Mast cell involvement in zymosan-induced peritonitis in C57Bl/6 mice

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Abstract

We have shown previously that mast cells (MC) and MC-derived mediators are crucial for early stages of zymosan-induced peritonitis in Swiss and Balb/c mice. Present results revealed the same phenomena in males of C57Bl/6 mice as in the MC-depleted animals (by application of a compound 48/80) the intraperitoneal accumulation of exudatory fluid and cells, measured at 0.5, 6 and 24 hours after zymosan injection, was significantly diminished. In particular, an early vascular permeability and PGE_2 content at 30 min of peritonitis, and exudatory leukocyte numbers and MCP-1 content at 6 hours of peritonitis were significantly lower in MC-deprived mice then in those with normal MC population. The peritoneal cavity of intact C57Bl/6 males contains less then 1% of mast cells defined as cells filled with metachromatically stained granules (counted in a haemocytometer) and those with MC-specific c-kit receptors (determined both by quantitative PCR at mRNA level and by flow cytometry on cell surface). After zymosan injection numbers of cells with metachromatically-stained granules significantly decrease reaching minimum at 6 hours of peritonitis with a parallel significant increase of c-kit positive MCs. These contradictory results might be explained by zymosan-induced degranulation of mast cells and concomitant influx of newly arriving mast cells through the omental milky spots and/or in situ MC proliferation.

Key words: *mast cells, peritonitis, peritoneal inflammation, vasopermeability, PGE_2, MCP-1.*

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Introduction

Since their discovery mast cells (MC) have been predominantly studied in the context of hypersensitivity reactions. It was just over the past decade that mast cells have gradually gained recognition as true immune effector cells, i.e. cells exerting direct effects and indirectly regulating other cells [1].

Mast cells are distributed in virtually all organs and vascularized tissues, including skin, gastrointestinal tract, peritoneum, and the airways thus MCs are strategically located at sites that interface with our external environment, closely associated with blood vessels and nerves [2-4]. This diverse distribution is associated with the heterogeneity of MCs that in mice can be divided into connective tissue mast cells (e.g. peritoneal MC) and mucosal mast cells (e.g. intestinal mucosal MC) that, apart of tissue localization, differ by content of intracellular proteoglycans [5]. A classical feature of mast cells is their strong metachromatic staining with cationic dyes (e.g. cristal violet). These staining properties are dependent on large amounts of negatively charged glycosaminoglycans (heparin and serglycin) that are present in mast cell secretory granules [6, 7]. When activated mast cells immediately exocytose their granule constituents and thus the numbers of granules alter and metachromatic stain is temporarily lost [8].

As long-lived cells, mast cells can have a great impact on the tissue microenvironment through the selective release of a wide variety of preformed and newly derived mediators from their granules which presence coined their name of *Mastzellen* i.e. mast cells [9]. The mediators include biogenic amines (histamine and serotonin), proteases (e.g. chymeases, tryptases), cytokines (e.g. TNF-α, IL-1β, IL-10), chemokines (e.g. IL-8, MCP-1) and arachidonic acid metabolites

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(e.g. LTC_4, PGE_2) that are effector molecules inducing allergic or inflammatory reactions that modulate innate and adaptive immune responses [2-4, 10].

Studies on a role of mast cells in acute inflammation included also investigations on a model of zymosan-induced peritonitis [11-15]. Murine peritoneum contains macrophages, lymphocytes and mast cells with highest numbers of the former and lowest of mast cells [16, 17]. The lymphocytes, however, do not play a significant role in zymosan peritonitis [18]. Mast cell numbers significantly differ between some murine strains, e.g. among investigated strains CBA mice had highest, Balb/c and C57Bl/6 mice moderate, and Swiss mice lowest MC counts [19, 20]. The studies on mast cell involvement in zymosan peritonitis included application of MC-deficient WBB6F1 mice or substantial reduction (>95%) of MC numbers in Swiss or Balb/c mice pre-treated with the MC activator compound 48/80 [11-14, 21]. The compound induces mast cell degranulation and the released substances/mediators are either cleared up from peritoneum or their half-life expires by day 4 post treatment as shown by lack of any signs of cytokines/chemokines and other mediators in the peritoneal cavity at that time [11, 14, 22]. Therefore the treatment leads to functional depletion of mast cells. The studies with compound 48/80 revealed that mast cells are important for vascular events [14] and leukocyte infiltration into the focus of inflammation [13] and this can be explained by a fact that MCs are critical for vasoactive mediator synthesis [14] and chemokine production in this model [11]. Interestingly, in other models of murine peritonitis, induced by LPS or thioglycolate in Swiss mice, mast cells did not produce the chemokines [11].

