The effect of mast cells and mast cell tryptase on B cells

Undergraduate thesis by Marcus Lundberg

Department of Cell & Molecular Biology
Supervisor: Professor Sandra Kleinau
30 credits
Abstract

B cells and mast cells are both involved in the autoimmune disease rheumatoid arthritis (RA). The effect of mast cells on B cells is poorly understood, and mast cells could contribute to the pathogenesis of RA through the activation of B cells. In this in vitro study we have investigated the effect of bone marrow derived mast cells (BMMCs) on B cells. This was done by co-culturing either unstimulated B cells or B cell receptor-stimulated B cells with resting, sensitised or degranulated BMMCs. Furthermore, the effect of the mast cell tryptase, mMCP-6, on the B cells were investigated by using mMCP-6-deficient BMMCs. We found that BMMCs induced B cell blast formation, elevated levels of MHC II, CD86 and CD80 expression, and increased IgM and IgG secretion. Compared to resting and sensitised BMMCs, degranulated BMMCs had a generally greater effect on the B cells. Thus, the BMMCs activate the B cells in several ways and can thus contribute to the pathogenesis of RA through the activation of B cells. The mMCP-6 deficient BMMCs induced less expression of MHC II and CD86 compared to the wt BMMCs. The mMCP-6 could thus play an important role in improving the B cell antigen presenting capacity stimulated by mast cells.

Sammanfattning

Både B-celler och mastceller är inblandade i den autoimmuna sjukdomen reumatoid artrit (RA). Mastcellers effekt på B celler är inte utredd. Mastceller skulle eventuellt kunna bidra till patogenesen av RA genom att aktivera B celler. I denna in vitro studie undersökte vi benmärgs-deriverade mastcellers (BMMCs) effekt på B-celler. Detta genomfördes genom att co-kultivera ostimulerade eller B cell receptor stimulerade B-celler med vilande, sensitiserade eller degranulerade BMMCs. Vi undersökte även om mMCP-6, ett tryptas i mastceller, hade en effekt på B-celler genom att använda BMMCs som saknade mMCP-6. Vi upptäckte att BMMCs inducerar B cell blastformation, ökat uttryck av MHC II, CD86 och CD80, samt ökad sekretion av IgM och IgG. Jämfört med vilande och sensitiserade BMMCs, hade degranulerade BMMCs en generellt högre effekt på B celler. BMMCs aktiverar B celler på ett flertal sätt och kan således bidra till patogenesen av RA genom aktivering av B celler. De mMCP-6 deficienta BMMCs inducerade lägre uttryck av MHC II och CD86 jämfört med vildtyps BMMCs. mMCP-6 kan således spela en viktig roll i mastcellens förmåga att förbättra B cellens antigenpresenterande kapacitet.
Introduction

The immune system defends the organism against pathogens such as viruses, bacteria and parasites as well as against tumor cells, while it at the same time avoids to attack the organisms own tissues and cells. However, a combination of several genetic predispositions and external triggers can turn the immune system towards the organism itself, and when it acts as such, an autoimmune disease emerges. The genetic background of autoimmune diseases is thought to be complex, with the interaction of several genetic products and environmental factors (see picture 1).

Rheumatoid arthritis (RA) is an autoimmune disease, which is characterised by persistent inflammation of the synovial membrane of the joints and systemic inflammation. Also antibodies targeting endogenous antigens (autoantibodies) can be found in the circulation of rheumatic patients. Active RA causes joint damage (by destruction of cartilage and bone), disability, decreased quality of life as well as cardiovascular and other comorbidities. Up to 1% of the population of the western world have RA, where 50 % of the risk for developing the disease is attributable to genetic factors. The main environmental risk in industrialised countries is smoking. Women and elderly people are more prone to develop the disease. Patients are treated with disease-modifying anti-rheumatic drugs e.g. methotrexate which reduce systemic inflammation, and biological anti-inflammatory agents such as tumor necrosis factor inhibitors.

RA was initially thought to be mediated mainly by T cells and macrophages. These cell types are important players in the pathophysiology of RA. However, increased evidence of the pivotal role of B cells in the dysregulation of the immune system in RA has been elicited. B cells contribute to the pathogenesis of RA by acting as producers of pro-inflammatory cytokines, as antigen presenting cells and activators of T cells. Furthermore, in the effector phase of RA, B-cells contribute to the pathology of RA as producers of autoantibodies. Even though autoantibodies role in humans is not completely understood, they have been proved to be strong contributors to the pathogenesis in animal models such as collagen-induced arthritis (CIA).

