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Mast cell-derived tumour necrosis factor-α mediates macrophage inflammatory protein-2-induced recruitment of neutrophils in mice

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1 Recent studies have indicated that mast cells play an intermediate role in chemokine-induced neutrophil recruitment *in vivo*.

2 The aim of the present investigation was to determine the role of tumour necrosis factor-α (TNF-α) in neutrophil recruitment provoked by the CXC chemokine macrophage inflammatory protein-2 (MIP-2). For this purpose, we used mast cell- and TNF-α-deficient mice and studied neutrophil adhesion to endothelial cells *in vitro* and neutrophil recruitment in the mouse cremaster muscle *in vivo*.

3 In contrast to the classical chemoattractant formyl-methionine-leucine-phenylalanin (fMLP), MIP-2 dose dependently increased neutrophil accumulation *in vivo*. This MIP-2-regulated neutrophil recruitment was abolished in mast cell-deficient mice.

4 TNF-α increased E-selectin mRNA expression in both wild-type (WT) and mast cell-deficient mice. In contrast, MIP-2 challenge increased gene expression of E-selectin in WT but not in mast cell-deficient animals. Moreover, MIP-2-provoked extravascular accumulation of neutrophils was reduced by 78% in mice lacking TNF-α.

5 In order to better define the role of mast cell-derived TNF-α in neutrophil responses to MIP-2, we used an *in vitro* endothelial cell adhesion assay with and without mast cells. Interestingly, MIP-2-induced neutrophil adhesion to endothelial cells was decreased by 58% using TNF-α-deficient compared to WT mast cells. Moreover, mast cell secretion of TNF-α increased by more than 71% in response to challenge with MIP-2.

6 Taken together, our results suggest that MIP-2-induced neutrophil recruitment is mediated by TNF-α released from local mast cells. These findings help to explain the complex molecular interactions between chemokines, mast cell activation and neutrophil infiltration *in vivo*.
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Abbreviations: CINC, cytokine-induced neutrophil chemoattractant; ELISA, enzyme-linked immunosorbent assay; h, hour(s); IL, interleukin; M, moles per litre; MIP-2, macrophage inflammatory protein-2; PBS, phosphate-buffered saline; PMNLs, polymorphonuclear leukocytes; RT–PCR, reverse transcriptase–polymerase chain reaction; s, second(s); TNF-α, tumour necrosis factor-α; WT, wild-type

Introduction

Chemokines constitutes a large group of small peptides with capacity to orchestrate leukocyte trafficking in many tissues. Chemokines are divided into two major subfamilies, CXC and CC, on the basis of structural properties (Zlotnik *et al*., 1999). In principal, CXC chemokines exert chemotactic influence on neutrophils, whereas CC chemokines mainly attract mononuclear leukocytes (Bacon & Oppenheim, 1998). CXC chemokines comprise macrophage inflammatory protein-2 (MIP-2) and KC, which are considered to be murine homologues of human interleukin-8 (IL-8) and growth-related oncogene chemokines (Oquendo *et al*., 1989; Tekamp-Olson *et al*., 1990). Leukocyte recruitment is a multistep process (rolling–adhesion–transmigration) supported by specific adhesion molecules expressed on leukocytes and endothelial cells (Butcher, 1991; Springer, 1994). It widely accepted that leukocyte rolling is mediated by the selectin family (P-, E- and L-selectin) of adhesion molecules (Butcher, 1991; Springer, 1994; Vestweber & Blanks, 1999), although subsets of integrins have been reported to modulate rolling under certain conditions (Carlos & Harlan, 1994). Endothelial cell activation and expression of P- and E-selectin are necessary for induction of leukocyte rolling (Vestweber & Blanks, 1999). One key aspect of leukocyte rolling is the reduction in velocity of circulating leukocytes, which enables leukocytes to detect chemokines on the endothelial surface (Jung *et al*., 1999). Thus, endothelial cell activation (expression of P- and E-selectin) and leukocyte rolling constitute a critical precondition for subsequent firm adhesion and extravascular accumulation of leukocytes (Vestweber & Blanks, 1999).