Here we show that mast cell numbers fluctuate during zymosan peritonitis in C57Bl/6 mice and the cells degranulate releasing content of their granules as shown by decreasing numbers of metachromatic cells. At the same time, however, numbers of c-kit$^+$ cells temporarily increase suggesting MC proliferation or infiltration into peritoneum. Moreover, we show that also in the investigated strain of mice mast cells participate in early vascular permeability and leukocytes infiltration due to release of PGE_2 and MCP-1, respectively.

Material and Methods

Peritonitis

Peritoneal inflammation was induced as described previously [13, 23]. Zymosan A (Sigma) was freshly prepared (2 mg/ml) in sterile 0.9% w/v saline, and 0.5 ml was *i.p.* injected per 25 g of mouse. Animals were killed by decapitation at the indicated time points. Control animals were left untreated. The peritoneal cavity was lavaged with 1 ml of saline and after 30 s gentle manual massage the exudate was retrieved and centrifuged at 3000 g for 3 min. Cells were counted with a hemocytometer following staining with Turk's solution (0.01% crystal violet in 3% acetic acid) [13]. The cell pellets were used for FACS staining. Peritoneal exudate fluid was frozen at –20°C prior to analysis.

Depletion of resident peritoneal mast cells

Resident peritoneal mast cells were functionally depleted by a single *i.p.* injection of compound 48/80 (Sigma) in a dose of 1.2 mg/kg (100 µl/mouse), 72 hours before induction of peritonitis [11, 15, 24]. The successful depletion was confirmed by microscopic analysis of safranin O positive peritoneal leukocytes (data not shown) as described previously [13]. The treatment led to degranulation of over 96% of mast cells. Mice with intact mast cell population are marked through the text as MC while animals depleted of functional mast cells as MCx.

Quantitative reverse transcription-PCR analysis

Total RNA from cells collected from normal or inflamed peritoneum was isolated using the QIAshredder and RNeasy Mini Kit (QIAGEN). The amount and purity of the total RNA was determined by spectophotometry (GENESYS 10 UV; Thermo Electron Corporation) at 260 nm. RNA was translated into single-stranded cDNA using the Superscript cDNASynthesis kit (Invitrogen) and random hexamers (Amersham Biosciences). Relative gene expression levels were determined using real-time PCR *Taq*Man technology (GeneAmp 5700 Sequence Detection System; Applied Biosystems) and SYBR green (Eurogentec) incorporation. The mouse hypoxanthine phosphoribosyltransferase (*HPRT*) gene served as an internal standard. The following mouse-specific primers (5' to 3') were used:

c-kit: CTGGTTGGCCTTCCCTTGT,
GAGAGATTTCCCATCACACTCGAT [25].

Staining of c-kit$^+$ mast cells

Peritoneal leukocytes were collected from the peritoneal cavity. The cell pellets (2-3 × 10^6 of total cells) were resuspended in FACS buffer (2% BSA in 0.9% w/v saline), preblocked with an Fc block (BD Bioscience PharMingen) for 20 min on ice and after washing (5000 rpm, 5 min, 4°C) incubated with rat anti-mouse anti-CD117 (c-kit) FITC-conjugated antibody diluted 1:100 (BD Bioscience PharMingen) on ice for 15 min [25]. After repeated washings in FACS buffer the cells were subsequently suspended in 50 µl of FACS buffer for flow cytometry analysis.

