Innate immune modulation in atherosclerosis and vascular

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Chapter 5

Mast cells mediate neutrophil recruitment during atherosclerotic plaque progression

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Abstract

Aims: Activated mast cells have been identified in the intima and perivascular tissue of human atherosclerotic plaques. As mast cells have been described to release a whole array of chemokines that mediate leukocyte fluxes, we propose that activated mast cells play a pivotal role in leukocyte recruitment during atherosclerotic plaque progression.

Methods and Results: Systemic IgE-mediated mast cell activation in apoE$^{-/-}$µMT mice resulted in an increase in atherosclerotic lesion size as compared to control mice, and interestingly, the number of neutrophils was highly increased in these lesions. In addition, peritoneal mast cell activation led to a massive neutrophil influx into the peritoneal cavity in C57Bl6 mice, while neutrophil numbers in mast cell deficient Kit(W^{-sh}/W^{-sh}) mice were not affected. Moreover, increased levels of CXCR2^{+} and CXCR4^{+} neutrophils were observed after mast cell activation. Indeed, mast cells were seen to contain and release CXCL1 and CXCL12, the ligands for CXCR2 and CXCR4. Intriguingly, peritoneal mast cell activation in combination with anti-CXCR2 receptor antagonist resulted in decreased neutrophil recruitment, thus establishing a direct role of the CXCL1/CXCR2 axis in mast cell-mediated neutrophil recruitment.

Conclusions: Our data suggest that chemokines, and in particular CXCL1, released from activated perivascular mast cells induce neutrophil recruitment to the site of inflammation, thereby aggravating the ongoing inflammatory response and thus possibly affecting plaque progression and destabilization.

Introduction

Acute cardiovascular syndromes such as myocardial infarction and stroke remain the principal cause of death in western society despite increasing insight in the mechanisms of atherosclerosis, which is the underlying cause of disease[1]. Atherosclerosis has been identified as a lipid-driven inflammatory disorder, in which various immune cells such as monocytes, macrophages but also mast cells and neutrophils have been implicated[2,3]. Although statin treatment has reduced the risk of acute cardiovascular events by its lipid-lowering and anti-inflammatory effects[4], this treatment is still insufficient for 70% of the patients, thus establishing the need for further research to obtain new therapeutic leads.

The mast cell, a potent inflammatory cell of the innate immune system, is currently mainly known for its role in allergy and asthma. However, evidence suggesting a detrimental role for the mast cell in atherosclerosis and acute cardiovascular syndromes is accumulating. For example, we and others have previously established that mast cells induce atherosclerotic plaque growth and destabilization in a number of different mouse models of atherosclerosis[5,6]. In human atherosclerotic plaques, mast cell presence has also been previously established[7,8]. More importantly, mast cell numbers were recently shown to correlate with plaque progression and were even demonstrated to associate with future cardiovascular events[9], thereby further emphasizing the crucial contribution of mast cells to plaque destabilization. Currently, most research is aimed at identifying the endogenous mast cell activators in atherosclerosis. Immunoglobulin E (IgE) is commonly known for its acute effects on mast cell activation in allergy. In men with hyperlipidemia and in patients with acute cardiovascular disorders[10], plasma IgE levels were shown to be increased as well. Further *in vitro* and *in vivo* evidence has also established a role for complement factors[11,12], neuropeptides[13,14], immune complexes[15] and lipid mediators[16] in mast cell activation during the development and progression of atherosclerosis.

Mast cells exert their detrimental effects on plaque stability by the release of a number of mediators, such as the mast cell specific proteases chymase and tryptase, histamine, and a vast amount of cytokines and chemokines[17]. We have previously established that chymase released from mast cells can induce plaque progression[18]. Additionally, it has been shown that mast cells can promote apoptosis of various cell types present in the plaque, such as vascular smooth muscle cells[19], endothelial cells[20] and macrophages[5], thereby contributing to plaque necrosis and destabilization. However, as mast cells secrete a whole panel of cytokines and chemokines, in this study we aimed to establish to which extent mast cells are capable of inducing leukocyte recruitment towards the plaque, thereby fuelling the ongoing inflammatory response and possibly aggravating plaque progression.