In RA mast cells (MCs) constitute as much as 5% or more of the cells in the synovium of the joints. Furthermore, MCs mediators, such as histamine and tryptase, are present at higher concentrations in the synovial fluid and tissue of the inflamed joints. Mice lacking MCs through a mutation in c-kit develop less severe symptoms in many models for autoimmune disease, and an inhibitor of c-kit and thus MCs, imatinib meylate, efficiently supresses CIA. MC mediators have many functions that can
be speculated upon to contribute to the RA progression. One of these is the chymase, mouse mast cell protease type 4 (mMCP-4), which is released during degranulation of the MCs. Compared to wild type (wt) DBA/1 mice, mMCP-4 deficient DBA/1 mice develop less severe CIA. However, the absence of mMCP-4 does not give full protection against disease, which indicates that other mechanisms can in part substitute or compensate for mMCP-4. The mouse mast protease-6 (mMCP-6) is a tryptase which is also released from BMMCs during degranulation. This tryptase works as an attractant and activator of lymphocytes. It has been shown that C57BL/6 mice lacking mMCP-6 develop less severe antibody-induced arthritis compared to the wt mice.

There is not much known about what effects MCs have on B cells. However, it has been shown that resting (unstimulated) bone marrow derived mast cells (BMMC) can induce proliferation of B cells. When the BMMC are degranulated they induce more proliferation of the B cells. If the B cells are first activated by anti-B cell receptor stimuli (anti-µ), the degranulated BMMC are even more efficient in inducing proliferation of the B cells. This effect is mediated by cell-cell contact (one of the interactions being CD40L and CD40), and by cytokines. Resting BMMC can also induce increased B cell blast formation, as well as stimulate increased IgM production. The BMMC must be metabolically active to have any effect on the B cells. It has been shown that mice injected with BMMCs have higher proportions of B cell blasts in the spleens which supports the in vitro studies that shows that BMMCs can induce proliferation of B cells.

Both B cells and MCs are important cell types in RA. It might be that MCs play an important role in RA as activators of B cells. In a previous pilot study performed by collaborators, it was shown that mMCP-6 deficient DBA/1 mice developed less CIA and had lower levels of IgM and IgG anti-collagen type II antibodies in the blood compared to the wt mice. This implied that mMCP-6 had a direct or indirect effect on the B cells which resulted in a lower antibody secretion and thus less arthritis development. The aim of this study was to investigate if MCs have a direct effect on B cells, and if there were effects, evaluate if these were mediated by mMCP-6. This would increase the knowledge about the interaction between B cells and MCs in general and in RA, and would further distinguish the role of mMCP-6 in this interaction.

Thus, B cells were co-cultured with resting (unstimulated), sensitised (IgE-anti TNP stimulated) and degranulated (IgE-anti TNP + TNP) BMMCs. Generally, the degranulated BMMCs had the highest effect on B cells. We found that BMMCs induce B cell blast formation and that the expression of CD80, CD86 and MHC II on the B cells increased after co-cultivation. Importantly, the mMCP-6 deficient BMMCs induced less MHC II and CD86 expression than wt BMMCs did. We also found that B cells produce more IgM and IgG when co-cultured with BMMCs compared to when they are cultured in medium alone.
Materials and Methods

Mice

Wild type DBA/1 mice were bred and maintained in the animal facility at the Biomedical Center at Uppsala University, Sweden. The mMCP-6 deficient DBA/1 mice were bred and maintained at the National Veterinary Institute, Uppsala, Sweden. The mice were used at 13 - 14 weeks of age and both sexes were used in the experiments. All mice were fed rodent chow and water ad libitum and all experiments were approved by the local ethics committee.

Spleens were removed from DBA/1 mice and mashed against nets. This was done over petri dishes which contained phosphate buffered saline (PBS). The obtained cell suspensions were centrifuged at 300g for 6 min at 4°C. The supernatants were discarded and 5 ml ammonium-chloride-potassium lysing buffer (ACK buffer) was added to the tubes for 3 min to lyse erythrocytes and then 5 ml cold PBS buffer was added to stop further lysis of cells. The suspensions were drawn into 10 ml pipettes and slowly released back into the tubes with a horizontal angle to make clumps stick to the wall of the pipettes. The suspensions were centrifuged at 300g for 6 min at 4°C. Supernatants were discarded and cell pellets were resuspended in PBS before the splenocytes were stained with trypan blue and then counted in Bürker chambers.

Magnetic-activated cell sorting (MACS)

The splenocytes were centrifuged at 300g for 6 min at 4°C. By discarding the supernatant and then pressing the tubes against a paper towel, the supernatants could be nearly completely removed. The cell pellets were resuspended in 90 µl MACS buffer (PBS 0.5 % BSA, 2mM EDTA) per 10⁷ cells. Ten µl of anti-CD43 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added per 2x10⁷ cells and the suspensions were then incubated on ice for 30 min. The suspensions were then centrifuged at 300g for 6 min at 4°C after which the supernatants were completely removed. If total cell number according to earlier count was below 10⁸ the pellets were resuspended in 500 µl MACS buffer whereas if the total cell number was higher than 10⁸, the pellets were resuspended in 1 ml MACS buffer. After this, the suspensions were run through a 25 LS column (Miltenyi Biotec) and the CD43-negative fractions (B cells) were saved and further used. After MACS sorting, the B cells were centrifuged at 300g for 6 min at 4°C and resuspended in PBS. The B cells were counted and concentrations were adjusted to 1x10⁷ cells/ml.