Flow cytometry

Samples were analyzed in a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems) by using CellQuest Pro software, and data were collected with 10.000 events being acquired. Leukocytes were initially gated by forward scatter/side scatter on the basis of their size and granularity. Subsequently, the data were collected in the FL1 flow cytometer channel (green channel) for FITC positive cells. The cells were analyzed in a histo-

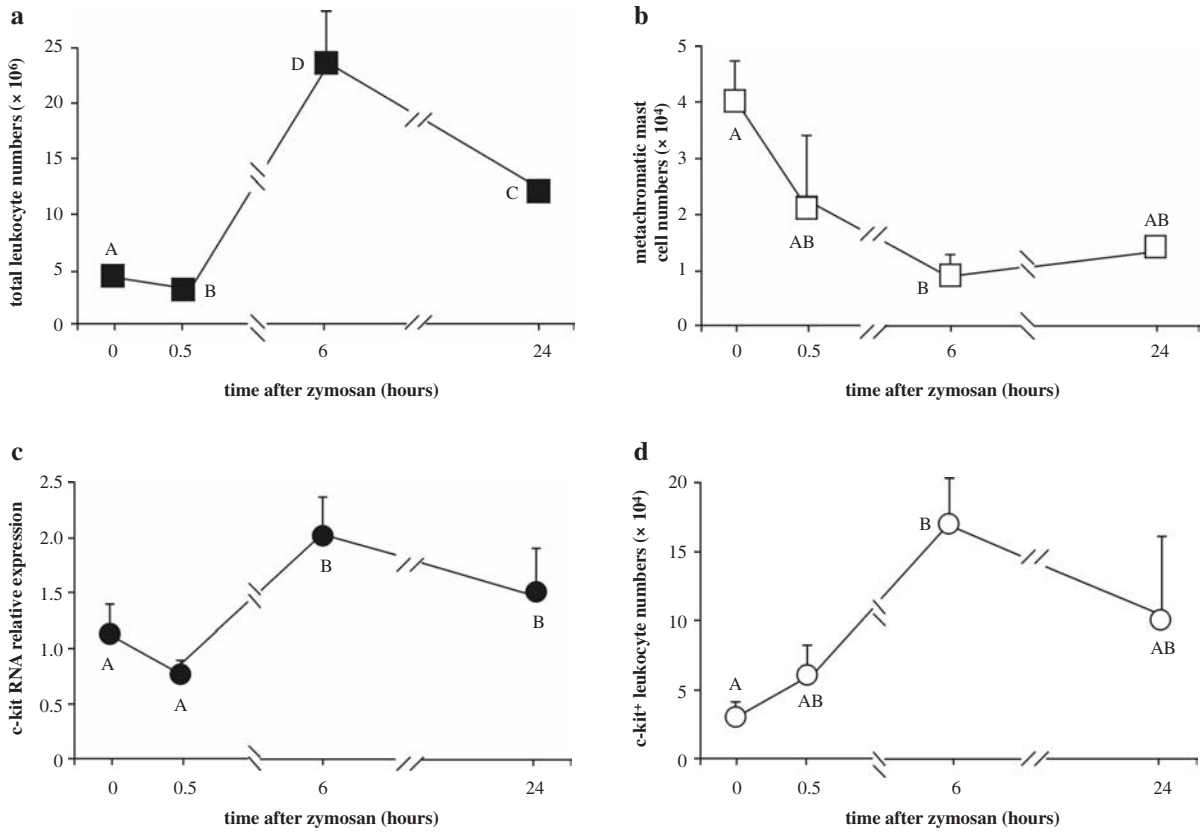

Fig. 1. Kinetics changes of leukocyte numbers during zymosan peritonitis in C57Bl/6 mice. Animals were *i.p.* injected with 1 mg of zymosan and numbers of total peritoneal leukocytes (PTLs) (A), metachromatically-stained mast cell (B), amount of c-kit mRNA (C) and counts of c-kit$^+$ cells (D) were estimated at the selected time points. Some mice were left untreated (intact). All results are shown as means ±SEM in groups of 3-4 mice. Mean values not sharing letters are statistically significantly different according to ANOVA ($P<0.05$)

gram displaying the FITC expression. The markers for negative and positive cells were denoted on cells that were not stained with c-kit FITC-labelled antibody (negative control) (Figure 2 uppermost).