Chemokine receptors are seven-transmembrane spanning, G-coupled proteins and at least six CXC chemokine receptors (CXCR1-6) have been reported in the literature (Zlotnik *et al*., 1999). CXCR2 (IL-8 receptor B) is the high affinity receptor on murine neutrophils for MIP-2 and KC (Huber *et al*., 1991; Cacalano *et al*., 1994; Jones *et al*., 1997). Interestingly, an accumulating body of evidence suggests that the expression of CXCR2 is not restricted to neutrophils but also is detected on a number of other cells, such as mast cells (Lippert *et al*., 1998;

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Nilsson *et al*., 1999) and tumour cells (Reiland *et al*., 1999), supporting the concept that chemokines exert effects beyond leukocyte trafficking. However, the data in the literature are complex and partly contradictory with respect to expression of CXCR2 on human endothelial cells. One study suggested the presence of CXCR2 in endothelial cells (Murdoch *et al*., 1999), whereas others could not find CXCR2 expression on endothelial cells (Petzelbauer *et al*., 1995; Schonbeck *et al*., 1995; Gupta *et al*., 1998). We have previously reported that murine endothelial cells do not express CXCR2 (Zhang *et al*., 2001). Recent observations have shown that CXC and CC chemokine can provoke all steps in the extravasation process, including P-selectin-dependent leukocyte rolling, adhesion and transmigration (Zhang *et al*., 2001; Wan *et al*., 2003). However, this capacity of chemokines was largely dependent on the presence of mast cells (Wan *et al*., 2003). Knowing that mast cells express multiple chemokine receptors, including CXCR2, make them potentially suitable for mediating effects of secreted chemokines (Lippert *et al*., 1998; Nilsson *et al*., 1999). However, it is not known which mast cell-derived mediator(s) that mediates MIP-2-induced neutrophil recruitment. Such a mast cell-derived mediator should have the capacity to upregulate selectins and leukocyte rolling on endothelial cells. The most obvious candidates include histamine and tumour necrosis factor-α (TNF-α), which both have receptors on endothelial cells and the capacity to upregulate P-selectin and leukocyte rolling (Weller *et al*., 1992; Yamaki *et al*., 1998a). One previous study has reported that cytokine-induced neutrophil chemoattractant (CINC) provokes mast cell-dependent leukocyte recruitment in a histamine-1-receptor dependent manner (Harris *et al*., 1996).

Thus, the aim of this study was to examine the mechanisms behind the intermediary role of mast cells in MIP-2-induced neutrophil recruitment, and to determine the potential role of histamine and TNF-α in this process.

Methods

Animals

Male C57/Bl6, TNF-α-deficient (Jackson Laboratory, Bar Harbor, ME, U.S.A.) and mast cell-deficient mice (WBB6F1; Jackson Laboratory) weighing between 22 and 27 g were used. Mice were maintained in 12-h dark and 12-h light cycles and given standard pellet food and water *ad libitum*. General anaesthesia was achieved *via* intraperitoneal injection of 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutical, Beerse, Belgium) per 100 mg body weight. All experiments were approved by the local ethics committee.

Neutrophil recruitment

In order to study neutrophil recruitment *in vivo*, MIP-2 (R&D Systems Europe, Ltd, Abingdon, Oxon, U.K.), TNF-α (R&D Systems) and fMLP (Sigma Chemical Co., St Louis, MO, U.S.A.) were administered at the indicated doses intrascrotally for 3 h. In separate experiments, mice were pretreated with 10 mg kg^{-1} of the histamine-1-receptor antagonist (diphenhydramine hydrochloride, Sigma Chemical Co.). Samples of intact cremaster muscle microvascular networks were fixed in 4% formaldehyde overnight and then stained with Giemsa stain for 1 h. After differentiation in acetic acid (0.01%), the samples were mounted on gelatin-precoated glass slides and covered with a coverglass by applying DPX-solidifying mountant for histology (Fluka, Buchs SG, Switzerland) as described in detail for the rat mesentery previously (Yamaki *et al*., 1998a). Neutrophil emigration was quantified by counting the number of polymorphonuclear leukocytes (PMNLs) per high power field observed along a randomly selected venule in each preparation and expressed as number of cells mm^{-2}. Mast cell content in the cremaster muscle was determined by counting the number of mast per high power field observed along a randomly selected venule in each preparation and expressed as number of cells mm^{-2}.

Bone marrow mast cells (BMMC)

Mice were killed and intact femurs and tibias were removed. Sterile medium without fetal calf serum was repeatedly flushed through the bone shaft. Harvested bone marrow cells were then passed through a sterile stainless steel mesh to remove bone fragments and debris. The cell suspension was centrifuged at 800 r.p.m. for 5 min at 4°C and then cultured at a concentration of 0.5–1 $\times$ 10^6 cells ml^{-1} in RPMI 1640 with 10% fetal calf serum, 50 μM of 2-mercaptoethanol and 20 ng ml^{-1} of IL-3 (BMMC medium). After 3 weeks, 40 ng ml^{-1} of stem cell factor was added to the BMMC medium. IL-3 was replaced three times a week with fresh BMMC medium. Mast cells were examined for purity after 4 weeks by 1% Toluidine blue staining of fixed cytocentrifuge preparations. Once the BMMC population reached high purity (>99%) after 5–10 weeks, they were used in subsequent experiments.

