

# Expression of membrane C1q in human monocyte-derived macrophages is developmentally regulated and enhanced by interferon- $\gamma$

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**Abstract** The present study investigated when during 'in vitro' maturation macrophages (M $\Phi$ ) express membrane C1q (mC1q), and whether cell activation affects expression and function of mC1q. Although C1q mRNA was repeatedly detected in freshly isolated monocytes using reverse transcriptase-polymerase chain reaction, C1q protein was observed only in developing M $\Phi$  from day 1 to 4 on using immunodetection and flow cytometry. However, the quantity of mC1q and other M $\Phi$  membrane proteins differed strikingly in cells from different donors. We report here for the first time that CD14<sup>+</sup> and CD14<sup>−</sup> mC1q-bearing M $\Phi$  can develop, and that interferon- $\gamma$  increases mC1q display at the cell surface, and mC1q-mediated phagocytosis. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Monocyte/macrophage; Differentiation; Complement; C1q; Phagocytosis

## 1. Introduction

Cells of the monocytic lineage are distributed throughout organisms and exhibit a high degree of heterogeneity, particularly in their mature forms, such as macrophages (M $\Phi$ ) and dendritic cells [1–3]. Monocyte-derived cells, in particular M $\Phi$ , appear to be the principal site of biosynthesis of the collagen-like complement protein C1q [4–7]. M $\Phi$  release a soluble form of C1q that has been found to be identical to its serum counterpart which in turn constitutes the activator-recognizing part of the first complement component, C1 [5,8–10]. C1q is a glycoprotein of about 460 kDa comprising 18 polypeptide chains, six each of which are from three different gene products [11]. The molecular structure and the biological function(s) of soluble C1q have been described in detail elsewhere [4,12–16].

Previously, we have identified C1q as a presumptive integral membrane protein (mC1q) of M $\Phi$  that (i) mediates endocytosis and (ii) is released from the extracellular side of the

plasma membrane to generate the soluble form [5,10]. Therefore, mC1q itself shares properties with other well established cell surface markers of M $\Phi$ , such as CD14 and CD16 of which soluble forms also exist [17–19].

The purpose of the present study was to detect when the expression of mC1q protein in developing M $\Phi$  ensues and to investigate whether or not activation of the cells influences expression and function of mC1q. Therefore we analyzed monocytes/M $\Phi$  isolated from human blood throughout in vitro differentiation for C1q synthesis at both the mRNA and protein level. C1q mRNA was often, but C1q protein never detected in freshly isolated monocytes using reverse transcriptase-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) or Western blot, respectively. Considerable C1q protein synthesis and membrane expression occurred in cultured monocytes/M $\Phi$ . Flow cytometry demonstrated that the amount of mC1q on the cell surface varied strikingly in cells from different donors.

For comparison with mC1q, we monitored the cell surface expression of two additional well established M $\Phi$  markers, namely CD14 and HLA-DR. CD14 is a glycosyl-phosphatidylinositol (GPI)-anchored M $\Phi$  membrane glycoprotein of 55 kDa. It serves as a receptor for a complex composed of lipopolysaccharide (LPS) and LPS-binding protein [17,20]. In addition, CD14 has been reported to mediate the phagocytosis of infectious agents, such as *Mycobacterium tuberculosis* [21], and apoptotic cells [22]. Furthermore, it is released from the cell membrane by enzymatic cleavage of the GPI anchor or its protein domain [17]. HLA-DR is a heterodimeric major histocompatibility complex (MHC) class II molecule and plays a crucial role in antigen presentation. Its plasma membrane expression in monocytic cells is generally upregulated under the influence of activating inflammatory cytokines, such as interferon (IFN)- $\gamma$  [23].

In the course of our investigation we observed previously unknown CD14-positive and CD14-negative subpopulations of mC1q-bearing M $\Phi$ . Furthermore, we demonstrate for the first time that activation of M $\Phi$  by IFN- $\gamma$  significantly enhances expression of mC1q on the cell surface and mC1q-mediated phagocytosis.

## 2. Materials and methods

### 2.1. Reagents

All chemicals, if not stated otherwise, were obtained in the highest quality from Merck, Darmstadt, Germany. Acrylamide, bisacrylamide, 5-bromo-4-chloro-indolyl-phosphate, bovine serum albumin (BSA), FITC, glycine, nitroblue tetrazolium, PMSF, SDS, SDS 7B

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**Abbreviations:** ABS, human AB serum; mC1q, membrane C1q; MFI, mean fluorescence intensity; rMFI, relative MFI; RT-PCR, reverse transcriptase-polymerase chain reaction

molecular mass marker kit, Tris-base and Tween 20 were purchased from Sigma Chemie GmbH, Deisenhofen, Germany.

## 2.2. Cells

The preparation of human monocytes and monocyte-derived MΦ was performed as previously described [5] with minor modifications. In brief, adherent monocytes were cultured for 8 days or indicated time periods in RPMI 1640 containing 25 mM HEPES, 2 mM glutamine (RPMI/HEPES; all Gibco BRL) and 10% either of human AB serum (ABS) or fetal calf serum (FCS) (PAA Laboratories GmbH, Coelbe, Germany). In some experiments on the fifth day in culture, the medium including serum was exchanged for serum-free CG medium (Vitromex GmbH, Vilshofen, Germany) with or without indicated amounts of recombinant human IFN-γ (Genzyme Corporation, Cambridge, MA, USA).