CFSE labelling of B cells

An equal amount of B cell suspension and 5 µM CFSE (CFDA SE Cell Tracer Kit, Invitrogen, Carlsbad, US) was incubated in room temperature for 5-7 min to stain cells with CFSE. The tubes were vortexed lightly once every minute. After incubation, an equal amount of complete DMEM medium with 20% fetal calf serum (FCS) to “cell suspension + CFSE” was added to stop the CSFE uptake. The tubes were centrifuged at 300g for 6 min at 4°C. Then the cells were washed 3 times with 3 ml of complete DMEM medium with 10% FCS. Next, the cells were resuspended in complete DMEM medium with 10% FCS and cells were counted after which the concentration was adjusted to 2x10⁶ cells/ml.

Preparation of BMMCs

Bone marrow cells were collected from the femur and tibia of wt and mMCP-6 deficient DBA/1 mice by flushing the bones with PBS. BMMCs were obtained by culturing the bone marrow cells in DMEM supplemented with 10% heat-inactivated fetal bovine serum ( SVA, Upppsala, Sweden), 100 IU/ml of
penicillin, 50 µg/ml of streptomycin, 2 mM L-glutamine, 10 ng/ml recombinant IL-3 and 30% WEHI-3B-conditioned medium (as an extra-source of interleukin-3). The cells were kept at a concentration of 0.5–1x10^6 cells/ml, and the medium was changed every third day. Cells were cultured for 4 weeks before being used. WEHI-3B-conditioned medium was produced by culturing WEHI-3B cells (0.5x10^6 cells/ml) for 3 days and collecting the culture supernatant.

The day before a co-culture, the concentrations of wt and mMCP-6 deficient BMMCs were calculated and 1x10^6 BMMCs were transferred to tubes which were then centrifuged at 300g for 5 min at 25°C. The cells were then resuspended in 3 ml medium. Two ml from each cell genotype were transferred to a well (in a 24-well plate) and IgE anti-2,4,6-trinitrophenyl (TNP) at 1 µg/ml were added and mixed before 1 ml of the BMMCs were transferred to another well. The remaining resuspended cell suspension without IgE (around 1 ml) was put into a third well and the plate was incubated overnight at 37 °C 5% CO₂.

Resting or IgE-sensitized BMMCs were transferred to tubes (the IgE-sensitised cells from two wells were pooled into one tube). The tubes were then centrifuged at 300g for 5 min at 25°C, supernatant was discarded, and the BMMCs were resuspended in 1 ml Tyrode’s buffer (130 mm NaCl, 5 mm KCl, 1.4 mm CaCl₂, 1 mm MgCl₂, 5.6 mm glucose, 10 mm 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid and 0.1% BSA, pH 7.4). The BMMCs were centrifuged at 300g for 5 min at 25°C and supernatants were discarded. The cells were resuspended in 1 ml Tyrode’s buffer, which were then transferred to a tube containing a further 9 ml Tyrode’s buffer.

Before co-culture, the BMMCs were centrifuged at 300g for 6 min at 4°C and supernatant was completely removed by pressing the tubes against a tissue. Next, the cells were resuspended in 250 µl DMEM 10% FCS and counted and adjusted to 2x10^6 cells/ml.

**Co-cultivation of B cells and BMMCs**

Unstimulated B cells or B cells which had been activated by anti-µ (5 µg/ml) were cultured alone (controls) or co-cultured with resting, sensitised or degranulated BMMCs. Each culture was prepared in four u-shaped wells in a 96-well plate (Sarstedt, Germany). Co-cultures were prepared by putting unstimulated 1x10^5 B cells into wells and then adding either 1x10^5 unstimulated BMMCs (resting BMMCs) or IgE-anti OVA-TNP sensitised BMMCs. To degranulate BMMCs, 2,4,6-trinitrophenyl ovalbumin (OVA-TNP) at 0.4 µg/ml was added to the wells containing the IgE-anti OVA-TNP sensitised BMMCs. Finally, complete DMEM 10% FCS was added to all wells to give a final volume of 200 µl. B cells cultured in complete DMEM 10% FCS with 5 µg/ml lipopolysaccharide (LPS) was used as a positive control. The plates were incubated for 72 hours at 37 °C 5% CO₂.