Materials and Methods

Systemic mast cell activation

This study was performed in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. All animal experiments were approved by the animal welfare committee of the Leiden University Medical Center (approval reference number 08014). Mice were obtained from the local animal breeding facility (Gorlaeus Laboratories, Leiden, The Netherlands). We used 10-12 weeks old male B cell deficient apoE$^{-/-}$µMT mice, kindly provided by Prof. B.H. Toh (Monash University, Melbourne, Australia) for our observational study. These mice lack endogenous IgE, which results in a more pronounced effect of IgE mediated mast cell activation. Mice were fed a western-type diet containing 0.25% cholesterol and 15% cacaobutter (SDS, Sussex, UK) for eight weeks. During these eight weeks, the mice were challenged with IgE (n=13) or PBS (n=10) control for 6 times (~every 1.5 weeks). In order to do so, mice were given 1 µg of antiDNP-IgE by intraperitoneal injection, and 24 hours later the mice received an intravenous injection containing 0.5 mg DNP. At sacrifice, mice were anaesthetized by subcutaneous injection of ketamine (60 mg/kg, Eurovet Animal Health, Bladel, The Netherlands), fentanyl citrate and fluanisone (1.26 and 2 mg/kg, respectively, Janssen Animal Health, Sauderton, UK). Adequacy of anaesthesia was monitored by regular visual inspection and toe pinch reflex. Mice were exsanguinated via orbital bleeding and *in situ* fixation through the left cardiac chamber was performed, after which the hearts were excised for further analysis. The hearts were dissected just below the atria and sectioned perpendicular to the axis of the aorta, starting within the heart and working in the direction of the aortic arch. Once the aortic root was identified by the appearance of aortic valve leaflets, 10 µm sections were taken and mounted on gelatin-coated slides. Mean lesion area (in µm^2) was calculated from six Oil-Red-O stained sections in distal direction starting at the point where all three aortic valve leaflets first appeared. Collagen content in the lesion was determined with a Sirius Red staining, while macrophages were visualized with a Moma-2 antibody (1:1000, Serotec, Puchheim, Germany). The necrotic core size was defined as the a-cellular, debris-rich plaque area as percentage of the total plaque area. The aortic roots were quantified by the Leica image analysis system (Leica Ltd, Cambridge, UK). T cell numbers in the intima and adventitia were determined by staining for CD3 (1:50, Neomarkers, Fremont, CA, USA) and were counted manually. Mast cells and neutrophils were visualized by staining of 10 µm cryosections with a naphthol AS-D chloroacetate esterase staining kit (Sigma, Zwijndrecht, The Netherlands) and counted manually. A mast cell was considered resting when all granula were maintained inside the cell, while mast cells were assessed as activated when granula were deposited in the tissue surrounding the mast cell. Neutrophils were identified as round cells with a characteristic lobular nucleus and pink granular cytoplasm. All morphometric analyses were performed by blinded independent operators.

Leukocyte influx

Mice were obtained from the local animal breeding facility (Gorlaeus Laboratories, Leiden, The Netherlands). Peritoneal mast cells of either male C57BL/6 or mast cell deficient male Kit(W^{-sh}/W^{-sh}) mice were activated by intraperitoneal injection of compound 48/80 (1.2 mg/kg). After 30 minutes and 3 hours (n=4 per group), mice were anaesthetized as described above, after which peritoneal cells were collected by flushing the peritoneal cavity with 10 ml PBS. After collection of the peritoneal fluid, mice were sacrificed via cervical dislocation. Total cell count and neutrophil, lymphocyte, monocyte and eosinophil counts in blood were analyzed using an automated XT-2000iV veterinary hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany). After centrifugation of the cells (1500 rpm for 5 minutes), supernatant was collected for protease activity as described below and for chemokine quantification by ELISA according to manufacturer's protocol. Subsequently, leukocyte suspensions were incubated with 1% mouse serum in PBS and stained for surface markers (0.25 µg/0.2*10^6 cells, eBioscience, San Diego, CA, USA),

after which surface marker expression was determined by FACS analysis (FACS Canto, BD Biosciences, Breda, The Netherlands).

An additional influx study was performed in order to investigate CXCR2 mediated neutrophil influx after mast cell activation. Male apoE$^{-/-}$ mice (n=8) were injected intraperitoneally with anti-CXCR2 (5 µg/mouse, R&D systems, Minneapolis, MN, USA) or PBS. One day later the mice received a second injection with anti-CXCR2 an hour prior to intraperitoneal mast cell activation with compound 48/80 (1.2 mg/kg) or PBS. 3 Hours later mice were anaesthetized and peritoneal fluid was collected as described above, after which mice were sacrificed via cervical dislocation. Subsequenlty, cells were stained for CD11b, NK1.1, Ly6C, Ly6G and CXCR2, after which they were analyzed by FACS.

β-Hexosaminidase activity was determined by adding 50 µL of peritoneal fluid to 50 µL 2 mM 4-nitrophenyl N-acetyl-b-D-glucosaminide (Sigma) in 0.2 M citrate (pH 4.5) and incubated at 37 °C for 2 hours. After addition of 150 µL 1 M Tris (pH 9.0), absorbance (optical density, OD) was measured at 405 nm. To measure chymase release after degranulation, 50 µL peritoneal fluid was added to 2 mM S-2586 (chymase substrate, Chromogenix, Llanelli, UK) in PBS supplemented with 100 U/mL heparin. After 24 hours at 37 °C, OD405 was measured. Values are expressed as percentage of total content. The CXCL1 ELISA was performed according to manufacturer's protocol (Life Technologies, Bleiswijk, The Netherlands).

Neutrophil isolation

Neutrophils were isolated by negative selection from bone marrow as described earlier[21]. In short, C57BL/6 mice were anaesthetized as described above and sacrificed via cervical dislocation after which bone marrow was isolated by flushing the femurs and tibias. Cell suspensions were incubated with an antibody cocktail containing α-CD5, α-CD45R, α-CD49b, α-CD117, α-F4/80 and α-TER119 (4°C, 10 minutes under constant shaking). After washing, cells were incubated with α-biotin microbeads (Miltenyi, Leiden, the Netherlands, 4°C, 10 minutes under constant shaking). Subsequently neutrophils were isolated by magnetic bead isolation (magnetic-activated cell sorting LS column, Miltenyi). We obtained neutrophils at ≈90% purity, as validated by flow cytometry and histology[22], which were used for further experiments.