## 2.3. RNA isolation, cDNA synthesis and RT-PCR

RNA from freshly isolated or cultured monocytes/MΦ was isolated according to the method of Chomczynski and Sacchi [24]. The final RNA concentration was estimated by spectrophotometry. First-strand cDNA synthesis was accomplished as previously described [25] using a Bioblock heater (Barnstead/Thermolyne, Dubuque, IA, USA). Table 1 lists the primers used for PCR in this study for amplification of specific cDNA templates, the applied annealing temperatures, the size of the respective PCR product, the restriction endonucleases used for specific cleavage, and the size of the cleavage products. The forward and reverse primers for amplification of each C1q chain react with sequence regions in the first and second exon, respectively. Therefore, these primers differentiate between PCR products originating from cDNA and genomic DNA. Amplification products of the latter are about 1300, 1500 and more than 2000 bp in size for A-, B-, and C-chains, respectively. Each reaction mixture included in a total volume of 50 µl 40.5 µl H<sub>2</sub>O, 5 µl 10× PCR buffer (500 mM KCl, 100 mM Tris, 15 mM MgCl<sub>2</sub>, 0.1% gelatin, pH 8.3), 1 µl dNTP (10 mM each; Appligene Oncor, Heidelberg, Germany), 1 µl of each primer (20 pmol final concentration; custom-built by Gibco BRL), 1 µl cDNA (equivalent to 300 ng of total RNA), 0.5 µl Taq polymerase (5 U/ml). The integrity of each cDNA sample was assessed by a PCR employing primers specific for human β-actin under the following conditions: initial denaturation at 94°C for 3 min followed by 33 cycles of (i) 45 s at 94°C for denaturation, (ii) 45 s at 60°C allowing annealing, (iii) 1 min at 72°C for extension and, at the end, another 5 min at 72°C. For amplification of the C1q A-, B-, and C-chains the same temperature profile was applied except that the annealing temperatures were changed according to the values listed in Table 1 and 35 cycles were performed. In each PCR run, genomic DNA (prepared as described previously [26]) and cDNA of 8 day old MΦ served as a positive control. Reaction mixtures lacking either cDNA or one of the primers provided the negative controls. A Hybaid Omnigene TR3 SM1 thermocycler (MWG Biotech, Ebersheim, Germany) was employed for all amplifications. The PCR mixtures were run on ethidium bromide-stained agarose gels. In order to check the specificity of the

PCR, amplification products of representative samples were extracted from the agarose gel, precipitated with ethanol and digested with one of the endonucleases listed in Table 1 (all purchased from New England Biolabs GmbH, Schwalbach, Germany) according to the supplier's instructions.

## 2.4. Preparation of cell lysates

1–2×10<sup>7</sup> cells were lysed in 1 ml phosphate-buffered saline containing 1 mM PMSF, 5 mM iodoacetamide, 5 mM EDTA, 10 mM sodium azide and 0.5% NP40 for 30 min at 4°C as described previously [5].

## 2.5. Affinity adsorption and immunoprecipitation

MΦ lysates were sequentially incubated with immobilized BSA (10 mg/ml; BSA-S4B) and anti-huC1q monoclonal antibody (mAb) 242G3 (0.5 mg/ml; murine anti-huC1q-S4B) coupled to CNBr-activated Sepharose (S4B; Pharmacia Biotech AB, Uppsala, Sweden). Precipitated proteins were eluted with SDS sample buffer [27], analyzed by SDS-PAGE and immunoblotting as detailed earlier [5,10].

## 2.6. Quantitation of C1q

The quantity of MΦ C1q in cell lysates was assessed in a sandwich ELISA. Anti-huC1q mAb 242G3 was used for detection and a goat anti-huC1q IgG (prepared in our laboratory according to standard procedures) served as the capture antibody (Ab) [28]. Serial dilutions of purified human serum C1q in cell lysis buffer provided the standard and buffer controls without C1q were included in each assay.

## 2.7. Flow cytometry

Flow cytometric analysis of cell surface molecules and phagocytosis was accomplished as previously reported [5]. The analyzed proteins and the employed mAbs were: mC1q: 242G3 (murIgG1; [29]), CD14: 12C3 (murIgM; Boehringer Mannheim, Mannheim, Germany), HLA-DR: L243 (murIgG2a; ATCC, Rockville, MD, USA). The respective isotype controls served: murIgG1: MOPC-21, murIgG2a: UPC-10, murIgM: MOPC-104E (all Sigma). The incubation step with the secondary Ab, a FITC-conjugated goat anti-murIgG/IgM F(ab')<sub>2</sub> fragment (Dianova, Hamburg, Germany), was omitted, if the primary Ab was conjugated to R-phycoerythrin. When cells of a donor were analyzed two or three times at a given day for reproducibility reasons, very similar results were obtained (data not shown).

For additive fluorescence measurements of C1q and CD14, cells were in a first step incubated with the primary mAb directed against one membrane molecule (anti-C1q or anti-CD14) or the respective isotype control (murIgG1 or murIgM). In a second step, the cells received the primary mAb recognizing the second antigen (anti-CD14 or anti-C1q) or its respective isotype control (murIgM or murIgG1) subsequent to one washing step. Therefore, the following samples were obtained: MΦ incubated with (i) murIgG1 and murIgM, (ii) anti-C1q and murIgM, (iii) murIgG1 and anti-CD14, and (iv) anti-C1q and anti-CD14. The mAbs are given in the order of use. In all experiments a set of samples was prepared with the inverse sequence

Table 1  
RT-PCR of C1q A-, B-, and C-chains

Sample	Primer		Annealing temperature <sup>a</sup> (°C)	Size of PCR product (bp)	Specificity check <sup>b</sup>	
		Sequence 5' → 3'			Enzyme	Products (bp)
β-Actin	for.	ATTTCGGTGGACGATGGAGGGGC	60	920		ND
	rev.	GGCATCGTCACCACTGGGACGAC				
C1q A	for.	ATGGTGACCGAGGACTTGTG	62	276	<i>Bsa</i> WI	158+119
	rev.	GTCTTGATGTTTCCTGGGC				
C1q B	for.	ACCCAGGGATAAAGGAGAG	60	216		
	rev. (1)	GGCAGAGAAGGCGATTTTCTG				
	rev. (2)	AGGTCAGGCCTCCATATCTG	60	602	<i>Pst</i> I <i>Xho</i> I	511+91 364+238
C1q C	for.	AAGGATGGGTACGACGGACTG	65	213	<i>Sma</i> I <i>Bcl</i> II	153+59 391+271
	rev. (1)	TTTCTGCTGTATCTGCCCTC				
	rev. (2)	GGTGGTCTTAGCAGAAGCTGAGGAGGTGGAAGGC <sup>c</sup>	65	663		

<sup>a</sup>Annealing temperature used in the PCR reaction.