Neutrophils

Mice were killed and intact femurs and humeri bones were removed. The bone marrow was aseptically flushed out of the bones with ice-cold phosphate-buffered saline (PBS) and neutrophils were isolated by use of Ficoll-PaqueTM Research Grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of bone marrow neutrophils was higher than 70% as assessed by Turk stain in a haematocytometer. Neutrophils were resuspended in culture medium until use in the adhesion assay.

Endothelial cells

The polyoma-transformed murine endothelioma cell line eEnd.2 was cultured in Dulbecco-modified Eagle's Medium supplemented with 10% fetal calf serum, L-glutamine, penicillin and streptomycin and subcultured twice weekly as described previously (Williams *et al*., 1989).

Neutrophil adhesion assay

Endothelial cells were plated at a density of 2 $\times$ 10^4 cells per well in 96-well plates. When confluent, the cells were coincubated with wild-type (WT) or TNF-α-deficient BMMC and stimulated with or without MIP-2 (300 ng ml^{-1}) or compound 48/80 (10 μg ml^{-1}, Sigma Chemical Co.) for 4 h. After washing, 2 $\times$ 10^4 neutrophils were added into each well and allowed to interact with the endothelial cells for 30 min.

Subsequently, the wells were washed with PBS three times in order to remove the nonadherent cells. Next, 50 μl of 5 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide was added into each well followed by 150 μl of 3,3′,5,5′,-tetramethyylbenzidine liquid substrate system and incubated for 10 min at 25°C for colour development. At the end, 100 μl of H_2SO_4 (0.5 M) was added in order to stop the oxidation reaction. The enzyme activity was determined spectrophotometrically as the myeloperoxidase-catalysed change in absorbance occurring in the redox reaction of H_2O_2-tetramethylbenzididne (450 nm, 25°C). A standard curve was constructed using defined quantities of neutrophils in the same 96-well plate.

Enzyme-linked immunosorbent assay (ELISA)

BMMC (1×10^6) were stimulated with PBS, MIP-2 (300 ng ml^{-1}) and compound 48/80 (10 μg ml^{-1}) for 24 h. Next, the BMMC were washed and the levels of immunoreactive TNF-α protein was determined in supernatants by means of double-antibody-specific Quantikine ELISA kits using recombinant murine TNF-α as standard (R&D Systems).

Reverse transcription–polymerase chain reaction (RT–PCR)

Total cellular RNA was extracted from the mouse cremaster muscle and BMMC using an acid guanidinium–phenol–chloroform method (TRIzol Reagent; GIBCO-BRL, Life Technologies, Grand Island, NY, U.S.A.) and treated with RNase-free DNase (DNase I; Amersham Pharmacia Biotech Sollentuna Sweden) in order to remove potential genomic DNA contaminants according to the manufacturer's protocol. RNA concentrations were determined by measuring in the absorbance at 260 nm spectrophotomertrically. RT–PCR was performed with SuperScrip One-Step RT–PCR system (GIBCO-BRL Life Technolgies). Each reaction contained 500 ng of cellular total RNA as a template and 0.2 μM of each primer in a final volume of 50 μl. Mouse β-actin served as an internal control gene. The RT–PCR profile was one cycle of cDNA synthesis at 50°C for 30 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, one cycle of final extension at 72°C for 10 min. After RT–PCR, aliquots of the RT–PCR products were separated on a 2% agarose gel containing ethidium bromide and photographed. The primers sequences of P-selectin and E-selectin and β-actin were as follows: P-selectin (f) 5′-ACG AGC TGG ACG GAC CCG -3′; P-selectin (r) 5′-GGC TGG CAC TCA AAT TTA CAG-3′; E-selectin (f) 5′-GGT AGT TGC ACT TTC TGC GG-3′; E-selectin (r) 5′-CCT TCT GTG GCA GCA TGT TC-3′; CXCR2 (f) TGT TCT TTG CCC TGA CCT TGC-3', CXCR2 (r) 5'-ACG CAG TAC GAC CCT CAA ACG-3'; β-actin (f) 5′-ATG TTT GAG ACC TTC AAC ACC-3′; β-actin (r) 5′-TCT CCA GGG AGG AAG AGG AT-3′.

Statistical analyses

Data are presented as mean ± s.e.m.. Statistical evaluations were performed using Kruskal–Wallis one-way analysis of variance on ranks followed by multiple comparisons vs control group (Dunn's method) and $P<0.05$ was considered significant.