Cell culture

C57BL/6 mice were anaesthetized as described above and sacrificed via cervical dislocation after which bone marrow was isolated by flushing the femurs and tibias. Bone marrow derived mast cells (BMMCs) were grown by culturing bone marrow cells at a density of $0.25*10^6$ cells in RPMI containing 10% fetal bovine serum (FBS), 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (all from PAA, Cölbe, Germany) and mIL3 supernatant (supernatant from WEHI cells overexpressing murine Interleukin (IL)-3) for 4 weeks in T175 tissue culture flasks (Greiner Bio-one, Alphen aan den Rijn, Netherlands). Total RNA was extracted from these cells with GTC, reverse transcribed using M-MuLV reverse transcriptase (RevertAid, MBI Fermentas, Leon-Roth, Germany) and expression of target genes (Supplemental table 1) was measured by qPCR on an ABI PRISM 7500 Taqman apparatus (Applied Biosystems, Foster City, CA, USA).

BMMCs ($5*10^5$) were activated by incubation with compound 48/80 (0.5 µg/mL, Sigma, Zwijndrecht, the Netherlands (n=4 per condition) for 15-30 minutes at 37ºC in HEPES-tyrode supplemented with 0.1% fatty acid free bovine serum albumin (BSA, Sigma). For total (100%) content measurements, mast cells were lysed with 10% Triton X-100 and untreated control cell supernatant served as 0% release controls.

Migration assay

BMMCs were degranulated as described above and the supernatant was collected. Neutrophils (10^5 per well) were applied to the upper chamber of a transwell system (24 wells, 8 µm pore size, PAA) in RPMI containing 10% fetal bovine serum (FBS), 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 µg/mL strep-

tomycin. Mast cell releasate was added to the basolateral chamber. To establish the role of neutrophil derived CXCR2 and CXCR4, anti-CXCR2 or AMD3100 (500 ng/mL) were added to the system. After 4 hours incubation, the number of migrated neutrophils was counted manually.

RNA isolation, cDNA synthesis and qPCR

Total RNA was isolated using a standard TRIzol-chloroform extraction protocol. RNA concentration, purity and integrity were examined by nanodrop (Nanodrop® Technologies). RNA was reverse transcribed by M-MuLV reverse transcriptase (RevertAid, MBI Fermentas, Landsmeer, The Netherlands) and used for quantitative analysis of mouse genes (Table 1) with an ABI PRISM 7700 Taqman apparatus (Applied Biosystems, Bleiswijk, The Netherlands). Murine HPRT and RPL27 were used as standard housekeeping genes.

Statistical analysis

Data are expressed as mean ± SEM. A 2-tailed Student's t-test was used to compare individual groups. Non-Gaussian distributed data were analyzed using a Mann-Whitney U test. Frequency data analysis was performed by means of the Fisher's exact test. A level of $P<0.05$ was considered significant.

Results

Mast cell activation correlates with increased neutrophil influx to the plaque

Repeated IgE treatment of apoE$^{-/-}$µMT mice did not significantly affect total mast cell numbers in the aortic root (controls: 15.8 ± 2.2 mast cells/section versus IgE: 20.5 ± 3.1 mast cells/section), but did result in a significant increase in mast cell activation (controls: 35.2 ± 3.9% versus IgE: 48.2 ± 3.4%, $P<0.05$, Figure 1A). Concomitantly, plaque size in the aortic root was increased by 40% from 2.0 ± 0.2*10^5 µm^2 in control mice to 2.8 ± 0.3*10^5 µm^2 in IgE treated mice ($P=0.05$, Figure 1B). Collagen content did not differ between the groups (controls: 12.0 ± 1.7% versus IgE: 9.6 ± 1.0%, P=NS, Figure 1C), while macrophage staining revealed a significant decrease in relative MOMA-2$^+$ area (controls: 27.4 ± 2.7% versus IgE: 9.9 ± 1.7%, $P<0.001$, Figure 1D). Necrotic core area was increased from 51.7 ± 14.2*10^3 µm^2 in the control mice to 82.6 ± 18.6*10^3 µm^2 in the IgE treated group (Figure 1E), which is suggested to be caused by increased macrophage apoptosis upon mast cell activation as established previously[5]. Furthermore, intimal and adventitial T cell numbers did not differ between the groups (Figure 1F). Interestingly, IgE mediated mast cell activation resulted in a striking increase in the number of neutrophils in the intima (controls: 12 ± 3 versus IgE: 39 ± 12 neutrophils/mm^2 tissue, $P=0.06$) and particularly in the perivascular tissue (controls: 58 ± 11 versus IgE: 183 ± 39 neutrophils/mm^2 tissue, $P<0.05$, Figure 1G,H). The number of neutrophils in the control mice remained below 25 neutrophils/mm^2 tissue (10 out of 10 plaques), while in IgE treated mice 6 out of 13 plaques had more than 25 neutrophils/mm^2 tissue ($P<0.05$). Similarly, within the perivascular tissue only 1 out of 10 control mice contained over 100 neutrophils/mm^2 tissue, while IgE treatment resulted in >100 neutrophils/mm^2 tissue in

9 out of 13 sections (P<0.01). Furthermore, mast cell activation status was seen to correlate with the number of perivascular neutrophils (R^2=0.28, P<0.05).