**Staining of cells and flow cytometry**

After incubation, 100 µl supernatant from each well was carefully removed, pooled in multiplicates, and saved in -20 °C for further analysis for IgM and IgG content. From the wells, the remaining B cells were pooled in multiplicates and transferred to FACS tubes (named A tubes). An equal volume of FACS buffer (PBS 1% BSA) to the volume of cell suspensions were added to the tubes which were then centrifuged at 300g for 6 min at 4°C. The supernatants were mostly discarded, and the pellets (containing cells) were thereafter resuspended in the remaining supernatant of the tubes. Half of the cell suspensions in each tube were transferred to a second tube (named B tubes). The B cells cultured in medium only were divided and used for fluorescence minus one (FMO) controls. One µl of Fc-block (CD16/CD32, BD, Franklin Lakes, US, 2.4G2) was added to all tubes and 1 µl of anti-i-Aβ-biotin (BD, KH116) was added to A tubes (to be further stained by streptavidin-APC at a later step) and all tubes were then incubated on ice for 30 min in the dark. The tubes were washed in 3 ml of FACS buffer
once. Two staining-mixes were prepared containing antibodies which were diluted 1:50. Staining mix A, contained anti-CD19-PE (BD, 1D3), anti-B220-APC-Cy7 (Biosite, Mera Misa, US) anti-CD80-Pacific Blue (Biosite, 16-10A1), Streptavidin-APC (Biolegend, San Diego, US) and staining mix B contained the same except that CD86-APC (BD, GL1) was added instead of streptavidin-APC. Fifty µl of the staining mixes were added to the respective A and B tubes. The tubes were then incubated on ice for 30 min in the dark and then washed twice in 3 ml FACS buffer.

Compensation controls CompBeads (anti-rat and anti-hamster Ig κ/Negative Control Compensation Particle Set, BD) were prepared by adding 100 µl FACS buffer and 1 µl antibody with a fluorophore to be used for compensation control (example, anti-B220-APC-Cy7). Compensation controls were then incubated on ice for 30 min in the dark, after which they were washed once in 3 ml FACS buffer.

Sample tubes, FMO-controls and compensation controls were brought to the flow cytometer LSRII, and 5 µl 7-AAD were added to all samples 5-15 min before analysis. This was done to stain dead cells. The samples were then analysed in the LSRII.

**Indirect ELISA for analysis of total IgM and total IgG**

Immune-plate F96 Maxisorp (Nalge Nunc International, Penfield, US) were coated with 50 µl of 0.05 µg/ml rabbit immunoglobulin targeting mouse immunoglobulin (DAKA-immunoglobulins, P161). This antibody had a peroxidase conjugation that was not necessary but did neither interfere with the ELISA. The plates were incubated over night at 4°C and were then washed 3 times with 0.05 % PBS-Tween (Tween 20). Next, the plates were incubated for 1 h in room temperature with 100 µl blocking buffer (PBS 1% BSA) per well. The plates were then washed 3x times in 0.05 % PBS-Tween. Mouse IgG (Jackson, Bar Harbor, US) or mouse serum was put into the positive control wells for analysis of IgG and IgM. Blocking buffer was and put into the negative control wells. Fifty µl of samples and controls were added in duplicates. The plates were incubated for 2 hours in room temperature and were then washed 3x times with 0.05 % PBS-Tween. Thereafter, 50 µl of secondary antibody - alkaline phosphatase sheep anti mouse IgM µ-chain specific (Sigma-Aldrich, St. Louis, US) diluted 1:1000 or alkaline phosphatase sheep anti-mouse IgG (Sigma-Aldrich) diluted 1:7000 - were added to the wells and plates were incubated in room temperature for 2 hours. The plates were then washed 3x times in 0.05 % PBS-Tween. Next, 50 µl of substrate solution were added to each well and the plates were incubated at room temperature in the dark and read every 5 min by Versamax spectrophotometer at 405 nm.

**Statistics**

The software GraphPad Prism was used for analysing flow cytometer and ELISA data. Statistical differences were calculated using 1-way ANOVA.
Results

Identification and gating of B cells in co-cultures using flow cytometry

The cultures containing B cells and BMMCs were stained with antibodies and further analysed by flow cytometry using a LSRII machine and the software program flowJo. Figure 1 shows the first step in a series illustrating how the B cells were gated from the co-cultures.

Figure 1. B lymphocytes were gated by flow cytometry according to size (FSC-A) and content of granula (SSC-A). Picture A shows B cells cultured in cell medium only, picture B shows B cells cultured with LPS and picture C shows B cells cultured with degranulated BMMC.

B cells cultured in medium only were used as a control to set the first gate. This gate mainly included the population of cells which were named “lymphocytes” (Figure 1A). In the culture where the B cells had been activated by LPS there appeared to be a higher amount of big and granulated cells (B cell blasts) which had to be included in the gate, and thus the gate was enlarged and set around a bigger area (Figure 1B). Next, the gate was adjusted in the co-culture of B cells and BMMCs (Figure 1C) to exclude as many highly granulated cells (mainly BMMCs) as possible. However, at this step of gating for B cells, the final gate contained many BMMCs which could not be separated from the B cells (Figure 1C).

Figure 2. Single cells were selected from the lymphocyte gate. Picture A shows the size (FSC-W/FSC-H) and picture B shows the granulation (SSC-W/SSC-H).

Next, single cells were gated from the gate set in figure 1C to exclude cell clusters and fragmented cells (Figure 2). Only single cells were to be further analysed.
Figure 3. Cell size (FSC-A) and expression of B220 were used for gating of B cells. Picture A shows B cells cultured in cell medium only, picture B shows B cells cultured with LPS and picture C shows B cells cultured with degranulated BMMC.