<sup>b</sup>The PCR products were cut off the agarose gel, extracted, precipitated with ethanol and finally resuspended in the reaction buffer of the respective enzyme.

<sup>c</sup>This primer contains 35 nucleotides.

of mAb application. Bound mAbs were detected with the FITC-labeled F(ab')<sub>2</sub> fragment mentioned above. The respective equivalent samples differed on average by 1.6% with respect to the percentage of positive cells.

For a phagocytosis assay, each  $5 \times 10^4$  MΦ were incubated with FITC-labeled Pansorbin<sup>®</sup> bacteria (fixed *Staphylococcus aureus*; Psb<sup>FITC</sup>) which had been coated before with the anti-huC1q mAb 242G3 or murIgG1 as an isotype control (20, 30, 40 or 50 μg per  $1 \times 10^8$  Psb<sup>FITC</sup>) in a total volume of 200 μl for 30 min at 37°C. Cells were then chilled on ice and washed three times with ice-cold buffer prior to immediate analysis by flow cytometry. To estimate the amount of ingested bacteria, trypan blue solution was added to a final concentration of 1 mg/ml to quench the fluorescence of adherent extracellular bacteria [5].

Flow cytometry analysis was performed using an EPICS Profile II, the EPICS Profile and ELITE software packages (versions 2.26 and 3.0, respectively; Coulter Electronics GmbH, Krefeld, Germany). MΦ were gated according to their characteristic forward and sideward light scattering properties [30]. The fluorescence data are expressed in relative mean fluorescence intensity (rMFI) which was calculated according to Te Velde et al. [31]:

rMFI =

$$\frac{\text{MFI of M}\Phi \text{ incubated with specific mAb (or coated Psb}^{\text{FITC}}\text{)}}{\text{MFI of M}\Phi \text{ incubated with isotype control mAb (or coated ctl Psb}^{\text{FITC}}\text{)}}$$

### 3. Results

#### 3.1. Expression of C1q in monocytes/MΦ throughout in vitro differentiation

Monocytes were isolated from blood of healthy donors and used either directly or following culture for indicated periods of time. In order to obtain sufficient amounts of RNA and/or cellular protein from all samples collected, cells of two to four

donors were processed in parallel and pooled immediately prior to RNA isolation or protein solubilization.

**3.1.1. RT-PCR of C1q A-, B-, and C-chains.** RT-PCR of the three chains of C1q and β-actin was performed as mentioned in Section 2 and the results are shown in Fig. 1. Whereas mRNA of all three C1q chains was always detected in cultured monocytes/MΦ, freshly isolated monocytes displayed only two out of three times (six samples) detectable amounts of the respective transcripts (Fig. 1, compare lanes 1 and 9). For B- and C-chains two different reverse primers were employed, each of which induced a reaction product of the expected, specific size (Table 1). Fig. 1 presents the RT-PCR products obtained with the reverse primer '1'. Substitution of genomic DNA for cDNA induced for each C1q chain a product of greater size, indicating the presence of the respective intron. Enzymatic digestion of the RT-PCR bands yielded the calculated cleavage products listed in Table 1, and provided additional evidence of specificity (data not shown).

**3.1.2. Determination by ELISA and immunoprecipitation of MΦ C1q in cell lysates.** Cell lysates of freshly isolated monocytes or cultured monocytes/MΦ were investigated either employing an ELISA system for quantitative estimation of C1q or by immunoprecipitation and immunoblotting to visualize MΦ C1q. Fig. 2A shows that the quantity of cellular C1q mostly increased throughout the course of culture but also considerably varied between lysates obtained from different cell preparations and thus donor pools. Furthermore, C1q was first detected in cultured monocytes/MΦ on day 1, 2 or 3 by use of the ELISA (Fig. 2A, lysates 5 and 6, 4, 2 and 3, respectively). The MΦ C1q displayed in Fig. 2B was precipitated from the respective samples of 'cell lysate 1' depicted in Fig. 2A. The immunoblot shows that C1q is present in the

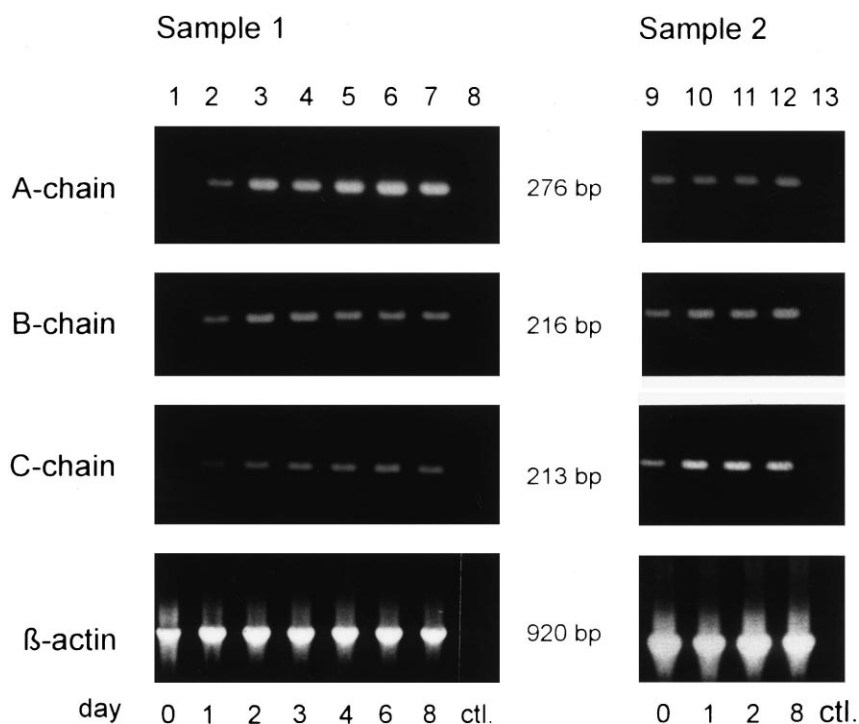


Fig. 1. RT-PCR of C1q A-, B-, and C-chains. Images of ethidium bromide-stained agarose gels displaying RT-PCR products of the indicated C1q chain (A–C) and β-actin. The sizes of the RT-PCR products are indicated (compare Table 1). In case of B- and C-chains, products are shown that were obtained using the respective reverse primer '1'. RNA was prepared of freshly isolated and 1–8 days cultured monocytes/MΦ and RT-PCR accomplished as described in Section 2. Samples shown in tracks 1–7 and 9–12 represent pools of cells obtained from four and two donors, respectively. Tracks 8 and 13 represent reaction mixtures in which cDNA was omitted.