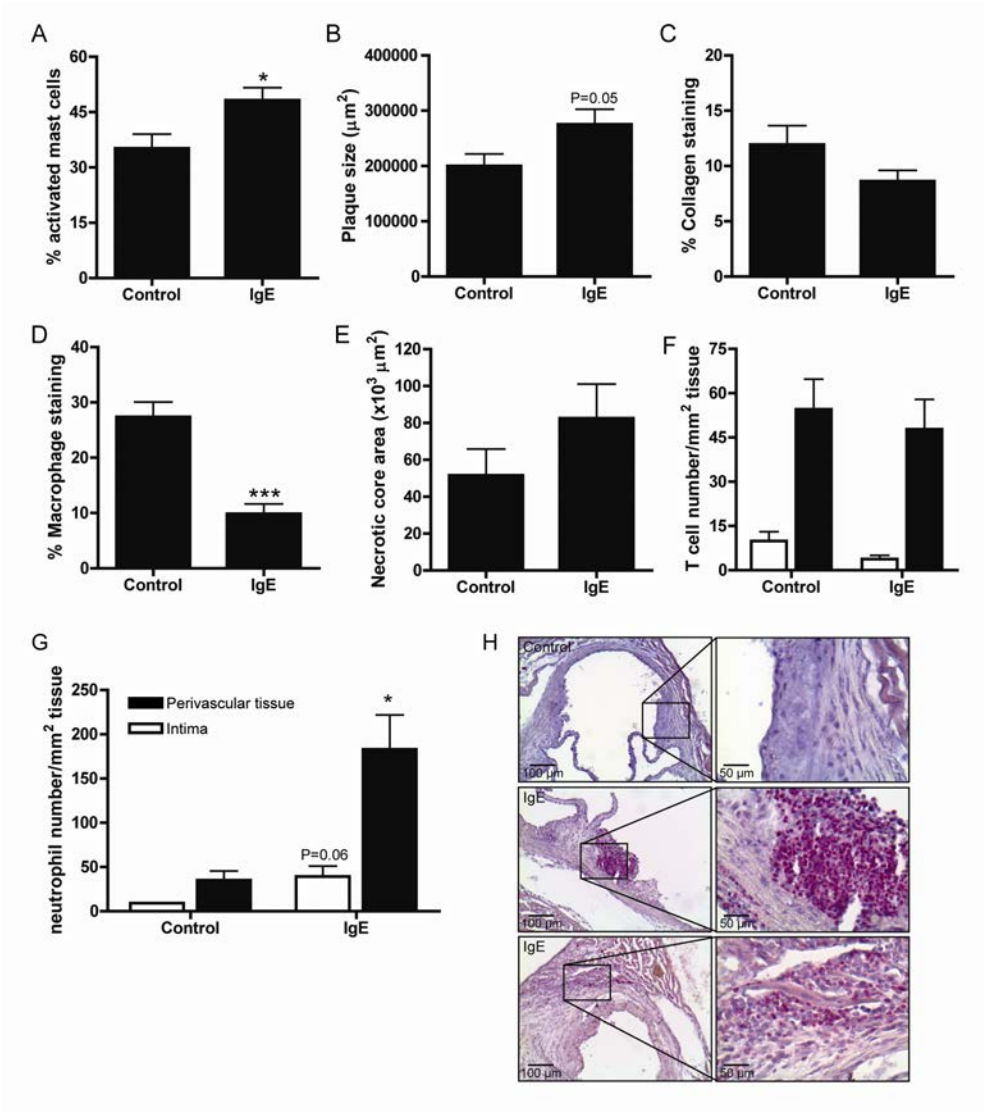

*Figure 1. IgE induced mast cell activation in apoE$^{-/-}$µMT mice. During eight weeks, male apoE$^{-/-}$µMT mice were challenged with IgE (n=13) or PBS (n=10) control for 6 times (~every 1.5 weeks). IgE treatment resulted in enhanced mast cell activation within the aortic root (A) and concomitant lesion progression (B). (C) Collagen content was not affected by the IgE induced mast cell activation, while macrophage content was significantly reduced (D). (E) Necrotic core area was somewhat, but not significantly increased in the IgE treated group. (F) T cell numbers in the intima (white bars) and the perivascular tissue (black bars) did not differ between the groups (G). Neutrophil numbers were increased in the intima (white bars) and even more pronounced in the perivascular tissue (black bars) after IgE mediated mast cell activation. (F) Representative images of aortic root sections stained with a naphtol chloroacetate esterase staining, illustrating large neutrophil accumulations within the lesion (middle panel) and the perivascular tissue (lower panel) after IgE treatment, but not in control mice (upper panel). Magnifications: left panels 100x, right panels 400x. *P<0.05, ***P<0.001.*