B cells are B220\textsuperscript{high} and thus a gate was set for the population of cells which expressed a high level of B220 (Figure 3A). The gate was widened to include B cell blasts which were recognized in B cells cultured with LPS (Figure 3B). This gate was adjusted to exclude B220\textsuperscript{low} cells in the co-culture of B cells and BMMC (Figure 3C).

Figure 4. Gating of B220\textsuperscript{+} B cells lacking high amounts of granula. Content of granula (SSC-A) was compared to the expression of B220. Picture A shows B cells cultured in cell medium only, picture B shows B cells cultured with LPS and picture C shows B cells cultured with degranulated BMMC.

In the next step of gating for B cells, the gate was set to exclude B220\textsuperscript{+} cells that had a different amount of granula in the cytoplasm (Figure 4). B cells have lower amount of granula compared to BMMCs. This was used to exclude BMMCs in the population of B220\textsuperscript{+} cells (Figure 4C).

Figure 5. Viable B cells were gated. Content of 7-AAD in cells was compared to cell size (FSC-A). Picture A shows B220\textsuperscript{+} cell sample where 7-AAD had not been added, and picture B shows the same cells where 7-AAD has been added. Cells were cultured in medium only.
7-ADD is a fluorescent staining compound with a strong affinity for DNA. Cells with compromised membranes will stain with 7-AAD which makes 7-AAD suitable to distinguish dead cells from live cells in flow cytometry. A gate was set that included the 7-AAD background (figure 5A) and excluded the cells which had been stained by 7-AAD and were thus dead cells (Figure 5B). Viability of cells was about 75%.

Figure 6. Gated cells were confirmed to be B cells. The expression of MHC class II was compared to the expression of B220 on the cells. Picture A shows B cells cultured in cell medium only, picture B shows B cells cultured with LPS and picture C shows B cells cultured with degranulated BMMC.

B cells express high levels of the major histocompatibility complex class II (MHCII). This is in contrast to BMMC that do not express MHCII. The final gating process was therefore made to select cells with high expression of MHCII in addition to B220 (figure 6). This was done on B cells cultured in medium only, with LPS or with BMMCs. All the final gated cells (Figure 6C) were MHC II$^{\text{high}}$ and B220$^{\text{high}}$ and thus this gate could be concluded to contain B cells only. Through this multi steps gating procedure the B cells in the co-cultures could be identified and selected for, to be further analysed for the size, proliferation and surface protein expressions.

Proliferation of B cells cultured with BMMC
Figure 7. Amount of B cells with different expression of CFSE as an indicator of proliferation. Picture A shows the proliferation of B cells cultured in medium only (red), with LPS (dark green) or with resting (blue), sensitised (orange) or degranulated (light green) BMMCs. Picture B shows the proliferation of anti-µ stimulated B cells cultured in medium only (dark green), or with resting (blue), sensitised (orange) or degranulated (light green) BMMCs compared to unstimulated B cells cultured in medium only (red). The figure is from one experiment and represents the results obtained from 4 experiments.

To identify proliferating B cells in the cell cultures the B cells had prior to cultivation been stained with CFSE, a fluorescent cell stain that with cell division is diluted in strength. Thus, proliferating cells can be detected by flow cytometry. The B cells cultured in medium only did not show any dilution in strength of CFSE and thus no proliferation, and the stimulated and unstimulated B cells cultured with either wt or mMCP-6 deficient BMMCs did neither show any proliferation (Figure 7A and 7B). B cells cultivated with LPS had two cell populations containing different amounts of CFSE, which indicated that the B cells had been proliferating (Figure 7A). This shows that the CFSE staining was successful and that B cells proliferated upon mitogen stimulation.

The amount of B cell blasts elevate when B cells are cultured with BMMCs.

Figure 8. The amount of B cell blasts according to cell size (FSC-A). Picture A shows B cells cultured in medium only and picture B shows B cells cultivated with degranulated BMMCs.

Blast formation was studied as an activation-marker of B cells. A gate for blasts was set in B cells cultured in medium only (Figure 8A) and the percentage of blasts in each co-culture of B cells cultured with either wt or mMCP-6 deficient BMMCs was measured and divided by the percentage of blasts obtained in B cells cultured in medium only. This gave ratios which are shown in figure 9. The ratio of B cells cultured in medium only, was set to 1.
Figure 9. B cell blastformation is stimulated by BMMCs. Picture A shows the blast formation of unstimulated B cells cultured with wt (white) or mMCP-6 deficient (pink) BMMCs. Picture B shows the blast formation of anti-BCR stimulated
B cells cultured with wt (black) or mMCP-6 deficient (green) BMMCs. Picture C shows picture A and B put together. All pictures show B cells cultured alone (yellow); unstimulated, anti-µ or LPS stimulated. The figure represents data from 4 separate experiments. Results are expressed as mean with SD.

