The role of C1q in (auto) immunity

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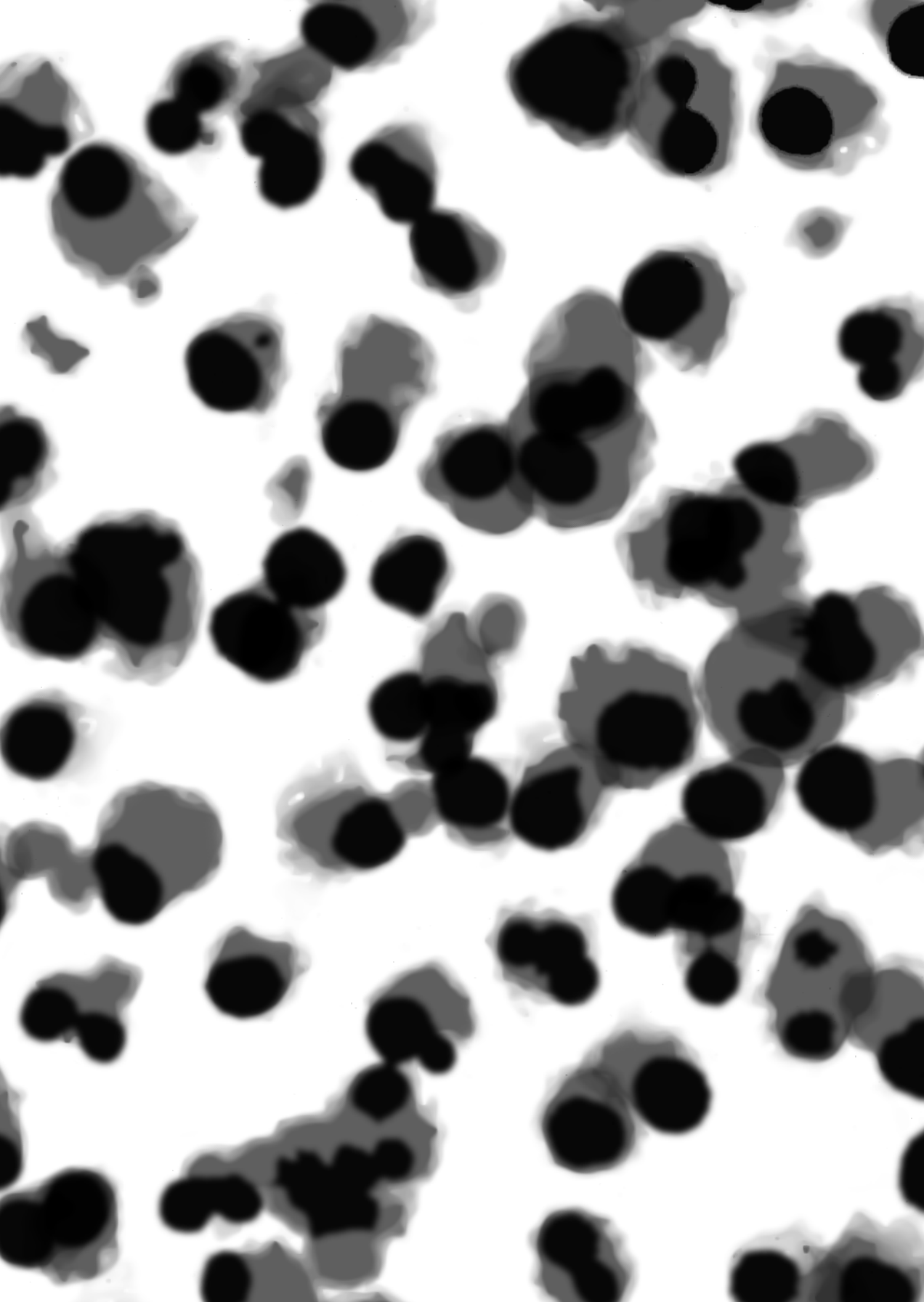
Part II

The local production of C1q by immune and non-immune cells



Chapter 6

The production and secretion of complement component C1q by human mast cells

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Abstract

C1q is the initiation molecule of the classical pathway of the complement system and is produced by macrophages and immature dendritic cells. As mast cells share the same myeloid progenitor cells, we have studied whether also mast cells can produce and secrete C1q.
Mast cells were generated in vitro from CD34+ progenitor cells from buffy coats or cord blood. Fully differentiated mast cells were shown by both RNA sequencing and qPCR to express C1QA, C1QB and C1QC. C1q produced by mast cells has a similar molecular make-up as serum C1q. Reconstituting C1q depleted serum with mast cell supernatant in haemolytic assays, indicated that C1q secreted by mast cells is functionally active. The level of C1q in supernatants produced under basal conditions was considerably enhanced upon stimulation with LPS, dexamethasone in combination with IFN- γ or via FcεRI triggering. Mast cells in human tissues stained positive for C1q in both healthy and in inflamed tissue. Moreover, mast cells in healthy and diseased skin appear to be the predominant C1q positive cells.
Together, our data reveal that mast cells are able to produce and secrete functional active C1q and indicate mast cells as a local source of C1q in human tissue.