In vivo mast cell activation results in neutrophil recruitment

To further investigate whether neutrophil recruitment is indeed mast cell mediated, we activated peritoneal mast cells in mast cell competent C57BL/6 mice and mast cell deficient Kit(W^{-sh}/W^{-sh}) mice, by intraperitoneal injection of the commonly used mast cell activator compound 48/80. This resulted in acute mast cell activation as indicated by β-hexosaminidase (Figure 2A) and chymase (Figure 2B) activity in the peritoneal cavity of C57BL/6 mice, but not in that of Kit(W^{-sh}/W^{-sh}) mice, at 30 minutes and up to 3 hours after injection. Leukocyte differentiation analysis using Sysmex revealed a striking influx of predominantly neutrophils in response to mast cell activation. This effect on recruitment was not observed for monocytes and lymphocytes. Total populations were even slightly decreased after mast cell activation with compound 48/80 and these numbers remained identical between C57BL/6 and Kit(W^{-sh}/W^{-sh}) mice (Figure 2C-E).

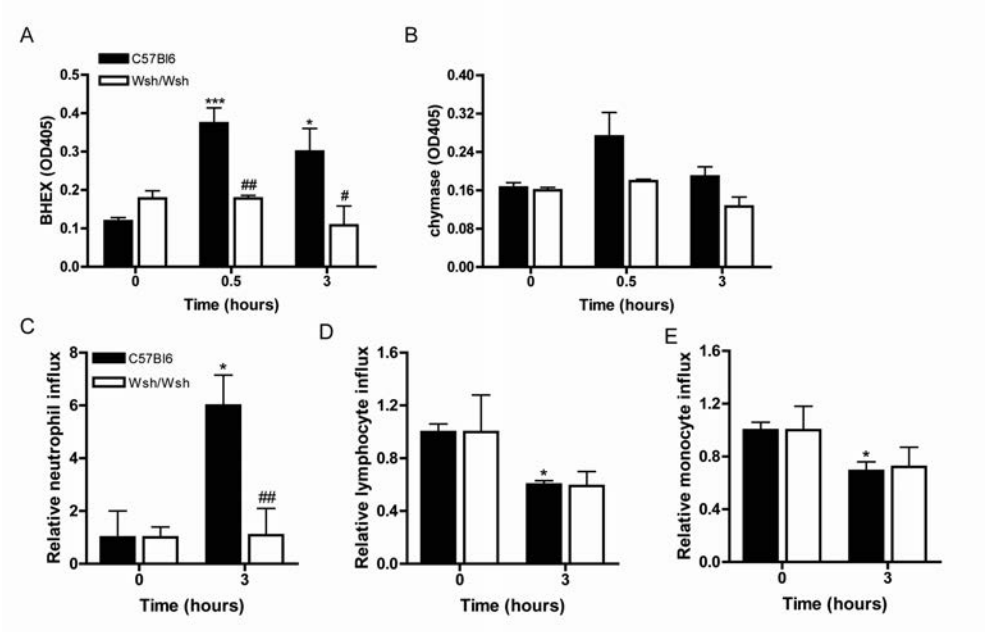

*Figure 2. Mast cell induced neutrophil recruitment in vivo. C57BL/6 or mast cell deficient Kit(W^{-sh}/W^{-sh}) mice (n=4) were intraperitoneal injected with compound 48/80, which resulted in acute mast cell activation as indicated by increased β-hexosaminidase (A) and chymase (B) activity in the peritoneal cavity of C57BL/6 mice (black bars) at 30 minutes and still at 3 hours after injection, which did not occur in mast cell deficient Kit(W^{-sh}/W^{-sh}) mice (white bars). Acute peritoneal mast cell activation by compound 48/80 induced recruitment of neutrophils (C), but not of lymphocytes (D) or monocytes (E) in C57BL/6 mice as measured by Sysmex cell differentiation analysis. *P<0.05 compared to T=0, ***P<0.001 compared to T=0, #P<0.05 compared to C57BL/6, ##P<0.01 compared to C57BL/6.*

By means of FACS analysis we confirmed the influx of $CD11b^{+}Ly6G^{high}CD71^{-}$ neutrophils in C57BL/6 mice as displayed in Figure 3A. Interestingly, the recruited neutrophils were CXCR2 and/or CXCR4 positive (Figure 3B,C), suggesting that the

ligands of these specific receptors, i.e. CXCL1 (or KC, the murine analogue of IL-8) and CXCL12, are involved in mast cell mediated neutrophil recruitment.