The amount of B cell blasts increase when unstimulated B cells are cultured with resting BMMCs, more so when cultured with sensitised BMMCs and most when cultured with degranulated BMMCs (Figure 9A). When B cells are first stimulated with anti-µ, this effect is enhanced. Degranulated BMMCs induce half the blast formation compared to the B cell mitogen, LPS, when the B cells are unstimulated. When the B cells are anti-µ stimulated the blast formation induced by degranulated BMMCs is almost the same as LPS. The mMCP-6 deficient BMMCs seem to induce a higher blast formation than that of wt BMMCs independent of the state of activation of the B cells and the BMMCs.

**Expression of surface proteins on B cells co-cultured with BMMCs**

The mean fluorescence intensity (MFI) of the expression of CD19, CD86, CD80 and MHC II on the B cells was measured in each co-culture of wt or mMCP-6 deficient BMMCs. The MFI value of the expressed cell protein on the B cells from each co-culture was divided by the MFI of the expressed cell protein on B cells cultured in medium only. This gave ratios which are shown in figures 11-13 for each respective cell surface protein. The ratio of B cells cultured in medium only was set to 1.
The expression of CD19 decrease when B cells are stimulated by anti-μ and this effect is enhanced by BMMCs.

Figure 10. Down regulation of CD19 on B cells by BCR stimuli and synergistic effect of BMMCs. Picture A shows the CD19 MFI ratio of unstimulated B cells cultured with wt (white) or mMCP-6 deficient (pink) BMMCs. Picture B shows the CD19
CD19 is part of a co-activation complex which is activated by the complement system. When activated, the complex enhances the B cell activation stimulated by the B cell receptor (BCR). B cells stimulated through the BCR (via anti-µ) down regulates CD19 (Figure 10). This effect is enhanced when BMMCs are present in the co-cultures (figure 10B) but BMMCs alone does not have this effect on the CD19 expression (Figure 10A). BMMCs does not induce expression of CD19 on B cells compared to B cells stimulated by LPS and does neither down regulate the expression of CD19 compared to when anti-µ is added to the culture. This suggests that BMMCs effect on CD19 expression is not mediated through the same pathway as LPS or anti-µ. There was no apparent difference in CD19 expression induced by mMCP-6 deficient or wt BMMCs.
B cells cultured with anti-µ and BMMCs express elevated levels of CD86, especially when BMMCs are wt.
Figure 11. B cells cultured with anti-μ and BMMCs express elevated levels of CD86. Picture A shows the CD86 MFI ratio of unstimulated B cells cultured with wt (white) or mMCP-6 deficient (pink) BMMCs. Picture B shows the CD86 MFI ratio of anti-BCR stimulated B cells cultured with wt (black) or mMCP-6 deficient (green) BMMCs. Picture C shows picture A and B put together. All pictures show B cells cultured without MCs (yellow); unstimulated, anti-μ or LPS stimulated. The figure represents data from 4 separate experiments. Results are expressed as mean with SD.

CD86 is a co-stimulatory molecule which induces the second activation signal to T-helper cells after an antigen has been presented to the T-helper cell through MHC II. Both anti-μ and LPS induce increased expression of CD86 on B cells (Figure 11). On unstimulated B cells, Wt BMMCs induce a higher expression of CD86 compared to the negative control (B cells cultured in medium only) (Figure 11A). Especially degranulated BMMCs induce increased CD86 expression on B cells. Anti-μ stimulated B cells cultured with BMMCs have a several times higher expression of CD86 compared to both the negative and positive control (Figure 11B). Independent of the active state of the B cells and the BMMCs, the mMCP-6 deficient BMMCs induce less expression of CD86 on B cells than wt BMMCs. Thus, mMCP-6 deficient BMMCs do not improve the antigen presenting capacity of B cells through CD86 to the same extent as the wt BMMCs.
Anti-BCR and BMMCs have a synergetic inducing effect on the expression of CD80 which is higher when the BMMC are mMCP-6 deficient.
CD80 is a co-stimulatory molecule with a similar function to that of CD86. When unstimulated B cells are cultured with either anti-μ, LPS or wt BMMCs there is nearly no change in the expression of CD80 on the B cells (Figure 12A). However, when unstimulated B cells are cultured with mMCP-6 deficient BMMCs, the expression of CD80 is elevated. The elevated level of CD80 on the B cells is the same independent of the active state of the mMCP-6 deficient BMMCs (Figure 12A). When anti-μ stimulated B cells are cultured with BMMCs (especially with mMCP-6 deficient BMMCs) the expression of CD80 is increased on the B cells (Figure 12B).
mMCP-6 deficient BMMCs induce less MHC II expression than wt BMMCs
Figure 13. MHC II expression on B cells is increased by BMMC, but not by mMCP-6 deficient BMMCs to the same degree. Picture A shows the MHC II MFI ratio of unstimulated B cells cultured with wt (white) or mMCP-6 deficient (pink) BMMCs. Picture B shows the MHC II MFI ratio of anti-BCR stimulated B cells cultured with wt (black) or mMCP-6 deficient (green) BMMCs. Picture C shows picture A and B put together. All pictured show B cells cultured without MCs (yellow); unstimulated, anti-µ or LPS stimulated. The figure represents data from 4 separate experiments. Results are expressed as mean with SD.