Introduction

Mast cells (MCs) are immune cells that are important in the first line of defence and are predominately present in tissues that are close to host/environment interfaces, like the skin and mucosal surfaces. MCs are important in host defence, innate and adaptive immunity, tissue homeostasis and immune regulation [1-3]. As effector cell in disease, MCs are well known for their anaphylactic effects in allergy. MCs are also described to play a role in other diseases like atherosclerosis, contact dermatitis, cancer and arthritis [4-7].
MCs can be activated via IgE receptor (FcεRI) cross-linking, resulting in degranulation of the MCs, but they can also be activated via other pathways. These pathways include activation via the C3a and C5a receptors resulting in degranulation, secretion of cytokines, chemokines and bioactive lipids [8, 9].
C1q is the first complement component in the classical pathway of the complement system [10]. Together with C1r and C1s it forms the C1 complex. C1q plays a prominent role in the clearance of immune complexes and by binding to apoptotic and necrotic cells and it can facilitate phagocytosis [11-13]. The interaction of C1q with immunoglobulins is of importance to protect against infections but it may also

contribute to tissue damage by targeting self-tissue [14].
The main producers of C1q have been reported to be macrophages and immature dendritic cells [15-18]. Also other studies have reported that other non-macrophage-like cells are able to produce C1q like trophoblast cells [19]. MCs are differentiated from the same myeloid precursor cells that can also give rise to macrophages and dendritic cells [20]. We have investigated whether also MCs can produce and secrete functionally active C1q as this would imply MCs to have a role in tissue homeostasis and innate immune defence via C1q.

Materials & Methods

MCs culture

CD34+ hematopoietic stem cells were isolated from either peripheral blood mononuclear cells or from mononuclear cells present in cord blood. Mononuclear cells were isolated from buffy coats (Sanquin, The Netherlands) or cord blood using Ficoll-Paque density gradient centrifugation. CD34+ hematopoietic stem cells were isolated from the PBMC's with CD34+ microbeads (Miltenyi Biotec, The Netherlands). The isolated CD34+ stem cells were differentiated into MCs by culturing the cells in culture medium as described before [21]. Heparinized cord blood was obtained through the department of Obstetrics of the Leiden University Medical Center (Leiden, The Netherlands), in accordance with the Declaration of Helsinki. CD34+ cells isolated from cord blood were cultured as described previously [22] [23]. After 8-9weeks the purity of the MCs was determined using Flow Cytometry, whereby CD117 (c-kit), FcεRI and CD203c were measured.

ELISA

MCs were cultured in a cell concentration of 2*106 cells/mL for 72h either in medium alone, or stimulated to increase C1q levels with LPS (Sigma, 200 ng/ml), Dexamethasone (Pharmacy LUMC, Leiden, The Netherlands, 10 μM) + IFN-γ (Peprotech, 200 U/mL). For FcεRI triggering, the MCs were sensitized with hybridoma IgE (non-immune, BPD-DIA-HE1-1, Enzo, Life Sciences, 0.1 μg/ml) overnight. Next, the MCs were washed to remove soluble IgE and activated using goat anti-human IgE (Nordic-MUbio, Susteren, The Netherlands, 10 μg/ml). Subsequently, culture supernatants were harvested and analysed by ELISA to determine C1q levels as described before [24].
IL-8 production was evaluated using the human IL-8 ready-set-Go!® (2nd generation) ELISA (eBioscience).

Haemolytic reconstitution assay

MCs were cultured for 72 with or without LPS (200 ng/ml). After 72h, The supernatant was harvested and used for a haemolytic reconstitution assay. Antibody opsonized sheep erythrocytes were prepared in sucrose containing veronal buffer and incubated with C1q-depleted serum (Quidel) diluted in veronal gelatin containing buffer and different dilutions of MC supernatant or different concentrations purified C1q (Quidel) as a positive control and as a negative control culture medium with or without LPS was used. After incubation the amount of lysis was measured compared to total lysis (sheep erythrocytes together with saponine and water) at 412 nm. The amount of lysis is corrected to medium control [25].

qPCR of C1qA, C1qB and C1qC

RNA was isolated from cultures mast cells after 7-8 weeks of cultured. The qPCR was performed as described previously [24]. Relative mRNA expression was calculated using the reference gene RPL5. The qPCR products were analyzed on a 2% agarose gel and visualized with Nancy 520 (Sigma).