Determination of PGE_2 and MCP-1 concentration by ELISAs

PGE_2 levels were estimated by the ELISA kit (Cayman Chemical) and MCP-1 by an Immunoassay Kit (BioSource Int.). The analyses were performed according to the manufacturer's instructions.

Statistical analysis

All values are reported as means ±SEM. Each experiment was repeated 2-3 times. Kinetic changes of each parameter were analysed by one-way analysis of variance (ANOVA), comparing the values recorded at the individual time points with that at time 0 (in control animals). Differences were considered statistically significant at $P<0.05$. When MC and MCx groups were compared the Student's t-test was used and the differences are marked accordingly: $0.01\leq P\leq 0.05$ (*), $0.01\geq P\geq 0.001$ (**).

Results

Total peritoneal leukocyte numbers

Injection of zymosan into C57Bl/6 mice initially decreased total peritoneal leukocyte (PTL) numbers (at 30 min) but after several hours an increase of PTL in peritoneum was observed (at 6 hours). At the time of the resolution of inflammation (at 24 hours) their numbers significantly decreased but were still above the levels detected in untreated intact animals (Figure 1A).

Mast cell numbers

Metachromatic staining

Mast cell counts were estimated by two approaches. Morphologically mast cells were distinguished, and counted,

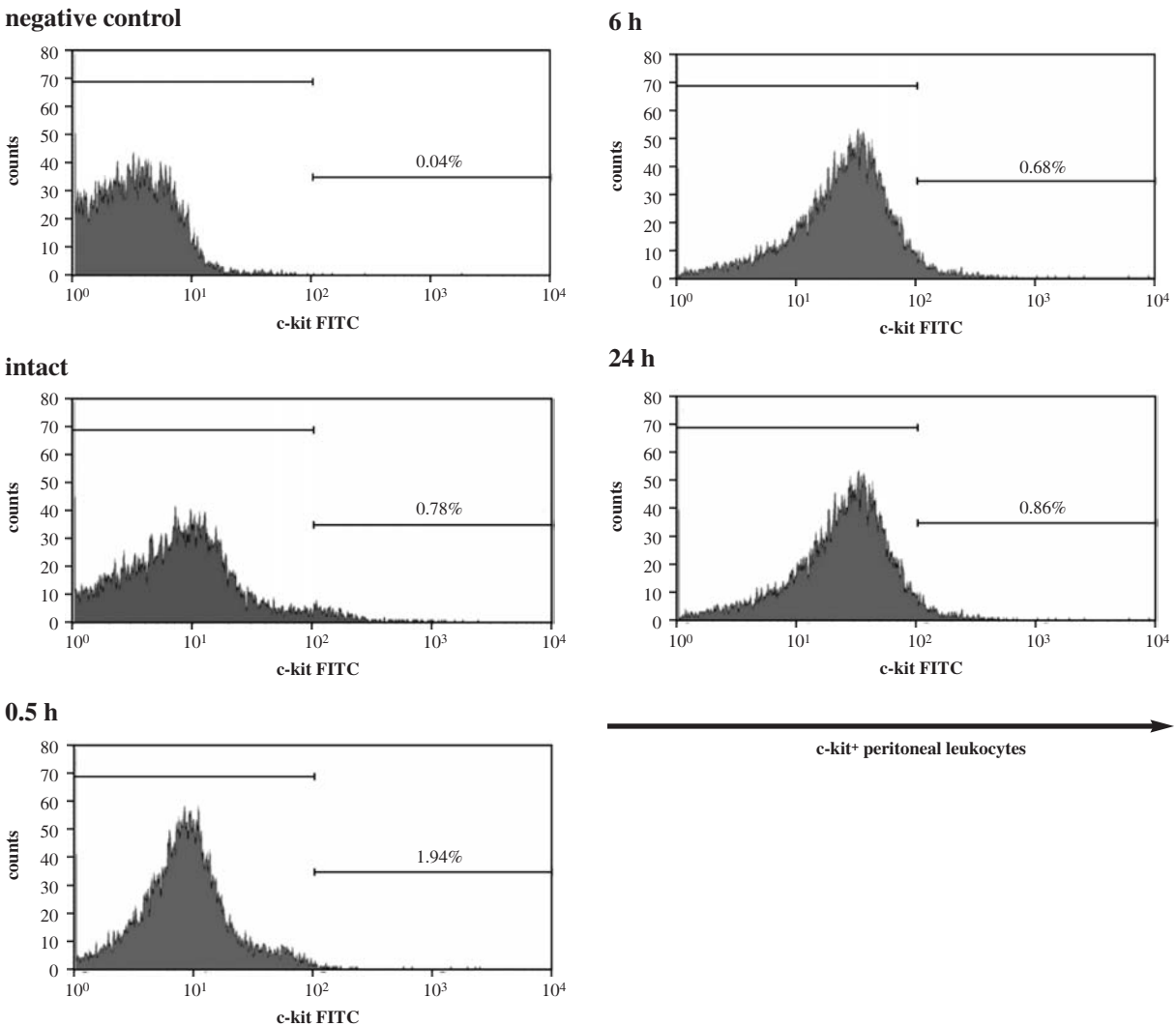

Fig. 2. Representative histograms of c-kit$^+$ cells. Peritoneal leukocytes were collected from either untreated (intact) mice or animals *i.p.* injected with 1 mg of zymosan and subsequently stained with anti-c-kit FITC-conjugated antibody. The markers for negative and positive cells were denoted on cells that were not stained with c-kit FITC-labelled antibody (negative control)