Results

MIP-2 is sufficient to provoke neutrophil recruitment

Challenge with MIP-2 alone dose dependently increased neutrophil recruitment into the extravascular space (Figure 1). In contrast, administration of fMLP alone had no significant effect on the tissue accumulation of neutrophils (Figure 2). Since fMLP does not increase leukocyte rolling (Lindbom *et al*., 1992), we combined fMLP treatment with TNF-α, which is a potent inducer of endothelial cell activation and leukocyte

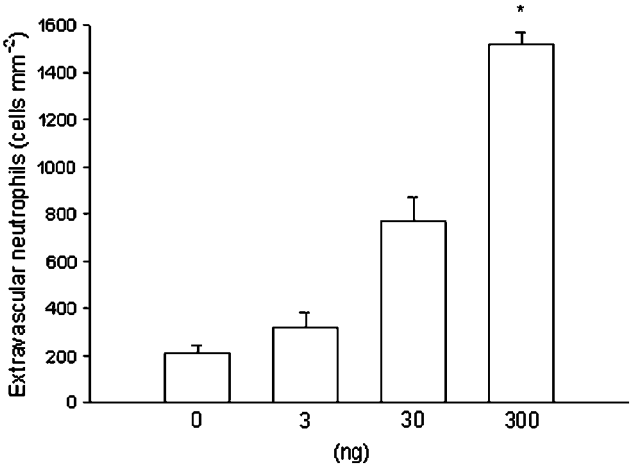

Figure 1 Neutrophil recruitment. The number of extravascular neutrophils per mm^2 was determined in the mouse cremaster muscle whole mounts (see Methods for a detailed explanation). Mice were challenged intrascrotally with the indicated doses of PBS (0) and MIP-2 for 3 h before harvesting the tissue. Data represent mean ± s.e.m. and $n=6$. *$P<0.05$ vs PBS (0).

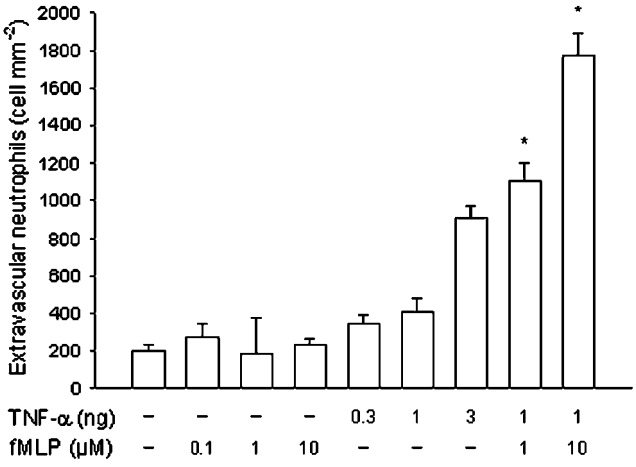

Figure 2 Neutrophil recruitment. The number of extravascular neutrophils per mm^2 was determined in the mouse cremaster muscle whole mounts (see Methods for a detailed explanation). Mice were challenged intrascrotally with indicated doses of PBS (−), fMLP and TNF-α for 3 h before harvesting the tissue. Data represent mean ± s.e.m. and $n=6$. *$P<0.05$ vs fMLP alone.

rolling (Weller *et al*., 1992; Månsson *et al*., 2000). For this purpose, different doses of TNF-α (0.3–300 ng) were evaluated and it was found that 1.0 ng of TNF-α did not increase neutrophil recruitment but higher doses (3.0 ng) provoked clearcut neutrophil extravasation (Figure 2). Thus, we noted that 1 ng of TNF-α represented a threshold dose of TNF-α with respect to triggering tissue accumulation of neutrophils. Interestingly, this threshold dose of TNF-α (1 ng) markedly potentiated fMLP-induced neutrophil recruitment (Figure 2), indicating that fMLP indeed can induce neutrophil migration *in vivo* when acting in concert with other mediators capable of activating endothelial cells.