Sequencing

RNA was isolated from cord blood derived mast cells (CBMC) from three independent donors. RNA was paired-end sequenced with Illumina Hiseq 2000, aligned to Hg38 human genome reference. Read were quantified per gene and RPKM (Reads Per Kilobase per Million) levels were calculated using EdgeR (as previously described Suurmond et al) [26].

Western blot analysis of C1q

Using western blot the composition of C1q was examined by detection of the three chains of the C1q protein. Supernatants of stimulated and unstimulated MCs were used in reduced and non-reduced SDS conditions. The western blot was performed using previously described methods [24].

ß-hexosaminidase release assay

In this assay the MCs were sensitized with IgE as described above in a cell concentration of 0.5 x 106 cells/ml in an 96-wells plate on 37°C/ 5% CO2. The next day, the cells were washed with Tyrode's buffer to clear unbound IgE and stimulated with mouse anti-human IgE or with compound 48/80 (Sigma, 50 µg/ml) for 15 minutes or with buffer only as a control. Triton-X100 (1%) was used as indicator of 100% degranulation. After incubation the supernatant was incubated at 37°C with substrate (2 mM 4-Nitrophenyl N-acetyl-b-D-glucosaminide, Sigma N9376). The reaction was stopped using stop solution (0.2 M Glycine, pH 10.7) after 1-hour incubation and extinction was measured at 405 nm.

Immunofluorescence staining on cytospins and human tissue

For the detection of C1q positive MCs in tissues, paraffin embedded skin tissue, psoriatic skin and synovial tissue of rheumatoid arthritis (RA) patients (5µm) was used. After treating the slides via standard methods to deparaffinise, tryptase and C1q were visualized using mouse anti-tryptase (Millipore) and rabbit anti-C1q (DAKO) or matching isotypes for 1 hour at RT. After washing the detection antibodies goat anti-rabbit ALEXA488 (Invitrogen) and donkey anti-mouse ALEXA568 (Invitrogen) were added to the slides for one hour at RT. After incubation the slides were washed and dried. Finally, the slides were covered with Vectashield containing DAPI (Vector laboratories) to stain nuclei and analysed on a Zeiss Axio ScopeA1 microscope and on a Confocal microscope (Leica SP8 confocal). Using cytospins, cultured MCs were pelleted onto poly-lysine coated microscope slides (Thermo scientific/Menzel-Glaser) in a cell concentration of 50.000 cells/slide and stained for immunofluorescence with minor adjustments. All participants in the study provided informed consent and the study was approved by the local medical ethics committee.

Stained sections were randomly analysed of three different slides per condition. The mean number and standard deviation in percentages of single- and double-positive cells in 10 high-power fields (magnification 400x) was scored blindly by three observers.

Statistical analysis

Statistical analysis on the ELISA data was performed using a Wilcoxon signed rank test. P-value's <0.05 were considered significant (Graphpad Prism software version 5.01).

Results

Cultured MCs spontaneously produce functionally active C1q

Mast cells were in-vitro differentiated from CD34-positive precursors. After 7-8 weeks of culture, the purity of the MCs population was determined by staining for CD117 (c-kit), FcεRI and CD203c, and was shown to range from 90% to 98% (Figure 1A). The expression of the mRNA's encoding the three C1q chains by unstimulated MCs was analysed by PCR. We could detect the expression of C1QA, C1QB and C1QC, all required to generate a functional C1q molecule (Figure 1B). With qPCR we analysed expression level of C1qA in PBMCs and MCs. Here we observe that PBMCs have a higher RNA expression level of C1qA compared to MCs (Figure 1C).

The expression of mRNA was confirmed using sequencing RNA extracted from CBMC. The RPKM values indicate that the expression of C1q genes in CBMCs are relatively high with an RPKM value >100 and only a 3 fold lower expression than the household gene RPL5. These data provide additional and independent evidence that at basal levels C1q is expressed by MCs (Figure 1D). Additionally, expression of C1q related genes were analysed on unstimulated CBMCs using RNA sequencing. This analysis demonstrates that MCs express several C1q receptors (Table 1). The C1q associated serine proteases C1r and C1s and also the inhibitor C1-INH show a low expression in CBMCs (Table 1).