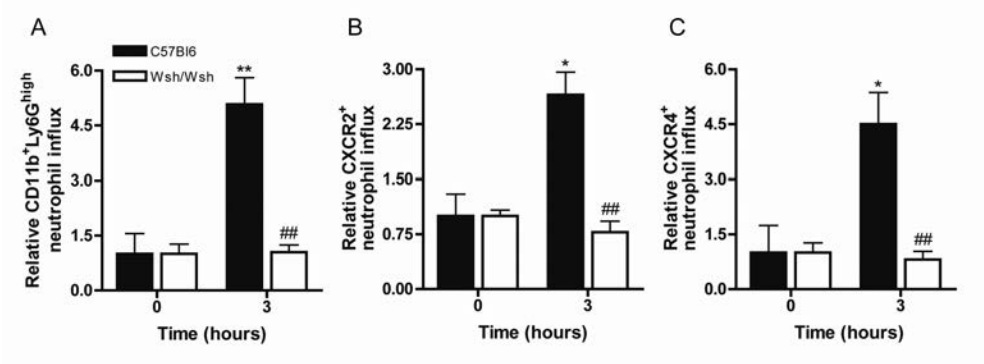

*Figure 3. Mast cells recruit CXCR2⁺ and CXCR4⁺ neutrophils. C57BL/6 or mast cell deficient Kit(W^{-sh}/W^{-sh}) mice (n=4) were intraperitoneally injected with compound 48/80 (A). FACS analysis confirmed the recruitment of $CD11b^{+}Ly6G^{high}CD71^{-}$ neutrophils in response to compound 48/80 induced mast cell activation in C57BL/6 mice but not in Kit(W^{-sh}/W^{-sh}) mice. The mast cell dependent neutrophil influx appeared to be CXCR2 (B) and CXCR4 (C) dependent. *P<0.05 compared to T=0, **P<0.01 compared to T=0, #P<0.05 compared to C57BL/6, ##P<0.01 compared to C57BL/6.*

Mast cells express and secrete CXCL1 and CXCL12

Next, we aimed to establish whether mast cell induced neutrophil recruitment was mediated via CXCL1 or CXCL12, the ligands for CXCR2 and CXCR4. First, mRNA expression of these chemokines was measured in cultured bone marrow derived mast cells (BMMCs). Indeed, we observed that both CXCL1 (relative expression: 0.005 ± 0.004) and CXCL12 (0.002 ± 0.001) were expressed by BMMCs. $5*10^5$ BMMCs were seen to contain 1.5 ± 0.3 ng of CXCL1 as measured by ELISA. After activation with compound 48/80, release of CXCL1 into the supernatant of BMMCs could be detected (102 ± 15 pg/mL compared to 6 ± 12 pg/mL in the releasate of unstimulated control cells, P<0.05). Similarly, we observed an increase in CXCL12 release after stimulation with compound 48/80 (OD 450 nm: 0.26 ± 0.008 versus 0.19 ± 0.004 in the releasate of unstimulated control BMMCs, P<0.05). These data indicate that mast cells, in accordance to previous literature, express and secrete chemokines such as CXCL1 and CXCL12 that can recruit neutrophils to the site of mast cell activation.

Neutrophil recruitment in vitro

To confirm our *in vivo* findings, we isolated neutrophils from bone marrow by negative selection as described previously[21] and allowed these cells to migrate towards supernatant of BMMCs stimulated with IgE. As expected, neutrophil migration towards the basolateral side of the migration chamber was significantly increased as compared to supernatant of unstimulated BMMCs ($32.3 \pm 4.7*10^3$ cells versus $11.6 \pm 2.5*10^3$ neutrophils, P<0.01, Figure 4). Blocking neutrophil CXCR2

with a specific mouse α-CXCR2 blocking antibody inhibited the mast cell induced neutrophil migration (18.8 ± 2.2*10^3 neutrophils, P<0.05 compared to IgE stimulated mast cells), while addition of the CXCR4 receptor antagonist AMD3100 was not as effective (24.8 ± 6.9*10^3 neutrophils, P=NS). These data illustrate that mast cells, when activated, can indeed directly induce neutrophil recruitment.

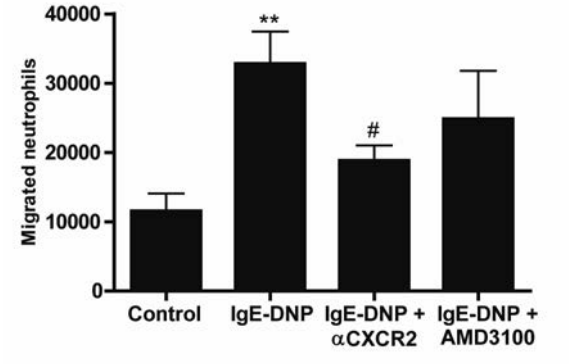

*Figure 4. Mast cell induced neutrophil recruitment in vitro. Supernatant from IgE stimulated BMMCs resulted in enhanced migration of freshly isolated neutrophils, which could be inhibited by a CXCR2 blocking antibody. **P<0.01 compared to supernatant of unstimulated control BMMCs. #P<0.05 compared to IgE stimulated BMMCs.*

αCXCR2 inhibits mast cell-mediated neutrophil recruitment in vivo

We aimed to validate our *in vitro* findings in an *in vivo* setting by activating peritoneal mast cells while blocking CXCR2 with αCXCR2 (Figure 5A). Similar to the previous results in C57BL/6 mice, compound 48/80-mediated mast cell activation in apoE$^{-/-}$ mice induced a striking 36-fold increase in CD11b^{+}Ly6G^{high} neutrophil influx to the peritoneum compared to control mice as measured by FACS analysis (Figure 5B; P<0.05). In line with these results, the peritoneal CXCL1 concentration was indeed increased after mast cell activation (Figure 5D). As observed in the previous influx study, total monocytes in the peritoneum were reduced after mast cell activation.