MIP-2-induced neutrophil is dependent on mast cells and TNF-α

We found clearcut gene expression of CXCR2 in BMMC (Figure 3). It was observed that neutrophil extravasation provoked by MIP-2 was significantly reduced by 97% in mice lacking mast cells (Figure 4, $P<0.05$ vs WT mice). In contrast, TNF-α-induced neutrophil recruitment was intact in mast cell-deficient animals (Figure 4). Knowing that histamine and TNF-α have the capacity to upregulate P-selectin expression and stimulate leukocyte rolling (Weller *et al*., 1992; Asako *et al*., 1994; Yamaki *et al*., 1998a; Månsson *et al*., 2000), it was also of interest to examine the potential role of these mediators in MIP-2-induced extravasation of neutrophils. Herein, we found that pretreatment with the histamine-1-receptor antagonist diphenhydramine (10 mg kg^{-1}), which effectively blocks histamine-provoked leukocyte rolling (Asako *et al*., 1994; Yamaki *et al*., 1998b), had no effect on MIP-2-induced neutrophil recruitment (Figure 5). Notably, we observed that neutrophil recruitment induced by MIP-2 was significantly reduced by 78% in TNF-α-deficient mice (Figure 6, $P<0.05$ vs WT mice). It was found that WT and TNF-α-deficient mice contained similar levels of mast cells in the cremaster muscle, that is, 64±7 mast cells mm^{-2} and 74±13 mast cells mm^{-2}, respectively ($P>0.05$). Thus, MIP-2-provoked accumulation of neutrophils is dependent on both the presence of mast cells and TNF-α. To determine whether MIP-2-induced neutrophil recruitment was mediated by TNF-α derived from mast cells, we adopted an *in vitro* adhesion assay. Confluent endothelial cells were coincubated with mast cells and MIP-2 (300 ng ml^{-1}) for 4 h, and after washing the endothelial cells were coincubated with neutrophils for 30 min. We observed that MIP-2

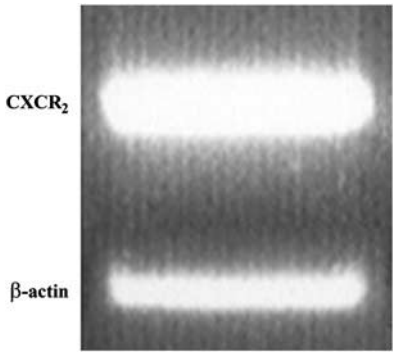

Figure 3 Expression of CXCR2 mRNA in mast cells. β-Actin serves as a housekeeping gene. The results presented are from one experiment, which is representative of three others performed.

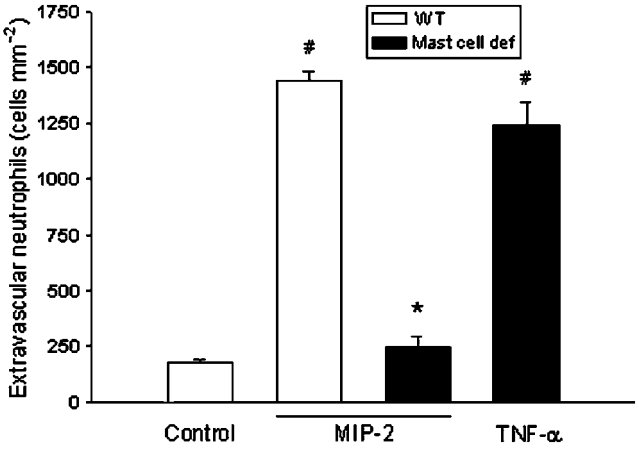

Figure 4 Neutrophil recruitment. The number of extravascular neutrophils (PMNLs) per mm^2 was determined in the mouse cremaster muscle whole mounts (see Methods for a detailed explanation). Wild-type (WT) and TNF-α- and mast cell-deficient mice were challenged intrascrotally with PBS (Control), MIP-2 (300 ng) and TNF-α (30 ng) for 3 h before harvesting the tissue. Data represent mean±s.e.m. and $n=6$. $^{\#}P<0.05$ vs PBS + WT (Control) and $^{*}P<0.05$ vs MIP-2 + WT.

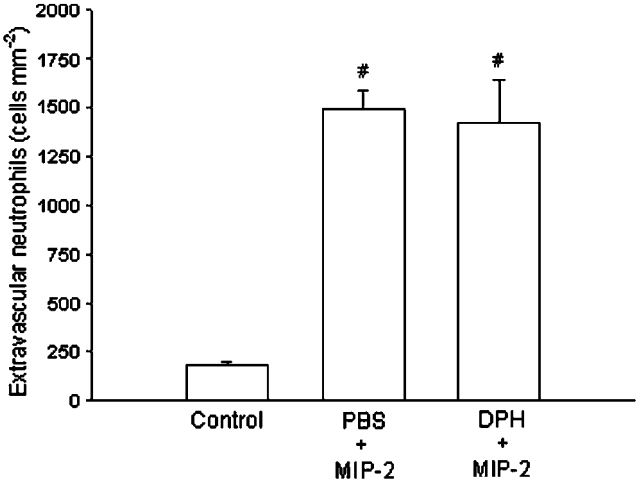

Figure 5 Neutrophil recruitment. The number of extravascular neutrophils per mm^2 was determined in the mouse cremaster muscle whole mounts (see Methods for a detailed explanation) 3 h after intrascrotal challenge with MIP-2 (300 ng). Negative controls received PBS intrascrotally (Control). Mice were either pretreated with PBS or the histamine-1-receptor antagonist diphenhydramine (DPH, 10 mg kg^{-1}). Data represent mean±s.e.m. and $n=5$–8. $^{\#}P<0.05$ vs PBS (Control).