To demonstrate that MCs are able to produce C1q, we performed a sandwich-ELISA for C1q using culture supernatant obtained from MCs cultures. In a time frame of 72 hours unstimulated MCs are able to secrete C1q as C1q was readily detectable in culture supernatants; mean 11.34 ng/ml; range 3.08-24.6 ng/ml (Figure 1E). To further confirm that mast cells produce C1q and to rule out the possibility that a few contaminating cells would be responsible for the C1q mRNA and C1q protein observed, we next performed a cytospin of cultured cells and analysed the presence of intracellular C1q. As depicted in figure 1E, all cells stained positive for tryptase (Figure 1F). In addition also a staining for C1q revealed that all cells stained positive for C1q compared to isotype controls (Figure 1F). This indicates that not contaminating cells but rather MCs are responsible for C1q production. Using western blot analysis we observed that C1q secreted from unstimulated MCs derived from either peripheral blood and cord blood contain all three C1q-polypeptides. These polypeptides display the same size as compared to C1q present in NHS (C1qA: 28 kDa, C1qB: 25 kDa, C1qC: 24 kDa). Likewise, the size of the secreted C1q protein is identical to C1q from NHS (Figure 1G). These results indicated that MCs are able to produce and secrete a complete C1q protein.

Next, we wished to examine whether C1q secreted by MCs is functionally active and is able to restore the activity of the classical pathway in C1q-depleted serum. Therefore we used a reconstitution experiment based on a haemolytic assay for the classical pathway. Antibody opsonized sheep erythrocytes were incubated with C1q depleted serum to which we added either buffer, unconditioned culture medium or culture medium in which mast cells had been cultured for three days. Whereas incubation with C1q depleted serum did not result in lysis of erythrocytes a limited degree of lysis was observed when culture supernatant of unstimulated mast cells was used. A substantial lysis was observed when culture supernatant of mast cells, stimulated with LPS to increase the C1q production, was used (Figure 1H). Overall these data demonstrate that C1q produced by mast cells is functionally active.