Mast cell activation in combination with αCXCR2 resulted in a strong reduction in neutrophil chemotaxis (Figure 5B; P=0.056), underlining the importance of mast cell derived CXCL1 in neutrophil recruitment. Previously, mast cell derived mediators have been described to influence neutrophil effector functions[22,23]. Interestingly, in our study we observed a similar increase in activation of recruited neutrophils after peritoneal mast cell stimulation, which was not affected by αCXCR2 treatment (Figure 5E).

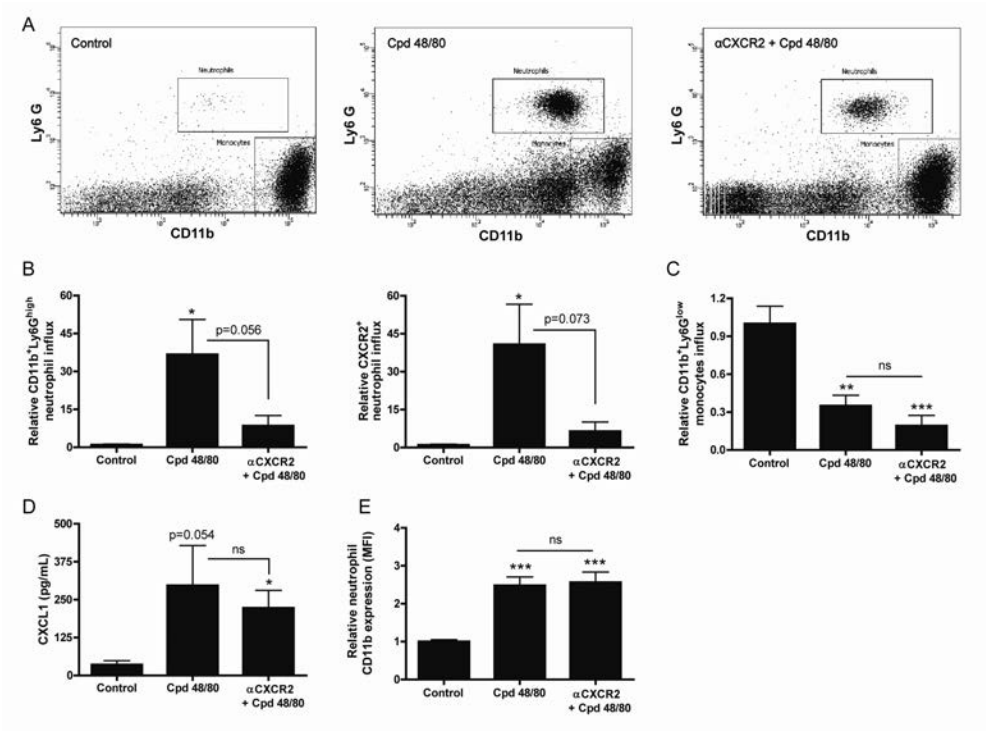

*Figure 5. In vivo mast cell mediated neutrophil influx is mediated by CXCR2. Intraperitoneal mast cell activation of apoE$^{-/-}$ mice (n=8) with compound 48/80 resulted in a significant influx of especially CXCR2^{+} neutrophils and this effect was strongly diminished by blocking CXCR2 (A, B). Monocyte influx was significantly decreased after mast cell activation in a CXCR2-independent manner (C). Mast cell activation caused a local increase in CXCL1 (D). Peritoneal neutrophil activation was significantly increased after mast cell activation, which was unaffected by αCXCR2 treatment (E). *P<0.05, **P<0.01, ***P<0.001 compared to control group.*

Discussion

The current study is the first to demonstrate a correlation between chronic IgE mediated mast cell activation and neutrophil influx into the atherosclerotic plaque. Moreover, mast cell activation was seen to directly cause neutrophil recruitment both *in vitro* and *in vivo*, in particular via the secretion of the chemokine CXCL1.
The mast cell is currently accepted as a potent contributing cell in the process of atherosclerosis. Mast cells can induce plaque destabilization by the release of a number of mediators such as chymase and tryptase, which can degrade matrix molecules that give rise to plaque stability and by the induction of plaque cell apoptosis[5,19,20]. Mast cells are also known for their capacity to store and produce a large number of cytokines and chemokines. Previously, we have provided evidence showing that MCP-1 release after either C5a[12] or lysophosphatidic acid-mediated[16] mast cell activation may result in the recruitment of monocytes towards

the plaque. Acute mast cell activation can also induce upregulation of adhesion molecules on endothelial cells, thereby enabling the influx of inflammatory cells into the subendothelial space[24]. Furthermore, it was previously shown that mast cell-derived IL-6 and IFNγ are crucial for the induction of mast cell dependent atherosclerotic lesion development[6], suggesting that the contribution of mast cell derived cytokines and chemokines to atherosclerotic lesion development as such is more important than previously thought. Chemokines are known for their function in the recruitment of leukocytes towards the site of inflammation, which may in turn aggravate the process of atherosclerosis. In this study, we therefore aimed to determine to what extent mast cell activation affects leukocyte recruitment, in specific to the atherosclerotic lesion.