increased the number of adherent neutrophils by more than three-fold when using WT BMMC (Figure 7, $P<0.05$ vs WT BMMC). Interestingly, it was found that MIP-2-induced neutrophil adhesion was significantly decreased by 58% using TNF-α-deficient BMMC (Figure 7, $P<0.05$ vs WT BMMC). As a positive control, we included the mast cell secretagogue compound 48/80 (10 μg ml^{-1}). Indeed, it was observed that compound 48/80-induced neutrophil adhesion was reduced by more than 60% using BMMC from TNF-α gene-targeted mice (Figure 7, $P<0.05$ vs WT BMMC). In separate experiments, we found that BMMC stimulated with MIP-2 and compound 48/80 significantly increased TNF-α secretion by 71 and 225%, respectively (Figure 8, $P<0.05$ vs control). In order to

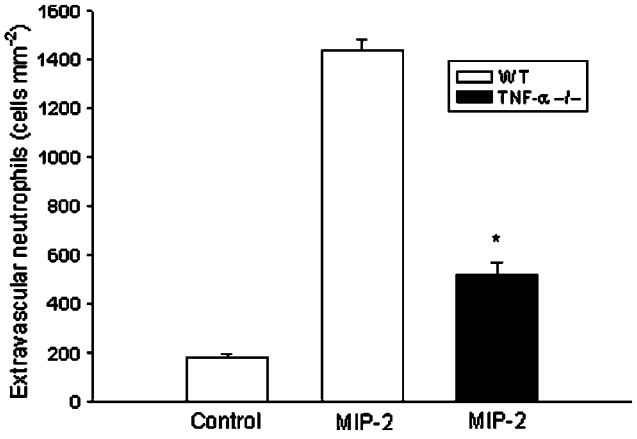

Figure 6 Neutrophil recruitment. The number of extravascular neutrophils per mm^2 was determined in the mouse cremaster muscle whole mounts (see Methods for a detailed explanation). Wild-type (WT) and TNF-α-deficient mice were challenged intrascrotally with PBS (Control) and MIP-2 (300 ng) for 3 h before harvesting the tissue. Data represents mean ± s.e.m. and $n = 5$–8. *$P < 0.05$ vs MIP-2 + WT).

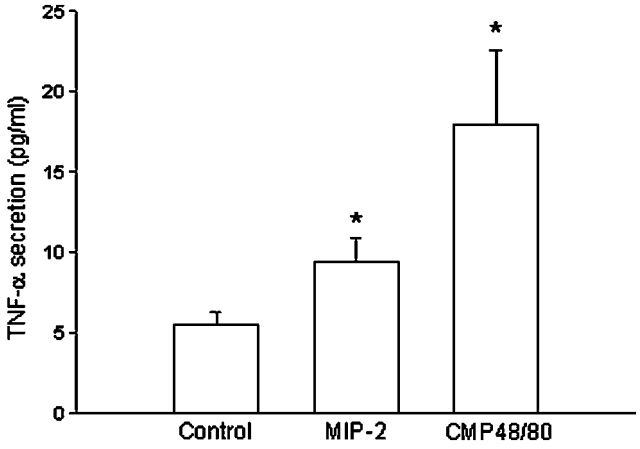

Figure 8 Mast cell secretion of TNF-α. BMMC (1×10^6) were stimulated with PBS (control), MIP-2 (300 ng ml^{-1}), compound 48/80 (10 μg ml^{-1}) and levels of secreted TNF-α was determined after 24 h by specific ELISA. Data represent mean ± s.e.m. and $n = 5$. *$P < 0.05$ vs PBS (Control).