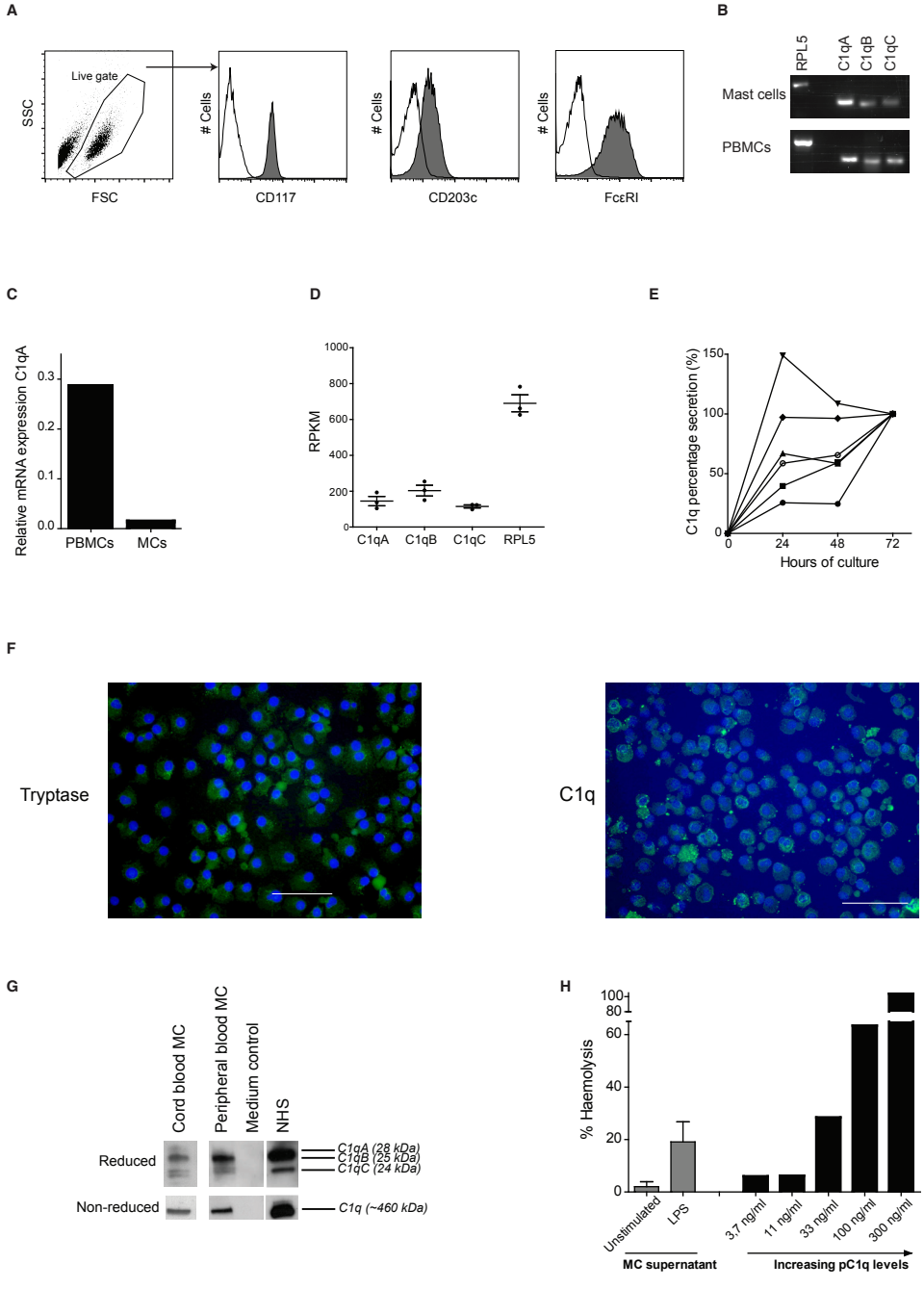

Figure 1. Production of C1q by cultured MCs.

A. Representative flow cytometric plots of characterization of MCs after 8-9 weeks of culture using CD117, CD203c and FcεRI staining on the cell surface. Histograms gated on live cells on forward scatter (FSC) and side scatter (SSC) Open histograms represents isotype controls, closed histograms represents the stainings. **B.** qPCR products from unstimulated MCs were analysed on a 2% agarose gel.

As positive control for C1qA, C1qB and C1qC DXM + IFN-γ stimulated PBMCs were used. RPL5 is used as housekeeping gene. **C.** Relative mRNA expression of C1qA in PBMCs and MCs. Data is relative to the house keeping gene RPL5 (N=1). **D.** Gene expression profile of C1q from three independent cord-blood derived mast cell donors. X axis depicts the three genes of C1q as well as RPL5. On the Y-axis, gene expression levels in reads per kilobase per million (RPKM) are depicted. **E.** C1q production during a time course of 72h (set at 100%) was analysed by ELISA (N = 6 different MCs donors). **F.** Cytospin staining of tryptase and DAPI together with matching isotype control of C1q (Rabbit Ig, ALEXA 568) on cultured peripheral MCs (400x magnification) and a of C1q and DAPI with matching isotype control of tryptase (Ms IgG1, ALEXA 568) on cytospins (400x magnification). Scale bars indicates 50 μm. **G.** Reduced and non-reduced supernatant of cultured MCs from peripheral blood and from cord blood was analysed on western blot. **H.** Haemolytic reconstitution assay of the classical pathway by adding MC supernatant to C1q depleted serum with the % heamolysis compared to a 100% lysis control as read-out (N=3) expressed as mean and standard deviation. As a positive control purified C1q ranging from 3,7 ng/ml to 300 ng/ml was used.

Gene	Conventional name	Mean RPKM	±SD
C1QA	C1qA	137.5	39.4
C1QB	C1qB	210.5	45.1
C1qC	C1qC	104.8	23.8
C1R	C1r	1.5	0.6
C1S	C1s	5	0.8
SERPING1	C1-INH	1.8	1
C1QBP	gC1qR	89.3	38.8
CALR	cC1qR	484	190.8
ITGB1	CD29	63.8	13.2
ITGA2	CD49B	4.5	1.7
LAIR1	CD305	15.5	7

Table 1. Gene expression levels of complement components in unstimulated CBMCs expressed in Reads Per Kilobase per Million (RPKM)

C1q production is upregulated after MC stimulation but does not correlate with IL-8 production or degranulation

We next wished to determine whether stimulation of MCs by triggers other than LPS could also alter C1q production by MCs. For this purpose, we stimulated MCs with LPS, DXM + IFN-γ or α-IgE for 72 hours. DXM in combination with IFN-γ are well-known stimulations to increase the C1q production in myeloid cells as described before for THP-1 derived macrophages [27]. All triggers enhanced C1q production as detected in culture supernatants harvested after 72 hours of stimulation (Figure 2A-C). Similar results were obtained using cord blood derived MCs (Figure 2D). However, IL-8 production, a prototype chemokine released by MCs was not increased upon triggering of MCs with DXM + IFN-γ (Figure 2E), indicating that C1q and IL-8 production are differentially regulated upon this stimulation.