Unstimulated B cells cultured with BMMCs express higher levels of MHC II compared to the negative control which is B cells cultured in medium only (Figure 13A). Degranulated BMMCs induced increased MHC II expression on B cells almost to the same extent as LPS did (Figure 13A). When anti-µ stimulated B cells were cultured with BMMCs the expression of MHC II was even further elevated, and surpassed the level of MHC II induced by LPS. Independent of state of the B cell or the BMMCs, the mMCP-6 deficient BMMCs did not induce increased expression of MHC II to the same degree as wt BMMCs did. This would make mMCP-6 deficient BMMCs less efficient than wt BMMCs at improving the antigen presenting capacity of B cells through MHC II.
Antibody secretion in co-cultures

The antibody secretion of IgM and IgG was measured in the supernatants gathered from cultures of B cells in medium only, B cells with LPS, or B cells with resting, sensitised or degranulated BMMCs. After an ELISA was performed, the optical density (OD) was measured to evaluate the concentration of antibodies in the culture, and the value obtained from each culture was divided by the OD value obtained in B cells cultured with LPS. This gave ratios which are shown figures 14-16. The ratio of antibody secretion by B cells cultured with LPS was set to 1.

B cells cultured with BMMCs, especially degranulated BMMCs, secrete more IgM and IgG

![Graphs showing antibody secretion ratios](image)

Figure 14. Elevated antibody secretion in B cells co-cultured with BMMCs. IgM (picture A) and IgG (picture B) secretion by B cells cultured in medium only, cultured with LPS or with resting, sensitised or degranulated BMMCs. The figure represents data from 4 experiments. Results are expressed as mean with SEM. *p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001

Resting and sensitised BMMCs induce an increased IgM and IgG secretion from B cells (Figure 14). There is no difference in induction of IgM and IgG secretion between resting and sensitised BMMCs. However, degranulated BMMCs induce IgM and IgG secretion from the B cells even further (Figure 14).
B cells cultured with mMCP-6 deficient BMMC secrete the same amount of IgM and IgG as B cells cultured with wt BMMC

Figure 15. Elevated antibody secretion in B cells co-cultured with mMCP-6 deficient BMMCs. IgM (picture A) and IgG (picture B) secretion by B cells cultured in medium only, cultured with LPS or with resting, sensitised or degranulated mMCP-6 deficient BMMCs. The figure represents data from 4 experiments. Results are expressed as mean with SEM. *p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001

To investigate whether mMCP-6 in the BMMCs granula mediated the increased IgM and IgG secretion from the B cells, supernatants from the co-cultures of B cells and mMCP-6 deficient BMMCs were investigated for antibody content. The mMCP6-deficient BMMCs, regardless of activation state, induced a similar pattern of IgM and IgG secretion to that of wt BMMCs, when cultivated with B cells (Figure 15). To further compare the secretion of IgM and IgG from B cells cultured with wt BMMCs or mMCP-6 deficient BMMCs the values obtained were put into the same figure (Figure 16). There was no significant difference in the induction of IgM and IgG secretion by wt or mMCP-6 deficient BMMCs (figure 16).
Figure 16. Elevated antibody secretion in B cells co-cultured with wt or mMCP-6-deficient BMMCs. IgM (picture A) and IgG (picture B) secretion of B cells cultured in medium only, cultured with LPS or with resting, sensitised or degranulated wt or mMCP-6 deficient BMMCs. The figure represents data from 4 experiments. Results are expressed as mean with SEM. *p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001
Discussion

The first aim of this study was to determine the effects of BMMCs on B cells. In this study, the BMMCs did not induce proliferation of the B cells. This was surprising since it contradicts results from earlier studies.\textsuperscript{11-13} To further confirm the results in this study, a different proliferation assay should be tried as a compliment to the CFSE staining.

The BMMCs cultured with B cells induced B cell blast formation, especially the degranulated BMMCs, and this effect was further enhanced when the B cells were first stimulated by anti-\(\mu\). This would suggest that MCs are better at stimulating antigen-activated B cells. It would be interesting to further analyse these B cell blasts to investigate if they are plasma cells or a subpopulation of big B cells which proliferate.

Concerning the effect of cell surface proteins on the B cells it was evident that a B cell mitogen like LPS could enhance the CD19 expression on the B cells. In contrast, anti-\(\mu\) stimulation of the B cells decreased the CD19 expression and BMMCs enhanced this effect. This implies that the signal transduction pathway stimulated by BMMCs at some point interacts or crosses with the signal transduction pathway stimulated by anti-\(\mu\) (mimicking antigen activation of the BCR). CD19 is a co-activation complex to BCR. If the B cell is stimulated through the BCR, the down regulation of CD19 might be a negative feedback mechanism to inhibit too high B cell activation.