after staining with Turk's solution using light microscopy. The Turk's solution contains cationic dye – crystal violet that stains mast cell granules metachromatically [26]. The analyses revealed that mast cell counts decreased significantly upon zymosan from the 30th min and reached lowest values at 6 hours of peritonitis (Figure 1B). By the end of peritonitis mast cell numbers started to come back to the levels detected in control intact mice (Figure 1B) but reached their levels after a week (not shown).

c-kit staining

Mast cells are unique mature leukocytes equipped with c-kit surface receptors [27, 28] therefore c-kit expression was assessed, both at the mRNA and protein levels (Figure 1C, Figure 1D and Figure 2). Percentage of c-kit$^+$ leukocytes detected by flow cytometric analysis (Figure 2) was recalculated per PTL numbers (Figure 1A) and is expressed as absolute c-kit$^+$ cell numbers (Figure 1D). The studies revealed that the changes at the transcript (Figure 1C) and protein (Figure 1D) levels followed the same pattern as they increased upon zymosan injection reaching highest values at 6 hrs of peritonitis and then started to slowly decline (Figure 1C and Figure 1D).

Vascular permeability and vasoactive PGE_2 synthesis

Injection of zymosan induced a dramatic increase of vascular permeability in peritoneum of C57Bl/6 mice that was detected 30 min post zymosan (Figure 3A). The