MCs are well-known for their ability to release granules, containing different compounds e.g. ß-hexosaminidase, tryptase, chymase and histamine [28].

Therefore we were interested whether C1q is released after MC degranulation. To this end, we performed a degranulation assay, by stimulating the cells via the FcεRI or compound 48/80, two well-known triggers for degranulation [29, 30]. As a marker of degranulation we measured the release of ß-hexosaminidase next to C1q. Following FcεRI triggering for 15 minutes, ß-hexosaminidase was readily detected in the supernatants, whereas only a low level of C1q was present. (Figure 2F). However, after stimulation with compound 48/80 for 15 minutes a different pattern was noted as a secretion of 40% of C1q is seen (Figure 2G). These observations suggest that C1q is present in preformed vesicles of MCs and is released upon compound 48/80 triggering.

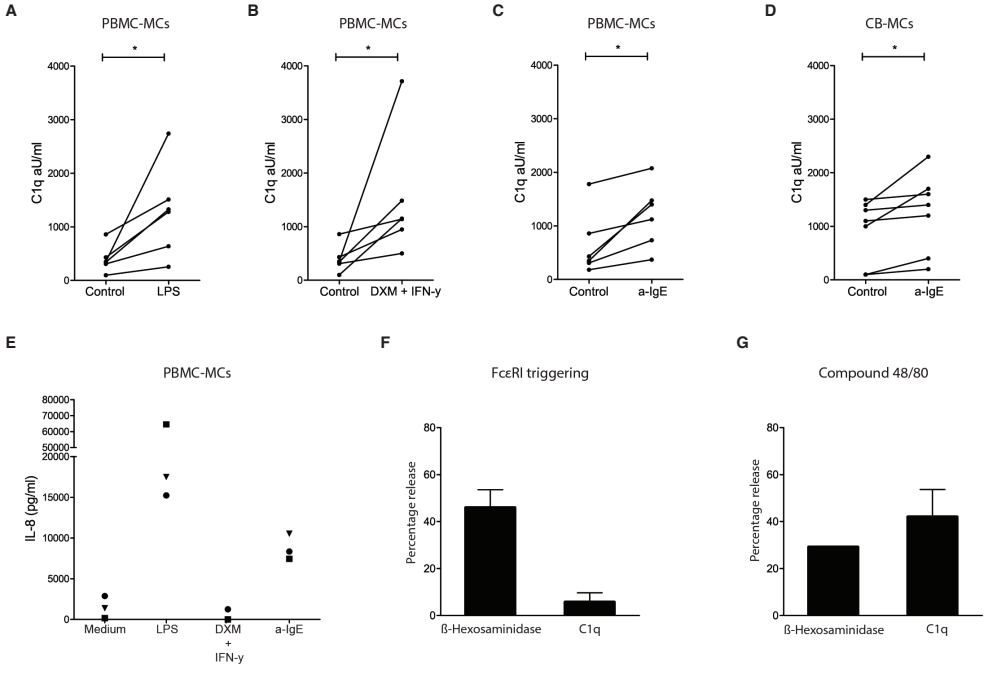

Figure 2. C1q production upon stimulation of MCs.

A. C1q production measured by ELISA in the supernatant of stimulated peripheral MCs for 72 h with LPS. **B.** DXM + IFN-γ. **C.** α-IgE. **D.** Stimulation of cord blood derived MCs with α-IgE. **E.** IL-8 ELISA on supernatant of peripheral MCs (N=3) after stimulation with LPS, DXM+IFN-γ and α-IgE. **F.** Percentages ß-hexosaminidase and C1q release assay after 15 min incubation of α-IgE defined by total release with Triton X100. **G.** Percentages ß-hexosaminidase and C1q release after 15 minutes of stimulation with compound 48/80 defined by total release with Triton X100.