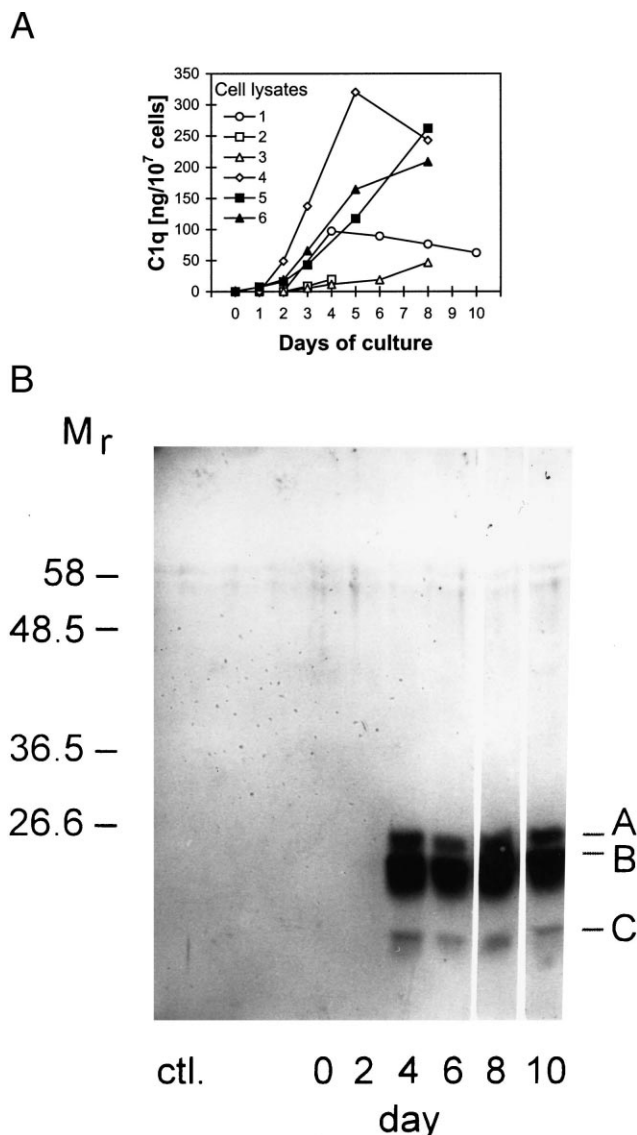


Fig. 2. Antigenic detection of MΦ C1q by ELISA (A) and immunoprecipitation and immunoblotting (B). Lysates were prepared of freshly isolated (day 0) and 1 and up to 10 days cultured monocytes/MΦ and subsequently subjected to analysis by either ELISA or immunoprecipitation and blotting as detailed in Section 2. Amounts of C1q per 10<sup>7</sup> cells depicted in (A) represent cell pools prepared from monocytes/MΦ of four (lysates 1–3), three (lysates 5 and 6), and two (lysate 4) donors. MΦ C1q displayed in (B) was precipitated from lysate 1. C1q A-, B-, and C-chains were separated by SDS-PAGE (12.5/1.2% acrylamide/bisacrylamide) prior to immunoblotting and are indicated on the right-hand side. 'Ctl' indicates a control sample eluted with SDS sample buffer from an aliquot of the immobilized anti-huC1q mAb that was not incubated with a cell lysate.

cells from day 4 onwards, but not immediately after separation from blood and, in this case, on day 2 of culture. The results obtained using the ELISA and the combination of immunoprecipitation with Western blotting confirm each other.

### 3.2. Flow cytometric analysis of monocyte/MΦ cell surface markers

In order to investigate the development and the phenotype(s) of the C1q-synthesizing MΦ population derived from

a single individual, monocytic cells of different donors were separately analyzed throughout in vitro culture by flow cytometry for cell surface expression of C1q and CD14. Fig. 3 shows that mC1q is hardly, if at all, discernible in freshly isolated monocytes (day 0; donors 1–3, 5 and 6 about 2% and donor 4 5.6% positive cells) but clearly occurs in cultured cells (donors 1–11). A major peak of mC1q appearance on the cell surface was observed on day 8 of culture (donors 1–3 and 7–11). However, monocytes/MΦ of some donors remained virtually negative for mC1q throughout day 2 and displayed only a weakly positive signal even on day 8 of culture (donors 5 and 6).

Cell surface expression of CD14 was always strong at the beginning and, as observed in cells of donors 1, 2, 4 and 5, for up to day 10 throughout in vitro culture. Sometimes however, the percentage of CD14-positive monocytes/MΦ clearly decreased in the course of the culture (donors 3 and 6).

