine mucosal-type mast cells can be distinguished from murine connective tissue-type mast cells (Jarrett & Haig, 1984).

In accordance with reports by others (Mosmann *et al.*, 1986; Smith & Rennick, 1986), IL-4 has been shown to synergistically enhance the proliferation of our IL-3-dependent mast cell lines (Hültner *et al.*, 1989). In pilot studies we found that not only proliferation but also IL-6 production of mast cells was significantly enhanced when IL-4 was used in addition to IL-3 (data not shown). Therefore, both IL-3 and IL-4 have been applied in the present investigation of IL-6 production by mast cells.

IL-6 was detected in test supernatants by the use of an IL-6-dependent mouse hybridoma (7TD1) (Van Snick *et al.*, 1986) as an IL-6 indicator cell line and of the neutralizing monoclonal anti-muIL-6 antibody 6B4 (Vink *et al.*, 1988). With these sensitive and specific tools a panel of primary murine bone marrow derived mast cell clones, permanent IL-3-dependent mast cell lines and autonomous tumourigenic sublines was screened for IL-6 production *in vitro* (Table 1). All 7TD1-stimulating activity could be attributed to murine IL-6 by means of the neutralizing monoclonal anti-muIL-6 antibody 6B4 (Fig. 3). The transcriptional activity of the IL-6 gene could be additionally demonstrated in all mast cell lines using a mouse IL-6-specific cDNA probe (Van Snick *et al.*, 1988) (Fig. 5). While IL-6 production of factor-dependent mast cells was significantly enhanced by IL-3 and IL-4 compared to medium controls, autonomous malignant mast cells were able to secrete high amounts of IL-6 also in the absence of exogenous mast cell growth factors (Fig. 4). The additional increase of activity observed in the presence of IL-3/IL-4 is in line with the fact that

although L138.8 auto A cells can grow in the absence of exogenous growth factors, their proliferation can be significantly increased by IL-4. In general we have made the observation that cloned autonomous malignant mast cell sublines reproducibly derived from non-tumorigenic IL-3-dependent parent lines (Hültner *et al.*, 1986) retained responsivity for both IL-3 and IL-4 for several weeks. Later (4 weeks up to 1 year) the autonomous mast cell clones failed to respond to IL-3, but they still responded to IL-4 with an increased rate of proliferation (L. Hültner, M. Welle, H. Szöts, P. G. Strauss, J. Moeller and P. Dörmer, manuscript in preparation). Considering these findings, we think that the increased IL-6 activity in the supernatant of IL-3/IL-4-stimulated autonomous mast cells (L138.8 autoA) may primarily reflect their retained IL-4-receptivity, resulting in the possibility of functional activation by IL-4.

The latter finding raises questions concerning the functional significance of IL-6 production by mast cells at different stages of leukaemogenesis. IL-6 secreted by IL-3-dependent mast cells may be considered a candidate autocrine growth factor, if the cells concomitantly have acquired the capacity to express functional IL-6 receptors. However, *in vitro* experiments with autonomous IL-6-secreting mast cells failed to demonstrate a proliferative effect of exogenous IL-6 or a significant inhibitory activity of neutralizing doses of monoclonal anti-IL-6 antibody (Hültner *et al.*, manuscript in preparation).

Based on the present finding that normal mouse bone marrow-derived primary mast cell clones as well as permanent IL-3-dependent mast cell lines express IL-6 mRNA and secrete biologically active IL-6 *in vitro*, studies have to be conducted to answer the question as to whether IL-6 is also released from mast cells *in vivo*. If this should indeed be the case, the key role of mast cells in the inflammatory process will be further substantiated.

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