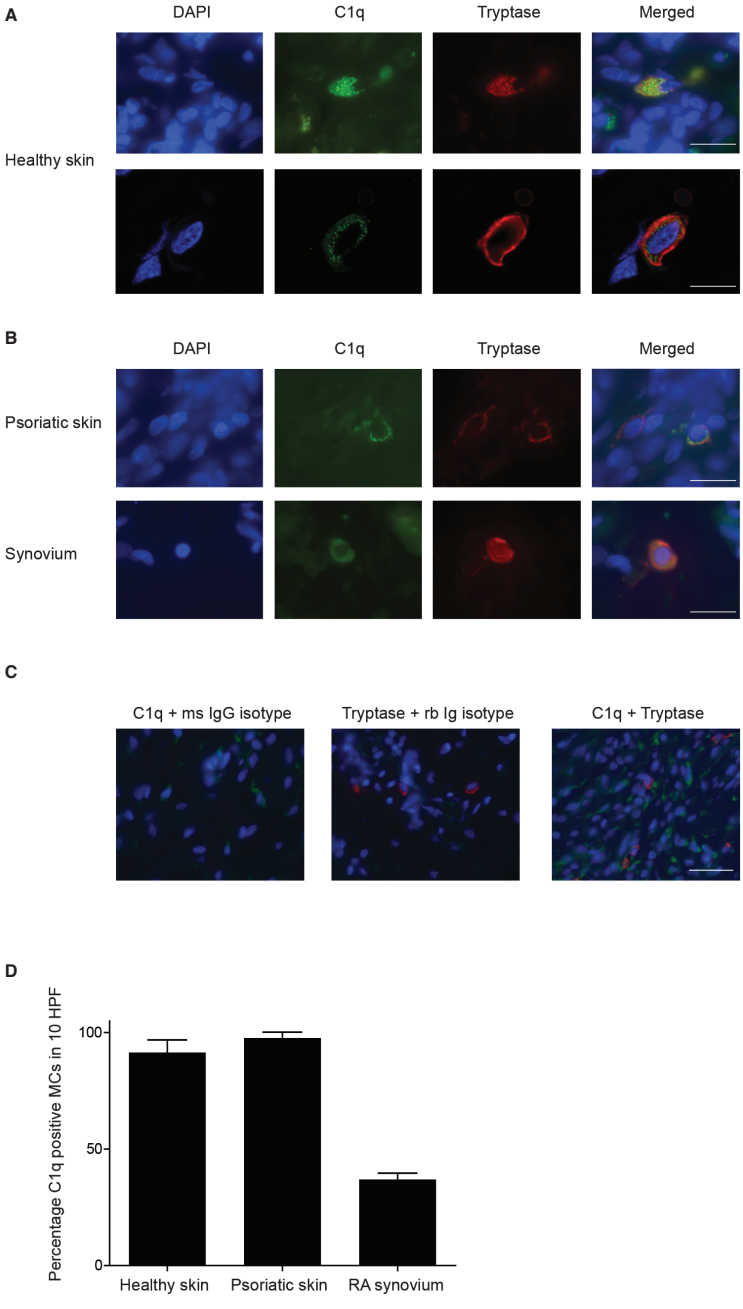

Figure 3. Distribution of C1q positive MCs in different tissue.

A. C1q and tryptase staining on healthy skin analysed on a conventional fluorescence microscope (upper images, 1000x magnification, scale bar is 10 µm) and on a confocal microscope (lower images, digital zoom 3000x magnification, scale bar is 3,3 µm). **B.** C1q and tryptase staining on psoriatic skin and on RA synovium (conventional microscope, 1000x magnification, scale bar is 10 µm). **C.** single staining C1q (green) with isotype control for tryptase (red), single staining tryptase (red) with isotype

control for C1q (green), double staining C1q (green), tryptase (red) on RA synovium. Conventional fluorescence microscope, 400x magnification. Scale bar is 50 µm **D.** Quantified percentages in 10 high power fields (HPF) of C1q positive mast cells in healthy skin (N=3), psoriatic skin (N=3) and RA synovium (N=3) expressed in mean and standard deviation.

C1q producing MCs are present in various tissues

ToW determine whether MCs present in tissue are positive for C1q we performed an immunofluoresence staining on healthy skin tissue using tryptase as a MC marker. As shown in figure 3A, tryptase positive MCs also express C1q as detected by immunofluorescence (Figure 3A), indicating that also tissue resident MCs express C1q (C1q positive MCs 91% ± 5%).

MCs have been indicated to play a role in several diseases. We wished to analyse whether MCs in diseased tissue express C1q. To this end, psoriatic skin and synovial tissue from RA patients was analysed for the presence of C1q positive MCs. As shown in figure 3B, all tryptase expressing cells in psoriatic skin are also positive for C1q (C1q positive MCs 98% ± 2%), resembling our observations made in the healthy skin. Likewise, in synovial tissue, tryptase positive MCs expressing C1q were present. However, in contrast to healthy and psoriatic skin, not all tryptase positive cells expressed C1q (C1q positive MCs 37% ± 3%). Moreover, also tryptase negative cells expressing C1q are observed in RA synovial tissue, indicating the presence of other C1q positive cells like macrophages and dendritic cells (Figure 3C).