Monocytes develop into MΦ when cultured for about 8 days in the presence of serum, which can be either FCS or ABS [32,33]. Therefore, we investigated MΦ following culture in the presence of ABS or FCS for the appearance of mC1q, CD14, and HLA-DR on their cell surface. HLA-DR was analyzed as an additional MΦ marker, as an increase of its expression indicates activation [23]. Fig. 4 summarizes the results and shows that the expression of all three plasma membrane molecules varies considerably between MΦ from different donors ( $n = 27$ ). The diversity in the cell surface phenotype is demonstrated by the difference between the percentage of positive cells and the rMFI as a measure of the molecule number on the cell surface. When monocytes/MΦ were cultured in parallel in media containing ABS (MΦ<sup>ABS</sup>) or FCS (MΦ<sup>FCS</sup>), the expression of mC1q and CD14 exhibited, on average, no significant differences between the respective cell populations. In contrast, the expression of HLA-DR was significantly higher in MΦ<sup>ABS</sup> than in MΦ<sup>FCS</sup> both in terms of

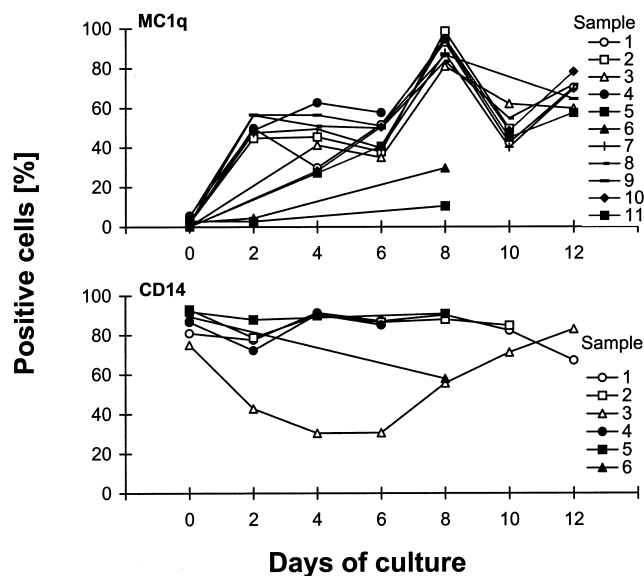


Fig. 3. Flow cytometric analysis of cell surface expression of mC1q and CD14 (top to bottom) in monocytes/MΦ throughout in vitro culture/differentiation. Each curve in a graph represents cell samples of an individual blood donation (1–11) assessed at the indicated time. Identical donor numbers in both graphs indicate cells originating from the same individual.

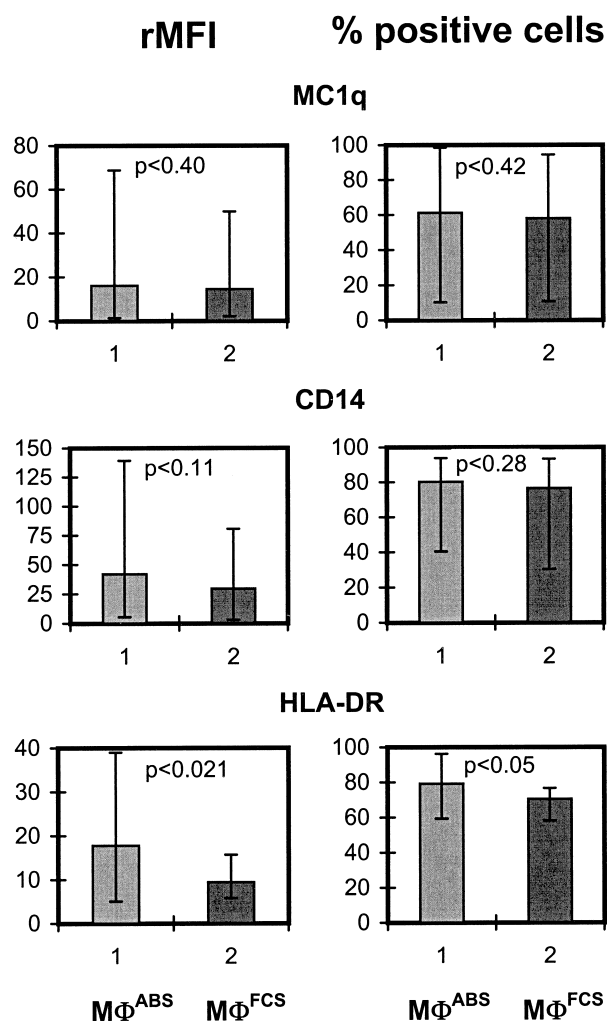


Fig. 4. Flow cytometric analysis of cell surface antigens of  $M\Phi$  on day 8 of culture. Cells obtained from 27 healthy donors were cultured in medium containing ABS ( $M\Phi^{ABS}$ ) or FCS ( $M\Phi^{FCS}$ ). Averages of rMFIs and percentages of positive cells are given. Note that the bars in the columns indicate the range (highest and lowest) of the respective values observed for a given antigen. A two samples *t*-test assuming an unidirectional hypothesis was performed for statistical analysis.

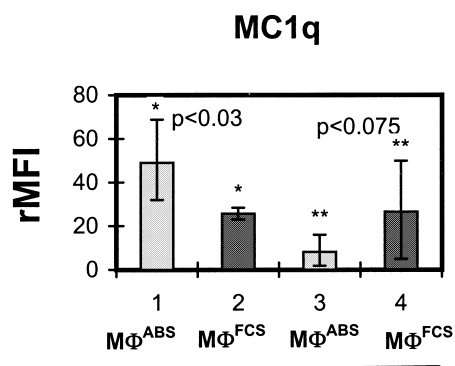


Fig. 5. Flow cytometric assessment of mC1q in matched pairs of  $M\Phi^{ABS}$  and  $M\Phi^{FCS}$  from eight donors. The rMFIs  $\pm$  S.D. are given. Statistical analysis applied a paired two samples *t*-test assuming an unidirectional hypothesis. MC1q expression of  $M\Phi^{ABS}$  was analyzed in comparison to  $M\Phi^{FCS}$  in both groups ( $n=4$ , respectively).

positive cells and antigen density (rMFI). However, when pairs of  $M\Phi^{ABS}$  and  $M\Phi^{FCS}$  of eight donors were investigated, we found that in 50% of the matched samples the level of mC1q expression was significantly higher in  $M\Phi^{ABS}$  compared to  $M\Phi^{FCS}$ . The opposite occurred in the remaining 50% (Fig. 5). Interestingly, the mean rMFI values for mC1q of  $M\Phi^{FCS}$  were almost identical in both groups. Although, the variability was much higher in the group in which the  $M\Phi^{ABS}$  expressed less mC1q compared with their  $M\Phi^{FCS}$  counterparts, and the difference did not reach statistical significance.