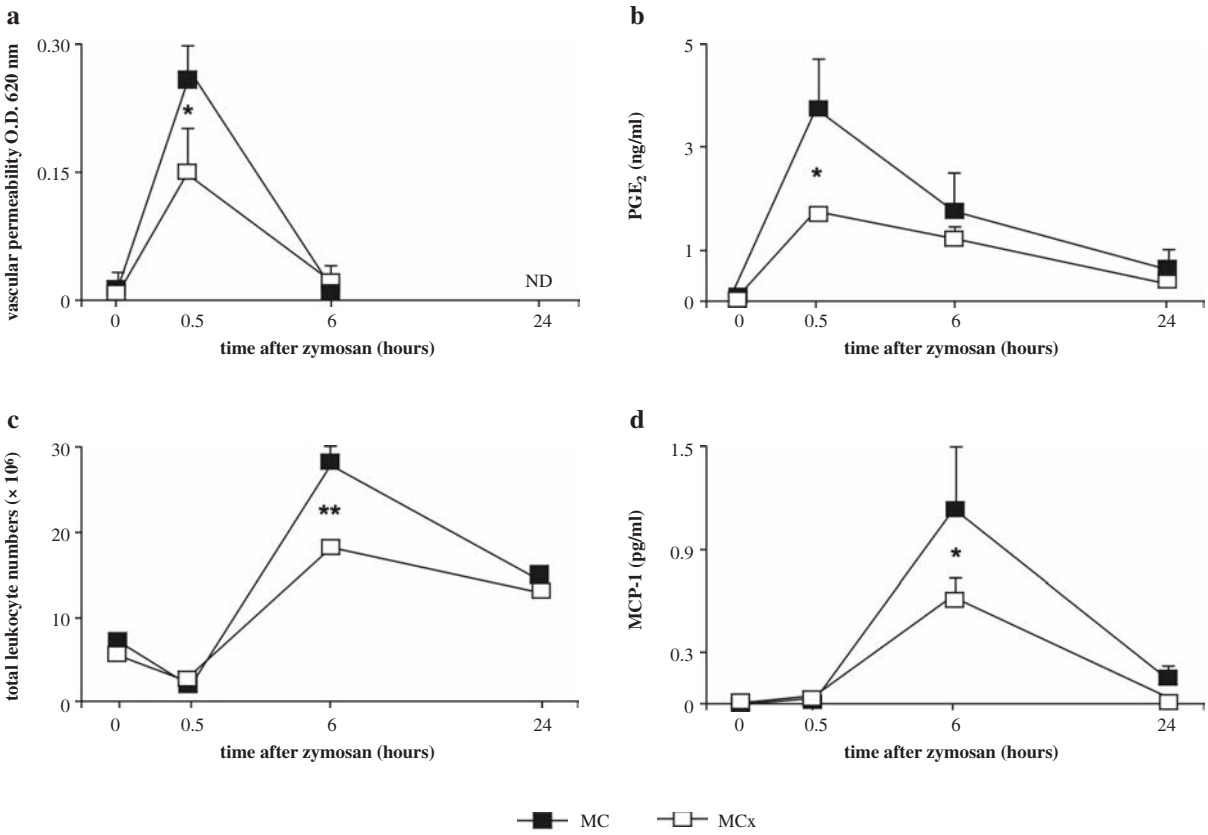

Fig. 3. Kinetics changes of some inflammatory events and mediator production during zymosan peritonitis in C57Bl/6 mice. Some animals were depleted of functional mast cells (MCx) prior to induction of inflammation while others had intact mast cell population (MC). Subsequently, some mice were either *i.p.* injected with 1 mg of zymosan or left untreated (intact) and decapitated at the selected time points. Vascular permeability (A) and PGE_2 levels (B) were estimated as well as peritoneal leukocyte numbers (C) and a MCP-1 chemokine release (D). All results are shown as means ±SEM in groups of 5 mice. Some differences between MC *vs*. MCx groups are significant at $0.01 \leq P \leq 0.05$ (*), $0.01 \geq P \geq 0.001$ (**)

vascular changes were temporary and thereafter rapidly decreased to the control levels (Figure 3A). In mice depleted of functional mast cells (MCx) the vascular permeability was significantly lower than in animals with normal mast cell population (Figure 3A). The change of vascular tone was accompanied by changes in vasoactive PGE_2 synthesis/release which highest levels were also detected at 30 min of inflammation (Figure 3B). At the later time points of peritonitis PGE_2 was still being produced, however, with significantly lower intensity. The depletion of mast cells significantly decreased PGE_2 synthesis and this difference was observed only at 30 min of inflammation (Figure 3B).

Leukocyte infiltration and MCP-1 chemokine production

Firstly zymosan injection induced a decrease of PTL counts (at 30 min) but then numbers of peritoneal leukocytes dramatically increased (6 hours). At the time of the resolution of inflammation PTL numbers significantly diminished but were still higher than those in intact mice (Figure 3C) and came back to the control level after a week (not shown). Removal of functional mast cells impaired leukocyte infiltration at the time of their maximal accumulation (at 6 hours) but not at 24 hours (Figure 3C). This corresponded with lowered levels of MCP-1 in MCx mice at that time (Figure 3D). The amount of the chemokine was also highest at 6 hours of zymosan peritonitis in both groups of mice (Figure 3D).