As shown in figure 2G C1q is released via degranulation. As shown in Figure 3A-C, C1q is stained in a granular pattern in all C1q positive mast cells in different tissues. Together, these results indicate that tissue-resident mast cells, analysed directly ex vivo in healthy skin, psoriatic skin and synovial tissue, stain positive for C1q, and in particular in the skin, are the main cell C1q-positive cell subset.

Discussion

Macrophages and immature dendritic cells have been described as the main producers of C1q [15, 16]. Because MCs are derived from myeloid precursors cells, which also give rise to macrophages and dendritic cells, we wished to determine if MCs are able to produce C1q. Here, we demonstrate that unstimulated MCs produce and secrete functional C1q. Moreover, our data show that C1q production by mast cells can be modulated by several immune stimuli. The amount of C1q produced by mast cells (mean 11.34 ng/ml; range 3.08-24.6 ng/ml) is lower compared to published values of macrophages (64.6 ng/mL; range, 2-148 ng/

mL, after 48h culture) and dendritic cells (318.4 ng/mL; range, 100-679 ng/mL, after 48h culture) [15] and also as compared to our own analysis (data not shown). To investigate if the secretion of C1q is differently regulated from secretion of cytokines produced by MCs, we compared IL-8 secretion, a prototype cytokine produced by MCs, with C1q secretion after stimulation. We observed that stimulation of MCs by LPS and FcεRI cross-linking induced both C1q- and IL-8 release whereas stimulation by DXM + IFN-γ induced only C1q secretion. These results are intriguing as they suggest that C1q and IL-8 production are differentially regulated and that C1q production is also boosted in situations that MCs are not primed for IL-8 production. It is conceivable that this is important in the case of, for example, apoptotic cell clearance. C1q can bind to apoptotic cells and promote ingestion of apoptotic cells by macrophages [12, 13, 31]. The clearance of apoptotic cells is of particular importance in tissues, where apoptosis is abundant because of normal tissue turnover/homeostasis such as in the skin. In this process, infiltration of inflammatory cells would not be desirable. MCs producing C1q can contribute to this process to maintain tissue homeostasis by the production of C1q without the concomitant production of inflammatory cytokines. We did not observe a clear effect of C1q stimulating MCs (data not shown).

Another possible role of C1q is promoting wound healing. As shown in mouse studies, C1q can be deposited on endothelial cells, leading to the enhancement of permeability, tube formation and angiogenesis [32]. It is conceivable that also under these conditions, the release of inflammatory cytokines is not beneficial and that C1q produced by MCs contributes to angiogenesis and normal tissue repair in situations where the production of inflammatory cytokines is less desired.

In our ELISA analysis we observed a spontaneous production of C1q by unstimulated MCs. Likewise, in human tissue we observed a granular type of staining of C1q in MCs suggesting that C1q is also present in the granules of the MCs. Indeed, degranulation using compound 48/80 led to an increase in C1q release, which coincided with the release of ß-hexosaminidase in supernatants suggesting that C1q can also be produced upon granule release. Furthermore, we did not obtain any evidence that C1q is expressed on the cell surface of MCs (data not shown).

In the immunofluorescence staining of C1q positive mast cells is a granular pattern seen. In addition of production of C1q by mast cells, another feature what we have to take into account is the possibility of the uptake of C1q from the extracellular milieu and store them in granules [33].

A limitation of this study is the lack of an in vivo model to define the role of C1q produced by MCs in healthy and disease conditions as mouse MCs do not appear to produce C1q. Using an ELISA, we could not detect C1q in the supernatant of

bone marrow derived mouse MCs after the same stimulation as used for the human MCs. In the same assay, we did detect C1q in the supernatant of bone marrow derived macrophages and dendritic cells from the mouse (data not shown). It is therefore difficult to translate our results using human cells and tissue in an animal model for further studies aiming to analyse in vivo relevance.
Together, we show that MCs are able to produce and secrete functionally active C1q and that MCs in healthy and in diseased human tissue are positive for C1q. Our findings suggest that C1q from MCs could have a previously unrecognized contribution in several processes such as inflammation, clearing of immune complexes, apoptotic and necrotic cells. Especially in human skin, tryptase positive cells are the most abundant C1q expressing cells which makes it tempting to speculate that C1q produced by MCs is playing an important role in tissue homeostasis and defence.

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