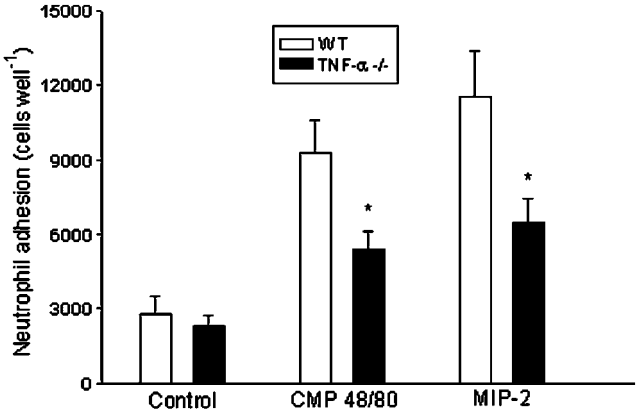

Figure 7 Neutrophil adhesion to endothelial cells. Endothelial cells were stimulated with WT or TNF-α deficient mast cells pretreated with MIP-2 (300 ng ml^{-1}) or compound 48/80 (10 μg ml^{-1}) for 4 h. Then 2×10^4 neutrophils were added into each well and incubated for 30 min. Nontreated mast cells served as negative control (control). Data represent mean ± s.e.m. and $n = 8$. *$P < 0.05$ vs WT.

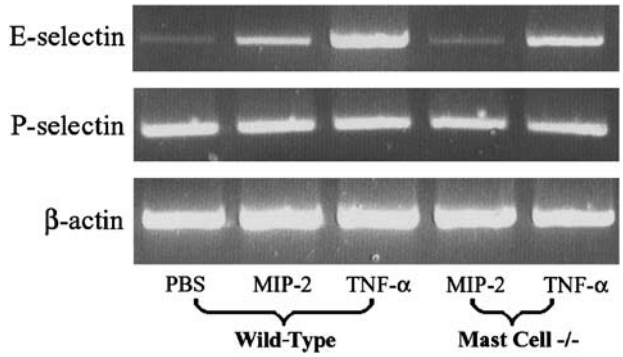

Figure 9 Expression of E- and P-selectin mRNA. β-Actin serves as a housekeeping gene. RNA was isolated from the cremaster muscle 3 h after treatment with PBS, MIP-2 and TNF-α in WT and mast cell-deficient mice. The results presented are from one experiment, which is representative of three others performed.

indirectly examine endothelial cell activation, we examined gene expression of P- and E-selectin in the cremaster muscle by the use of RT–PCR. As described previously (Wan *et al.*, 2003), we observed that P-selectin mRNA is expressed at baseline in the cremaster muscle (Figure 9). Thus, we did not observe any increase in P-selectin gene expression in response to TNF-α and MIP-2 challenge (Figure 9). In contrast, E-selectin was absent at baseline but increased readily after challenge with MIP-2 and TNF-α (Figure 9). Interestingly, MIP-2-induced gene expression of E-selectin was abolished in mast cell-deficient mice (Figure 9). On the other hand, we found that TNF-α caused clearcut expression of E-selectin mRNA also in animals lacking mast cells (Figure 9).

Discussion

This study demonstrates that mast cell-derived TNF-α mediates MIP-2-induced neutrophil recruitment into the extravascular space. It is widely held that endothelial cell activation and selectin expression is a precondition for leukocyte recruitment. Indeed, MIP-2 provoked mast cell secretion of TNF-α *in vitro* and induced E-selectin expression *in vivo*, which was completely dependent on the presence of mast cells. These findings help to explain the mechanisms behind CXC chemokine-induced neutrophil recruitment *in vivo* and elucidate an important part of the complex interactions between chemokines, tissue resident cells and circulating leukocytes.

Leukocyte recruitment to sites of inflammation is coordinated by a complex interplay between specialized tissue resident cells and endothelial cells regulating the expression of specific chemokines and adhesion molecules (Butcher, 1991; Springer, 1994; Vestweber & Blanks, 1999). In the present study, we confirmed previously published data showing that the neutrophil response to MIP-2 *in vivo* is largely dependent on the presence of mast cells (Mercer-Jones *et al.*, 1999; Wan *et al.*, 2003). Moreover, we found clearcut expression of CXCR2 mRNA in the BMMC, supporting a role of MIP-2

