CR3 is the dominant phagocytotic complement receptor on human dendritic cells

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Abbreviations:

ADCC: antibody dependent cellular cytotoxicity

APC: antigen presenting cell

C3d(g): C3 d(g) fragment of C3

iC3b: inactivated complement C3b fragment

CR1: complement receptor type1

CR2: complement receptor type 2

CR3: complement receptor type 3

CR4: complement receptor type 4

CRIg: complement receptor of the Ig superfamily

DC: dendritic cell

HI: heat inactivated

imDC: immature dendritic cell

imMDC: immature monocyte-derived dendritic cell

maMDC: mature monocyte-derived dendritic cell

MDC: monocyte-derived dendritic cell

Abstract

Dendritic cells (DCs) play a decisive role in immunity; they interact with various pathogens via several pattern recognition and different opsonophagocytotic receptors, including Fc- and complement-receptors. β2-integrins, including complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) participate in many immunological processes, especially those involving cell migration, adherence, and phagocytosis. Human monocyte derived dendritic cells (MDCs) are known to express CR3 as well as CR4, however possible differences regarding the role of these receptors has not been addressed so far. Our aim was to explore whether there is a difference between the binding and uptake of various complement-opsonized microorganisms, mediated by CR3 and CR4. Studying the expression of receptors during differentiation of MDCs we found that the appearance of CD11b decreased, whereas that of CD11c increased. Interestingly, both receptors were present in the cell membrane in an active conformation. Here we demonstrate that ligation of CD11b directs MDCs to enhanced phagocytosis, while the maturation of the cells and their inflammatory cytokine production are not affected. Blocking CD11c alone did not change the uptake of opsonized yeast or bacteria by MDCs. We confirmed these results using siRNA; namely downregulation of CD11b blocked the phagocytosis of microbes while silencing CD11c had no effect on their uptake. Our data clearly demonstrate that complement C3-dependent phagocytosis of MDCs is mediated mainly by CR3.

Introduction

The complement system is a key element of an efficient immune response. Its activation leads to the cleavage of the central complement component, C3, generating C3a and different fragments fixed covalently to the activating surface - such as C3b, iC3b, C3c, and C3d(g). These activation products are the ligands of various complement receptors, namely C3aR, CR1, CR2, CR3, CR4 and CRIg, expressed by a wide variety of immune cells (Bajtay et al., 2006; Carrol, 1998; Erdei et al., 2009; Li et al., 2011; Liu and Niu, 2009). The most abundant complement receptors present on the surface of neutrophils, monocytes, macrophages, NK cells and dendritic cells (DCs) are CR3 (CD11b/CD18) and CR4 (CD11c/CD18). Both receptors are members of the β_2 integrin family, and the heterodimeric receptors consist of one α and one β subunit. Integrins mediate important cellular functions, like adhesion, especially during the formation of the immunological synapse, transendothelial migration of immune cells and interaction with the extracellular matrix. The natural ligand of CR3 and CR4 is the inactivated fragment of C3, namely iC3b (Rosen and Law, 1990; Ross and Vetvicka, 1993). One of the most important functions of these complement receptors is that they mediate phagocytosis, which results in the clearance of pathogens, apoptotic- and tumor cells. Integrin signalling is also exploited by microbial pathogens for entry into host cells (Bajtay et al., 2004; Dupuy and Caron, 2008; Oliva et al., 2008). Important functions of macrophages and DCs are the processing and presentation of antigens to initiate adaptive immunity by the activation of T lymphocytes. These professional antigen presenting cells express different phagocytic receptors including FcRs, scavenger receptors, C-type lectins, CRIg and integrins. Among these latter structures CR3 was the first to be demonstrated to mediate phagocytosis (Dupuy and Caron, 2008). In addition CR3 has also been shown to play a critical role in antibody dependent cellular cytotoxicity (ADCC) against various targets

including different tumors and parasites (Capron and Dessaint, 1985; Gelderman et al., 2004a; Gelderman et al., 2004b; van Spriel et al., 2001; van Spriel et al., 2003; Vignali et al., 1990).

Pathogen microbes entering the body become opsonized by complement proteins, mainly by the larger fragments of C3, which help eliminate antigens by the phagocytes, including immature dendritic cells (imDCs). CR3 and CR4 are generally thought to mediate overlapping functions; however the possible distinctive role of these receptors has not been investigated so far.