Discussion

Mast cells possess several characteristics that make them the important cell population controlling both physiological and pathophysiological functions of the body.

They are (I) long-lived cells (II) numerously located at surfaces where host and pathogens interact with each other and, (III) capable of inflammatory mediator release through IgE- and non-IgE-dependent pathways. Moreover, in some circumstances MCs can (IV) phagocytose and (V) act as antigen presenting cells [1, 2, 5, 29].

The most important effects caused by mast cells are induced by their mediators. The mediators are either pre-stored in mast cell granules or produced *de novo* and released upon activation [2, 3]. Morphologically this can be observed after MC staining with cationic dyes as during degranulation the metachromatic stain is lost from mast cell granules [30, 31]. This phenomenon was also observed in the current study as numbers of metachromatically-stained mast cells diminished during the course of zymosan peritonitis in C57Bl/6 mice. And this indicates that their degranulation occurred. A characteristic feature of mast cell exocytosis is that it does not involve release of a few individual secretory granules, but rather, a large fraction of the granular content is released due to fusion of granule-to-granule and granule-to-plasma membrane [32]. And moreover, the secretory granules, considered lysosome-related organelles, might be phagocytosed by peritoneal macrophages once released [31, 32]. Furthermore, we showed here that there is also further mobilization of mast cells into zymosan-inflamed peritoneum as staining of c-kit$^+$ cells revealed that mast cell numbers increase during zymosan peritonitis in C57Bl/6 mice. Till recently migratory properties of mast cells were questioned. However, it is now recognized that mast cell numbers might increase in local tissues under different pathophysiological conditions, including asthma, allergic rhinitis, inflammatory bowel disease, rheumatoid arthritis angiogenesis, and host defense mechanisms against parasites and microbes [33]. This chemotactic response can be induced by SCF, chemokines, anaphylatoxins, acute-phase proteins and cytokines such as TNF-α [33, 34]. Alternatively or additionally the increase in MC numbers observed in our studies might be explained by proliferation of zymosan stimulated peritoneal mast cells. In fact, proliferation of mast cells in inflamed tissue has been reported previously [35] and also preliminary data from our laboratory suggest mast cell proliferation upon zymosan treatment (Wypasek, in progress).

The role of mast cells in some aspects of zymosan peritonitis was studied previously in different murine strains. The studies on genetically mast cell-deficient WBB6F1-W/Wv mice with a defect of the hemopoietic stem cells due to lack of the c-kit receptor revealed a significant impairment of most stages of inflammation and the intraperitoneal transfer of wild-type bone marrow-derived mast cells fully restored all the impaired factors [13]. These included release of mast cell histamine and plasma exudation as well as the influx of exudatory leukocytes, accumulation of plasma and exudate chemoattractants and the release of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) [13, 14]. Some other studies on Balb/c mice pretreated with cromolyn, a mast cells stabilizer protecting against degranulation, also caused an impairment of plasma exudation while pharmacological blockade of histamine receptors impaired not only the early vascular events but also exudatory leukocyte influx [13]. The studies on Swiss and Balb/c mice pretreated with compound 48/80 revealed that mast cells are important sources of eicosanoids [14] and chemokines [11] in this model. And the current study showed that also in C57Bl/6 mice peritoneal mast cells are important for early vascular permeability and this is connected to PGE_2 release by those cells. Furthermore, we show that mast cells are also crucial for normal leukocyte infiltration into zymosan-inflamed peritoneum of C57Bl/6 mice by synthesis of chemokines such as MCP-1 (CCL2). Interestingly, it was reported that PGE_2 dose-dependently induces MCs to release the MCP-1 and this release is not accompanied by MC degranulation [36].

In conclusion, the studies show that mast cells are important for the course of zymosan peritonitis in C57Bl/6 mice. Moreover, zymosan injection induces not only activation of peritoneal mast cells but also their influx and/or infiltration into the inflammatory focus.

Acknowledgements

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