In the cells of the majority of the donors the percentage of CD14-positive  $M\Phi$  was greater than that of C1q-positive cells (19 of 27 samples, Fig. 4). However, in several cases the opposite was observed (eight of 27 samples shown in Fig. 4), suggesting the existence of a C1q-positive but CD14-negative

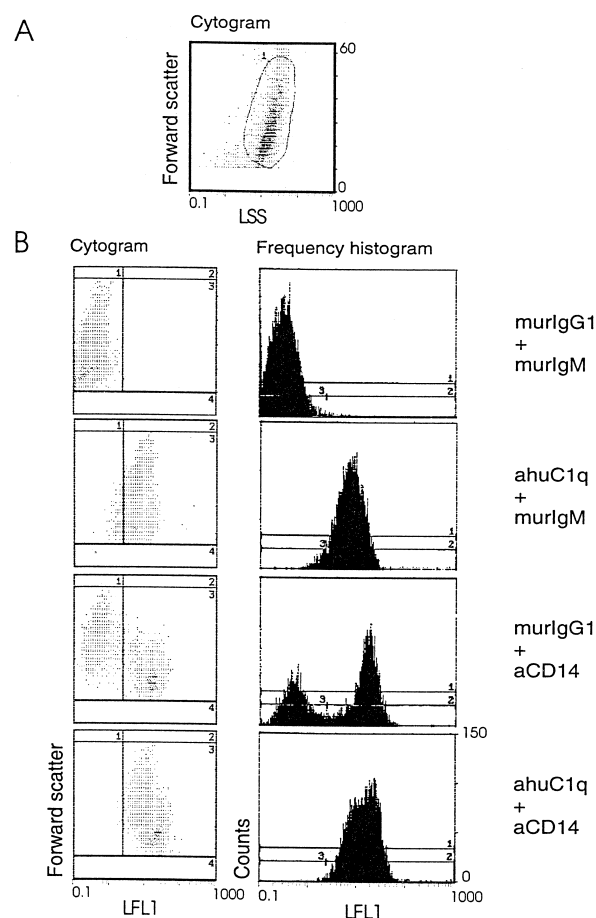


Fig. 6. Assessment of CD14-positive and CD14-negative mC1q-bearing  $M\Phi$  subpopulations by flow cytometry. LSS: logarithmic side scatter; LFL1: logarithmic fluorescence 1 (FITC). (A) Gating of monocyte-derived  $M\Phi$  on day 8 of culture based on forward and side scatter properties (cells shown here were cultured in medium containing FCS). (B) Additive fluorescence measurement was performed after sequential incubations of the cells with the two specific Abs (anti-huC1q mAb and anti-CD14 mAb) or the respective isotype controls (murIgG1 and murIgM) or combinations of these as indicated on the right. The order in which the mAbs are given represents the sequence of application before the final staining with the FITC-labeled secondary Ab. The cytograms in the left column display cell size and fluorescence. Note the difference in size of the CD14-negative and CD14-positive cells (second cytogram from the bottom) and the shift in fluorescence intensity of the CD14-negative cells when stained with the anti-huC1q mAb (bottom). One representative out of nine samples is shown.

Table 2  
Percentages of MC1q<sup>+</sup>/CD14<sup>−</sup> MΦ: additive fluorescence measurement

Donor	MC1q <sup>+</sup> /CD14 <sup>−</sup> MΦ (%) Cell culture type <sup>a</sup>	
	MΦ <sup>ABS</sup>	MΦ <sup>FCS</sup>
1	<b>25.0</b>	<b>36.9</b>
2	<b>15.8</b>	<b>20.0</b>
3	<b>1.5</b>	<b>15.6</b>
4	ND	11.3
5	14.6	2.4

Additive fluorescence measurement was performed as described in Section 2.

Within the bold samples, the difference of the percentage of MC1q<sup>+</sup>/CD14<sup>−</sup> cells in MΦ<sup>ABS</sup> and MΦ<sup>FCS</sup> is statistically significant ( $P < 0.04$ , paired two samples for means *t*-test).

<sup>a</sup>Cells obtained from a donor were cultured in parallel in medium containing ABS (MΦ<sup>ABS</sup>) or FCS (MΦ<sup>FCS</sup>).

**MΦ subpopulation.** Assessment of CD14-positive and CD14-negative mC1q-bearing MΦ was performed in additional samples by additive fluorescence measurement. Fig. 6 shows a representative example. Fig. 6A displays both the forward and logarithmic side scatter properties of a typical MΦ population on day 8 of culture. Fig. 6B demonstrates that a subpopulation of these MΦ staining negative for CD14 (see cytogram and frequency histogram labeled: murIgG1+aCD14) shifts into the gate of positive cells, if incubated with the anti-huC1q mAb. Table 2 summarizes the assessment of the CD14-negative and mC1q-positive MΦ subpopulation in nine samples derived from five donors. In three out of four pairs of MΦ<sup>ABS</sup> and MΦ<sup>FCS</sup>, the number of C1q-positive/CD14-negative MΦ was significantly higher in the MΦ<sup>ABS</sup>. In terms of percentage, the CD14-negative subpopulation was always smaller than the CD14-positive counterpart, whereas with respect to cell size, the opposite was observed (Fig. 6B). When expressed in arbitrary forward scatter units (FSU), the CD14-positive and CD14-negative MΦ had an average size of  $24 \pm 3$  and  $39 \pm 3.1$ , respectively. The whole MΦ population displayed an average size of  $27.8 \pm 1.5$  (FSU  $\pm$  S.D. from six determinations).