The aim of the present work is to reveal whether CR3 and CR4 mediate different functions on human monocyte-derived DCs, and to monitor if the expression and function of these complement receptors changes during DC maturation. Studying their functions on MDCs we clearly demonstrate that CR3 – but not CR4 - plays a key role in the phagocytosis of iC3b-opsonized particles. We found that CR3 expression is downregulated on MDCs during maturation, in contrast to CR4 which is significantly enhanced in the same time. Investigating the effect of the common ligand, iC3b and a CD11b specific antibody we found that the phenotype of MDCs did not change significantly after treatment, suggesting that CD11b/CD18 transduces signals which affect phagocytosis only.

Materials and Methods

Reagents and antibodies

For the isolation and culture of cells the following materials were used: Ficoll-Paque (Amersham, Uppsala, Sweden), CD14+ Microbeads from Miltenyi Biotec (Bergisch Gladbach, Germany), CellGro serum free DC Medium (CellGenix, Germany), recombinant human (rHu) IL-4 (R&D systems), rHu GM-CSF (R&D systems). LPS was purchased from Sigma (Hungary). Carboxy-fluorescein-succinimide-ester (CFSE) and FITC were obtained from Molecular Probes (Invitrogen, USA), saponine from Fluka (Switzerland), iC3b from Merck (Germany).

The following primary and secondary antibodies were used for FACS and confocal microscopic analysis: anti-hu CD4-A647 from SantaCruz (USA), FITC-labeled anti-biotin mAb from Sigma (Hungary), streptavidine-Alexa546, streptavidine-Alexa647, streptavidine-Alexa488, Alexa488 labeled goat-anti-mouse Ig from Molecular Probes (Invitrogen, USA). FITC-labeled anti-hu-MHCII, FITC-labeled anti-hu-CD83, FITC-labeled anti-hu-CD86, APC-labeled anti-hu CD14 mAb and biotynilated anti-hu CD11b from BD PharMingen (USA), PE conjugated mouse-anti-hu mannose receptor (mIgG1, 3.29B1.10) from Immunotech (France), biotinilated anti-hu CD40 from Serotec (UK), monoclonal anti-hu CD11b-RPE from Dako (Denmark), monoclonal anti-hu CD11c and anti-hu CD11c-A647 and mIgG1 from BioLegend (USA), anti-hu CD35-FITC from BD PharMingen (USA), anti-hu CD21-PE from Immunotools (Germany), anti-hu iC3b from Quidel (USA). Biotinilated anti-hu-CD11c and FcR blocking was purchased from Miltenyi Biotec (Germany). The CD11b ligand bindig site specific monoclonal antibody TMG6-5 (mIgG1) was kindly donated by István Andó, BRC, Szeged, Hungary

Generation of human MDCs

Monocytes were isolated from buffy coat obtained from healthy donors and provided by the Hungarian National Blood Transfusion Service by magnetic separation of the CD14+ monocytes from PBMC or by adherence to gelatin as previously described (Sandor et al., 2009). Informed consent was provided for the use of blood samples according to the Declaration of Helsinki. Cells were cultivated in CellGro serum-free medium supplemented with 100 ng/mL rHu GM-CSF and 15 ng/mL rHu IL-4.

On day five $CD14^-$ immature MDCs (imMDCs) were washed and treated with 50 µg/ml iC3b or with 10 µg/ml CD11b specific mAb TMG6-5, for 30 minutes at 37°C. As control an isotype-matched antibody was used. After incubation cells were washed extensively and cultured in fresh medium supplemented with cytokines for additional 2 days. For control samples imMDCs were left untreated. To generate maMDCs, cells were resuspended in serum-free medium containing 1 µg/ml LPS. Phenotypical (flow cytometry) and functional (MLR, cytokine measurement) studies were carried out at day seven.

Monitoring DC maturation

The maturation of MDCs was assessed by monitoring the expression of the following cell membrane molecules: MHCII, CD40 and mannose receptor (MR). To detect these markers cells were stained with the relevant antibodies and analyzed by cytofluorimetry.