The BMMCs induced increased expression of MHC II and CD86 on the B cells. When B cells were first stimulated by anti-\(\mu\) before the addition of BMMCs, the increase was enhanced, and the expression of CD80 was also elevated. Since MHC II is used for presenting antigens to T-helper cells, and CD80 and CD86 are co-activation molecules responsible for the second activation signal for T-helper cells, an elevation of these proteins would mean that the B cell becomes better at presenting antigen. Thus, MCs can improve the antigen presenting capacity of B cells.

The BMMCs also induced increased IgM and IgG secretion from the B cells. Especially degranulated BMMCs had this effect. Since antibodies are targeting microorganisms such as bacteria and viruses, one of the normal functions of MCs in a healthy individual would be to make the B cells secrete more antibodies and thus eliminate the pathogens more efficiently. However, in RA, MCs would activate B cells that target endogenous antigens and which would lead to higher secretion of autoantibodies and thus a more severe disease.

The second aim of this study was to investigate whether the BMMCs effect on B cells was mediated through mMCP-6. The results of CD80 expression on unstimulated B cells were interesting. A difference between the expression of CD80 induced by wt BMMCs and mMCP-6 deficient BMMCs was evident regardless of the activation stage of the BMMC. The level of CD80 was higher on B cells cultured with mMCP-6 deficient BMMCs than on B cells cultured with wt BMMCs. mMCP-6 is a tryptase released during degranulation and thus there should be no effect on the CD80 expression on the B cells in the cultures with resting or sensitised BMMCs. One could speculate that mMCP-6 leaked from the wt BMMCs and that this caused the CD80 to be down regulated on the B cells cultured with resting and sensitised wt BMMCs. Further, this would not occur where B cells and mMCP-6 deficient BMMCs were co-cultured because no mMCP-6 was present. In line with this speculation there would be an enhanced decrease of CD80 when the wt BMMCs were degranulated since this is when the tryptase is released. However, this was not the case. There was no difference in CD80 expression regardless of the active state of the BMMCs. Thus, it can be concluded that there is some characteristic of the mMCP-6 deficient BMMCs which makes them prone to induce CD80 expression which cannot be attributed to the lack of mMCP-6. Further experiments where soluble mMCP-6 is added to B cell cultures or mMCP-6 inhibitors are added to wt BMMC and B cell co-
cultures are warranted. This would demonstrate which effects are mediated by the tryptase on the B cells.

Regarding further differences between the effects obtained by wt BMMCs and mMCP-6 deficient BMMCs, the mMCP-6 deficient BMMCs induced increased blast formation and CD80 expression compared to wt BMMCs. This would make mMCP-6 deficient BMMCs better at activating the B cell and improve its antigen presenting capacity (through CD80) compared to wt BMMCs. However, wt BMMCs induced higher expression of both CD86 and MHC II on the B cells than mMCP-6 deficient BMMCs did. Since the expression of CD86 and MHC II was several times higher on B cells cultured with BMMCs, and because the blast formation and CD80 expression was not increased to the same extent, one can conclude that the difference in effects induced by wt and mMCP-6 deficient BMMCs on CD86 and MHC II would have a higher impact on the B cells function than the difference in blastformation and CD80 expression. The MHC II and CD86 are important for antigen presentation, thus it can be concluded that wt BMMCs improve the antigen presenting capacity of B cells to a higher extent than mMCP-6 deficient BMMCs.

To further investigate the antigen presenting capacity of B cells induced by wt and mMCP-6 deficient BMMCs in arthritis, B cells collected from actively immunised wt and mMCP-6 deficient mice can be co-cultured with T helper cells with T cell receptors specific for the antigen used for immunisation. The proliferation of the T helper cells would then work as an indicator of the antigen presenting capacity of the B cells.

Regarding the antibody secretion, there was no significant difference in IgM and IgG secretion when B cells were cultured with wt BMMCs or mMCP-6 deficient BMMCs. Thus, the previous noted effect of reduced anti-collagen type II levels in mMCP-6 deficient mice immunized for CIA may not be directly mediated between B cells and BMMCs, but rather indirectly, possibly through the enhanced antigen-presenting capacity of B cells stimulated by wt MCs.

In this study, there were only 4 animals/experiments assessed. Data gathered from animal experiments tend to be varied and to obtain statistical significance the experiment must be repeated several times. More experiments would contribute to help confirm the results based on the findings made in this study.

In conclusion, BMMCs activates B cells in several ways. This would benefit a healthy individual in the defence against pathogens, but could also possibly on the other hand contribute to the induction and pathogenesis of RA. The tryptase mMCP-6 may contribute to the induction of RA by improving the antigen presenting capacity of B cells. Further studies are needed to fully understand the effects of BMMCs and tryptase on B cells in healthy and arthritic subjects. By doing this, knowledge can be obtained which might lead to the discovery of new therapeutic targets in the treatment and prevention of RA as well as in other diseases where MCs and B cells may play a role.
References