To establish a pronounced effect of mast cell activation, we used apoE$^{-/-}$ mice that lack B cells and thus not produce endogenous IgE, and chronically injected these mice with IgE. Besides increased mast cell activation and lesion size, we observed a striking increase in neutrophil numbers in the intima, and even more pronounced in the perivascular tissue. We then aimed to determine whether these effects are mast cell specific by investigating leukocyte influx to the peritoneum in mast cell deficient and control mice. Indeed, mast cell activation resulted in exaggerated neutrophil influx to the peritoneum, which was absent in mast cell deficient mice. Neutrophils were seen to be primarily CXCR2 and CXCR4 positive, suggestive of involvement of the CXCR2 ligand CXCL1 and the CXCR4 ligand CXCL12. We then confirmed that mast cells produce and secrete both CXCL1, as previously established[15,25], and CXCL12, and investigated the contribution of these chemokines to neutrophil migration *in vitro*. Blockage of CXCR2, but not that of CXCR4, in an *in vitro* setup was seen to significantly reduce mast cell induced neutrophil migration, indicating that mast cell derived CXCL1 may be more important in neutrophil recruitment than mast cell derived CXCL12. Previously, it has been described that systemic disruption of the CXCL12/CXCR4 axis aggravates atherosclerosis by expansion of neutrophils in the blood and in the plaque[26]. Also, functional blockade of CXCR4 in later stages of atherosclerosis was seen to exacerbate plaque progression, accompanied by hyperactivation of circulating neutrophils[27]. Taking into account these previous findings, and the lack effects we observed on neutrophil migration *in vitro* after blocking CXCR4, we consider it unlikely that mast cell derived CXCL12 is a major contributor in neutrophil influx to the atherosclerotic plaque. However, blockage of CXCR2 did significantly reduce mast cell mediated neutrophil migration *in vitro*, which we then aimed to confirm *in vivo*. Again, after peritoneal mast cell activation, a massive neutrophil influx was observed, which could be partially inhibited by αCXCR2 pretreatment. Based on these data we postulate that mast cell derived CXCL1 is a major contributor to neutrophil migration to the site of inflammation, which is in line with a previous report demonstrating that mast cell and macrophage derived CXCL1 and CXCL2

induce neutrophil recruitment in an LPS-induced peritonitis model[28]. Furthermore, in the current study, we made use of compound 48/80 and IgE mediated mast cell activation, which are well-known to cause mast cell degranulation. We showed that both these general mast cell activators are capable of inducing neutrophil recruitment to the site of inflammation. Also, these mast cell mediated effects are not strain specific, as we demonstrate neutrophil recruitment after mast cell activation in both apoE$^{-/-}$ and C57BL/6 mice.

In advanced atherosclerosis, we have previously established that mast cells can activate endothelial cells, thereby inducing the adhesion of leukocytes to the endothelial layer in a CXCR2 and VCAM-1 dependent fashion[5]. Direct mast cell dependent neutrophil recruitment has been previously associated with diseases such as EAE[29], skin diseases[30] and rheumatoid arthritis[31], either via CXCL1 or other mechanisms such as the tryptase/heparin complex. We now postulate that also in atherosclerosis mast cells may directly induce neutrophil recruitment via the CXCL1/CXCR2 axis, thus providing another mechanism by which mast cells can fuel the ongoing inflammatory response. This mechanism may also explain the massive increase in perivascular neutrophils, which can be caused by activation of perivascular mast cells and subsequent neutrophil recruitment via perivascular microvessels instead of influx through the endothelium.

In the observational *in vivo* study, both increased mast cell activation as well as increased neutrophil numbers were observed. It is therefore difficult to distinguish between effects on plaque formation caused by either the mast cell itself or indirectly by the neutrophil. However, a detrimental role for neutrophils in both early and late stage atherosclerosis has been previously described. Hypercholesterolemia in apoE$^{-/-}$ mice was shown to increase the amount of circulating neutrophils, which correlated with early atherosclerotic lesion size[32]. The direct presence of neutrophils has been readily detected in early fatty streaks and in advanced atherosclerosis they accumulate especially in shoulder regions of the plaque[33]. In human atherosclerotic plaques neutrophils are detected as well, and moreover, increased numbers of neutrophils correlated with rupture-prone lesions[34]. Markers of so-called neutrophil extra-cellular traps are even associated with adverse cardiac events[35]. Furthermore, neutrophils release granules containing large amounts of matrix-degrading proteases, they produce vast amounts of reactive oxygen species and go rapidly into apoptosis[36]. Thus, there are a number of mechanisms via which neutrophils can contribute to atherosclerotic plaque growth and destabilization. In our study, we have provided evidence that stimulation of mast cells resulted in increased activation of recruited neutrophils, which may cause additional detrimental effects on local inflammation.

In conclusion, systemic mast cell activation results in neutrophil accumulation within the vessel wall, and enhanced atherosclerotic lesion development. Recruitment studies revealed a direct role for mast cell derived CXCL1, which attracts

CXCR2$^+$ neutrophils. These data may thus provide a novel mechanism by which mast cells can aggravate the ongoing inflammatory response in atherosclerotic lesion development and progression.

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