### 3.3. Influence of IFN- $\gamma$ on mC1q and mC1q-dependent phagocytosis

Since mC1q-bearing MΦ originating from different donors exhibited a striking diversity, we wondered whether or not this could be a consequence of activation and thus, if cytokine stimulation of the cells affects mC1q expression. Monocytes/MΦ were cultured for 8 days but between day 5 and 8 were in

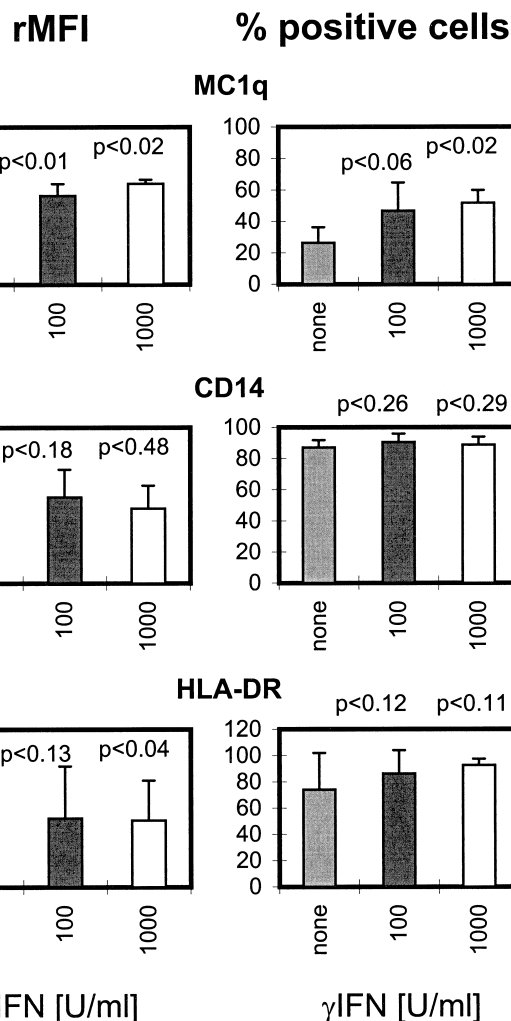


Fig. 7. Influence of IFN- $\gamma$  on the expression of mC1q, CD14 and HLA-DR in MΦ. Results of the flow cytometric analysis on day 8 of culture. Cells were cultured in the presence of ABS until day 5. The culture in the presence or absence of IFN- $\gamma$  was performed in serum-free CG medium from day 5 to 8.

the presence or absence of the indicated amounts of recombinant human IFN- $\gamma$ . Subsequently, the cells were assayed by flow cytometry for the cell surface expression of mC1q, CD14 and HLA-DR and for their ability to perform mC1q-dependent phagocytosis (Fig. 7 and Table 3). Expression of MHC class II molecules generally increased when the MΦ received the stimulus. However, a significantly higher antigen density

Table 3  
Flow cytometric assessment of mC1q-dependent phagocytosis by MΦ: enhancing effect of IFN- $\gamma$

Cell culture		rMFI ( $\pm$ S.D.)	
Type	Additive	Adherent and ingested coated Psb <sup>FITC</sup> <sup>a</sup>	Ingested coated Psb <sup>FITC</sup> <sup>b</sup>
MΦ <sup>ABS/CG</sup>	none	2.25 ( $\pm 0.83$ )*	2.40 ( $\pm 1.19$ )**
	IFN- $\gamma$	3.29 ( $\pm 0.81$ )* $P < 0.009$ * ( $n = 9$ )	3.14 ( $\pm 0.60$ )** $P < 0.04$ ** ( $n = 9$ )

Culture in the presence or absence of IFN- $\gamma$  (1000 U/ml) was performed in CG medium from day 5 to 8 (MΦ<sup>ABS/CG</sup>). The phagocytosis assay was performed as described in Section 2 and the rMFI is given.

\*, \*\*: A paired two samples for means *t*-test was employed for statistical analysis of the differences of the rMFI values observed for IFN- $\gamma$ -stimulated and untreated cells (nine pairs).

<sup>a</sup>Adherent, extracellular and ingested, coated Psb<sup>FITC</sup> (Psb<sup>FITC</sup>- $\alpha$ C1q mAb versus Psb<sup>FITC</sup>-murIgG1).

<sup>b</sup>Ingested, coated Psb<sup>FITC</sup>, fluorescence of extracellular adherent, coated Psb<sup>FITC</sup> quenched with trypan blue solution (final concentration 1 mg/ml).

(represented by rMFI) occurred only under treatment with the highest concentration of IFN- $\gamma$  (1000 U/ml). Notably, although the mean percentage of HLA-DR-positive cells increased, the all over change was not significant. However, Fig. 7 shows that the expression of mC1q always increased under the influence of IFN- $\gamma$ . The improvement of the antigen density as indicated by the rMFI was always statistically significant, whereas the increase of the percentage of positive cells was only significant if the cells had been treated with 1000 U/ml of IFN- $\gamma$ .

In contrast to mC1q and HLA-DR protein, the expression of CD14 on the plasma membrane remained basically unchanged by IFN- $\gamma$ .

The analysis of mC1q-mediated phagocytosis of IFN- $\gamma$ -treated and untreated cells is summarized in Table 3. The results demonstrate that the fluorescence signals evoked by the adherent and ingested FITC-labeled Pansorbin® bacteria (Psb<sup>FITC</sup>) and the ingested material alone are 2.25 and 2.40 times higher, respectively, if the Psb<sup>FITC</sup> had been coated with the anti-huC1q mAb that links the particles to mC1q. The fluorescence signals for this specifically mC1q-dependent adherence and uptake increased significantly, when the M $\Phi$  were stimulated with IFN- $\gamma$  ( $P < 0.009$  for adherent and ingested Psb<sup>FITC</sup>,  $P < 0.04$  for ingested particles,  $n = 9$  sample pairs; Table 3).

#### 4. Discussion

Previously, we have shown that the collagen-like C1q molecule is a presumably integral membrane protein of M $\Phi$  [5,10]. The apparent lack of mC1q on monocytes, the precursors of M $\Phi$ , suggested that mC1q may be a marker of maturing M $\Phi$  [5]. In the present study, we investigated the expression of M $\Phi$  C1q/mC1q on the mRNA and protein level throughout in vitro differentiation of M $\Phi$ . Formation of a functional C1q molecule appears to depend critically on the production of three mRNA species and the simultaneous occurrence of the respective protein chains as concluded from known C1q-deficiencies [26,34]. Therefore, we designed RT-PCRs for detection of mRNA of all three C1q chains. Interestingly, mRNA of C1q A-, B-, and C-chains was not only detected in cultured cells but also in two out of three fresh monocyte preparations (Fig. 1). In contrast, C1q protein was never detected in lysates of freshly isolated monocytes by ELISA and immunoprecipitation combined with Western blotting. This result is in accordance with previous studies performed in our laboratory [5,10,35] and by other investigators [36]. However, detectable expression of C1q protein/mC1q ensued in 1–4 day old cultured monocyte-derived M $\Phi$ . This suggests that the three mRNAs for C1q could be transcribed in advance of the onset of C1q protein synthesis.