Flow cytometry

For the phenotypic analyses, DCs were incubated with the indicated antibodies for 20 minutes at 4°C according to the manufacturer's instruction, and then washed twice in FACS buffer (PBS, 1% FCS, 0.1% Na-azide). Isotype-matched antibodies were used as control in each case. For intracellular staining cells were fixed in PBS containing 2% formaldehyde and permeabilized by 0.2% saponine in the same buffer. Intracellular staining was performed the

same way as described for extracellular staining. Samples were analysed using a FACSCalibur or FACSAriaIII flow cytometer employing CellQuestPro (Becton Dickinson) or FACS Diva software for data aquisition and FCS Express software for data analysis. Results are expressed as ΔMFI calculated as follows: $(MFI_{sample} - MFI_{isotype\ control}) / MFI_{isotype\ control}$.

Confocal laser scanning microscopy

Cells were treated with 50 μg/ml iC3b for 1 hour at 37°C, then fixed, permeabilized and stained with anti-iC3b antibody. For visualization a secondary anti-mouseAlexa488 antibody was applied. Double staining of cell bound iC3b and CD11b or CD11c was performed by using anti-iC3b followed by anti-mouseAlexa488, anti-CD11b-biotin followed by strepatvidineAlexa647 or anti-CD11c biotin followed by strepatvidineAlexa647, respectively. To follow internalization, CD11b was labeled using biotinylated anti-CD11b and streptavidine-Alexa546, while CD11c was visualized using anti-CD11c-Alexa647. After these staining steps iC3b opsonized Staphylococcus aureus was added to the cells for 1 hour. Labeling the receptors prior phagocytosis was necessary because the epitopes of CD11b and CD11c recognized by the antibodies are lost upon fixation. The antibodies were tested for interference with iC3b binding, and were found negative. Samples were analyzed by an Olympus IX81 confocal microscope applying Fluowiev500 confocal workstation.

MLR studies

MDCs treated as described above were transferred to 96 well plates at day seven and further cultured with allogeneic T cells at a DC:T cell ratio of 1:5. T lymphocytes were labeled with 0.5 μM CFSE prior to use. After 4 days of coculturing, the proliferation of T cells was assessed by analyzing CFSE staining of CD4-Alexa647 labelled cells by

cytofluorimetry. MFI+/- SD of the dividing cells is calculated from three independent experiments.

Cytokine measurements

The culture supernatants of MDCs were analyzed 24 hours after the various treatments. Measurements for IL-6 and TNF-α were carried out using the R&D duoset sandwich ELISA system, while IL-8 production was assesed using the BenderMedsystem Instant ELISA kit. Data are presented as mean +/- SD of three independent experiments.

Phagocytosis of opsonized yeast (Saccharomyces cerevisiae), Staphylococcus aureus and sheep red blood cells (SRBC)

Staphylococcus aureus bacteria were kindly provided by Department of Microbiology at Eötvös Loránd University. Yeast and bacteria were heat-killed and labelled with FITC according to the manufacturer's (Molecular probes) instructions. For testing the phagocytic capacity of imMDCs, yeast and Staphylococcus were opsonized with normal human serum for 1 hour at 37°C, then washed extensively and offered to the cells under different conditions. To generate S.aureus that is opsonized only with IgG but not complement, bacteria were incubated in heat inactivated (HI) serum for 1 hour at 37°C. In some control experiments non-opsonized yeast and bacteria were also used. In blocking experiments the anti-CD11b or anti-CD11c antibodies were used at 100 μg/ml concentration and the human FcR blocking Miltenyi reagent was employed according to the manufacturers instructions. ImMDCs were incubated with opsonized yeast particles at a DC:yeast ratio of 1:5 for 2 hours, or with Staphylococcus aureus at 200 μg/ml concentration for 2 hours in a CO_2 incubator. The amount of the ingested particles were determined by cytofluorimetric analysis using trypane blue for

quenching surface bound particles, or by determining the phagocytic index using an invertoscope. Data are presented as mean +/- SD of three independent experiments.

RNA silencing

RNA silencing was performed according to the method of Prechtel (Prechtel et al., 2007). We used commercially available predesigned Qiagen (Germany) AllStar Negative control siRNA and Qiagen Genome Wide predesigned siRNA for CD11c (Hs_ITGAX_6) and CD11b (Hs_ITGAM_5). Cells were transfected on day0 and day3 of differentiation with 20µg siRNA to generate CD11c silenced, CD11b silenced or negative control silenced MDCs at day5. The expression of CD11c and CD11b was analyzed on day5 by cytofluorimetry and subsequent experiments were carried out on the same day.