in mast cell-dependent inflammation. In fact, this expression of CXCR2 on murine mast cells is in line with previous observations showing that human mast cells also express CXCR2 (Lippert *et al*., 1998; Nilsson *et al*., 1999). Mast cells are considered to play an important role in early and late phases of pathological inflammation as an intermediate cellular source of inflammatory mediators (Thorlacius *et al*., 1994; Biedermann *et al*., 2000; Schramm *et al*., 2002). However, the precise role of mast cells in the crosstalk between chemokines and endothelial cells has not been understood. Activated mast cells release a number of substances, including histamine, leukotrienes and TNF-α with the ability to increase P-selectin expression on endothelial cells and in turn promote neutrophil rolling and extravascular recruitment (Kanwar *et al*., 1995; Yamaki *et al*., 1998a). Our present data suggest that histamine is not a critical mediator of CXC chemokine-regulated neutrophil infiltration. Thus, the histamine-1 receptor antagonist diphenhydramine, which completely blocks histamine-induced leukocyte rolling (Asako *et al*., 1994; Yamaki *et al*., 1998b), had no effect on MIP-2-induced neutrophil recruitment. However, we found that MIP-2-regulated neutrophil recruitment was severely compromised in TNF-α-deficient mice. Together, these findings suggest that TNF-α is a critical substance mediating MIP-2-provoked accumulation of neutrophils *in vivo*. However, these experiments do not tell whether the TNF-α was derived from mast cells or other tissue resident cells. Therefore, we adopted an *in vitro* adhesion assay to test whether mast cell-derived TNF-α was involved in mediating MIP-2-induced neutrophil–endothelium interactions. It was observed that MIP-2-provked neutrophil adhesion was significantly reduced when endothelial cells were coincubated with TNF-α-deficient mast cells compared to WT mast cells. These findings strongly suggest that MIP-2-regulated neutrophil recruitment is mediated by mast cell-derived TNF-α. This notion is also supported by separate experiments, in which we could show that MIP-2 challenge increased the level TNF-α secretion from mast cells although less potently than that of the classical mast cell secretagogue compound 48/80. In this context, it should be mentioned that one study showed that the CC chemokine monocyte chemotactic protein-1 indirectly provokes peritoneal recruitment of neutrophils *via* production of leukotriene B_4, although the cellular origin of this lipid remains to be determined (Matsukawa *et al*., 1999). Thus, a potential additional role of mast cell-derived leukotrienes in CXC chemokine-induced neutrophil recruitment may be of value to examine in future studies.

Endothelial cell activation is a critical step in the extravasation process of neutrophils *in vivo* due to the important role of endothelial selectins in supporting leukocyte rolling (Weller *et al*., 1992; Månsson *et al*., 2000). In contrast to fMLP, which is a prototype of classical chemoattractants, MIP-2 dose dependently increased neutrophil recruitment. However, by titrating out a threshold dose of TNF-α, which did not alone provoke clearcut neutrophil recruitment, we could show that fMLP could provoke substantial neutrophil recruitment *in vivo* when coadministered with this suboptimal dose of TNF-α. These findings show that fMLP can induce tissue migration and accumulation of neutrophils *in vivo* when acting in concert with other proinflammatory mediators capable of activating endothelial cells. We have previously shown that CXC chemokines, including MIP-2, have the capacity to provoke all steps in the transmigration process of leukocytes, that is, leukocyte rolling, firm adhesion and extravasation (Zhang *et al*., 2001; Wan *et al*., 2003). This MIP-2-provoked accumulation of neutrophils is mediated by endothelial cell expression of selectins (Miotla *et al*., 2001; Zhang *et al*., 2001; Wan *et al*., 2003). Our present data suggesting that TNF-α derived from mast cells mediates MIP-2-induced neutrophil extravasation in multicellular tissues *in vivo*. Indeed, TNF-α has the capacity to upregulate P- and E-selectin on the endothelium and support leukocyte rolling, which is a precondition for subsequent tissue recruitment of leukocytes (Weller *et al*., 1992; Kunkel *et al*., 1996; Månsson *et al*., 2000). Herein, we found that challenge with MIP-2 increased expression of E-selection (a marker of endothelial cell activation) *in vivo*, suggesting that MIP-2 indeed caused activation of endothelial cells *in vivo*. Interestingly, this MIP-2-regulated expression of E-selectin was absent in mast cell-deficient mice, indicating that MIP-2-induced activation of endothelial cells is mediated *via* mast cells. In contrast, we observed that TNF-α-induced gene expression of E-selectin was intact in mast cell-deficient mice. P-selectin mRNA was found to be expressed at baseline, which is in line with previous studies (Riaz *et al*., 2002; Wan *et al*., 2003) and explained by the continuous production of P-selectin in endothelial cells for storage in Weibel Palade bodies (van Mourik *et al*., 2002). Thus, neither TNF-α nor MIP-2 challenge increased P-selectin mRNA production in the cremaster muscle.

In conclusion, our data demonstrate the TNF-α is a critical mediator secreted by mast cells in response to MIP-2 stimulation. This mast cell-derived TNF-α plays an important role in MIP-2-induced recruitment of neutrophils to the extravascular space. Thus, these findings help to explain the complex interactions between specialized tissue resident cell, endothelial cells and circulating leukocytes *in vivo*.

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