Flow cytometry allows to study the expression of proteins on the single-cell level and was used to investigate separately mC1q expression in cells of individual donors.

This approach revealed in six of 11 cases some 2% C1q-positive cells within the freshly isolated monocytes (Fig. 3; MCIq, day 0). In the present study, all experiments were performed in buffers resembling physiological conditions, and we and others have shown that monocytes show no specific C1q-binding activity in buffers of physiological ionic strength [35,36]. Indeed, addition of soluble C1q to fresh monocyte preparations did not increase the population of

C1q-positive cells (data not shown, but see [35]). Therefore, the low number of C1q-positive cells could be explained by the presence of some ‘contaminating’ cells, such as granulocytes, which, in contrast to monocytes, bind serum C1q [35,37]. However, it is also possible that some mature M $\Phi$  which other investigators have reported to be present in the circulation [38] had been co-purified with monocytes. C1q originating from a small number of mature M $\Phi$  or C1q-binding granulocytes would probably not be detected by ELISA or immunoblotting due to its low concentration. However, a low number of mC1q producing circulating M $\Phi$  could account for the C1q mRNA detected by RT-PCR in freshly isolated monocytes.

Assessment of mC1q in cell lysates and flow cytometry demonstrated that the expression occurred and increased throughout M $\Phi$  maturation with striking differences between cells from different donors (Figs. 2 and 3). Under our experimental conditions, the quantity of cellular C1q was the highest at days 4 or 5 (Fig. 2: lysates 1 and 4) or increased throughout culture until termination of the experiment (Fig. 2: lysates 2, 3, 5 and 6, respectively). The percentage of M $\Phi$  exhibiting mC1q on the cell surface was maximal on day 8 of culture in eight out of 11 samples (Fig. 3). An earlier report by Kaplan and Gaudernack [32] that most cultured monocytes develop within 8 days into mature M $\Phi$  may explain our observation: at day 8 of culture most cells may have reached the stage of mature M $\Phi$  that express mC1q.

Interestingly, human ABS, when compared to FCS, appeared to exert an either enhancing or suppressing effect on mC1q expression in cells from different donors (Fig. 5). This suggests that human serum components indeed regulate C1q production. However, the reason(s) for this diverging reaction of monocytes to human serum which imply a predisposition of the donor remain to be elucidated.

Taken together, our results demonstrate that synthesis of mC1q in monocyte-derived M $\Phi$  is developmentally regulated. Therefore, mC1q shares features with other cell surface molecules of M $\Phi$ , such as CD16 [39].

In the case of CD14, the cells maintained a strong cell surface expression or displayed a reduction in the course of the culture (Figs. 3 and 4), both of which are in good accordance with reports of other groups (reviewed in [17]). However, closer analysis of M $\Phi$  populations by additive fluorescence measurement showed for the first time the existence of distinct CD14-positive and CD14-negative mC1q-expressing M $\Phi$  subpopulations. The two cell populations differ in relative numbers and morphological measures: CD14-negative cells are usually smaller in terms of counts but greater in terms of cell size. Interestingly, these subpopulations appeared only in cells from some donors, but if so then in both M $\Phi$ <sup>ABS</sup> and M $\Phi$ <sup>FCS</sup>, and without the addition of any exogenous stimulus. This suggests that occurrence of CD14-positive or CD14-negative mC1q-expressing M $\Phi$  is determined independently of the culture media, but rather by a predisposition of the donor. At presence the factor(s) which drive the development of the subpopulations remain to be elucidated and the physiological function of the observed M $\Phi$  subpopulations will be subject of future investigations.

Monocytes develop into M $\Phi$  regardless of the presence of ABS or FCS in the culture medium [32]. However, differences occurred in the present study between M $\Phi$ <sup>ABS</sup> and M $\Phi$ <sup>FCS</sup> not only in the percentage of positive cells and the amount of

mC1q (see above), but also with respect to HLA-DR molecules residing on the membrane (Fig. 4). The relatively higher expression of the MHC class II molecules in  $M\Phi^{ABS}$  could be due to the presence of stimulating factors in ABS which FCS might lack and/or species differences.

We also wondered whether or not activation of the  $M\Phi$  influences the appearance and function of mC1q. IFN- $\gamma$  is a stimulus of  $M\Phi$  which has been reported to increase the expression of MHC class II molecules [23]. Interestingly, a significant enhancement of HLA-DR expression was only detected when we applied 1000 U/ml of IFN- $\gamma$  (Fig. 7). Since  $M\Phi^{ABS}$  showed a strong and significantly higher MHC class II molecule expression than  $M\Phi^{FCS}$  (Fig. 4) even without addition of IFN- $\gamma$ , a further massive increase in the number of positive cells could not be expected. But a significant improvement of the antigen density on the cell surface also required a high dose of IFN- $\gamma$ . However, our results demonstrate for the first time that activation of  $M\Phi$  influences significantly the expression and function of mC1q as IFN- $\gamma$  increased the expression of mC1q (Fig. 7) and enhanced the mC1q-mediated phagocytosis (Table 3). Our finding and the observation by others that C1q-deficient mice show a reduced capacity to produce IFN- $\gamma$  [40] suggest that C1q and IFN- $\gamma$  are engaged in a mutual immunoregulatory mechanism. In contrast, expression of CD14 was not significantly affected by IFN- $\gamma$ . This and the  $CD14^{-}/mC1q^{+}$   $M\Phi$  indicate that CD14 is independently regulated from mC1q.

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