Internalization of cell-bound iC3b and CR3

To follow the fate of cell-bound iC3b-fragments, MDCs were incubated with 50 µg/ml iC3b for 30 minutes at 4°C. To test the integrins' activation state iC3b binding was also measured in the presence of 5mM Mg^{++} (Varga et al., 2007), which is known to induce the active conformational state (data not shown). After removing unbound iC3b by washing, the cells were kept in medium at 37°C in a CO_2 incubator for the indicated time periods. Similar treatment was applied using TMG6-5 antibody when the motion of CR3 was studied. MDCs were treated with the antibody at 10 µg/ml for 30 min on ice to prevent internalization, then washed and incubated at 37°C for the indicated time periods. After fixation, the cells were stained for residual surface-bound iC3b and CR3 and analyzed by flowcytometry.

Statistics

Student's t-test was performed with GraphPad software; $p<0.05$ was considered significant.

Results

1. Expression of CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) on human MDCs

Previously we demonstrated that MDCs – similarly to macrophages and B cells - are able to fix C3b fragments covalently on their cell surface which enhance the antigen presenting capacity of these cells (Kerekes et al., 1998; Papp et al., 2008; Sandor et al., 2009). Regarding the expression of complement receptors on DCs, so far only CR3 and CR4 have been investigated on MDCs (Kacani et al., 1998; Pickl et al., 1996), but not CR1 (CD35) and CR2 (CD21). Thus, we set out to analyze the expression of various complement receptors and compared the appearance of CR1, CR2, CR3 and CR4 on immature and mature MDCs. We found that imMDCs express very small amounts of CR1, which is completely downregulated upon maturation (Figure 1A). In contrast to this, we could detect no CR2 expression either on imMDCs or on maMDCs (Figure 1B). Analyzing CD11b and CD11c we found that both integrins are expressed constitutively on MDCs (Figure 1C and 1D). During LPS-induced maturation of the cells, CR3 was found slightly downregulated in contrast to CR4, which was significantly ($p=0,018$) upregulated (Figure1C, 1D, 1E). Although the absolute number of CR3 and CR4 showed high individual variability, the change in their expression during the differentiation was always significant (Figure 1E). As shown in the figure, the expression of CD11b decreases to 65%, whereas that of CD11c increases to more than 200% on maMDCs compared to imMDCs (Figure 1E).

2. Binding and uptake of iC3b and iC3b-opsonized particles by imMDCs

Integrins are known to require activation for ligand-binding (Abram and Lowell, 2009), therefore we tested the cells’ capacity to bind ligands via CR3 and CR4. ImMDCs

were incubated with iC3b, the natural ligand of both CR3 and CR4, at 4°C for 30 minutes, than binding was assessed using Alexa488 labeled antibody. As shown in Figure 2A, in the beginning of the measurement (i.e. at 0 min) we detected strong iC3b deposition on the surface of the cells. Since Mg^{++} is known to initiate the activation of integrins (Varga et al., 2007), we performed the above experiment on Mg^{++} pretreated MDCs as described in the section of Materials and Methods. We found that this treatment did not enhance the binding of the ligand, suggesting that both CR3 and CR4 are already in an active conformation in the cell membrane (data not shown).

Next we analyzed the fate of receptor bound iC3b. To this end the cells were treated with iC3b at 4°C followed by incubation at 37°C to allow internalization, as described in Materials and methods. Analyzing cell-bound iC3b after 2 hours we found a significant (p=0,0039) decrease of the ligand on the cell surface, which was further diminished by 48 hrs (Figure 2A). To investigate whether the cells internalize the ligand or shed it from the cell membrane, we analyzed the cells by confocal microscopy. Our data show that after 2 hours surface-bound iC3b is engulfed by the cells. The distribution of C3-fragments is not even neither on the cell membrane nor inside the cells; staining appears in small patches (Figure 2B). We also tested whether both CR3 and CR4 are able to bind the ligand. We treated the cells with iC3b at 4°C for 30 minutes and then detected cell-bound iC3b along with CD11b or CD11c. As shown in Figure 2C the ligand colocalizes with both CD11b and CD11c.

Since we observed a strong expression of both CR3 (CD11b/CD18) and CR4 (CD11c/CD18) by MDCs (Figure 1C and 1D.) we aimed to clarify the role of these receptors in the phagocytosis of iC3b opsonized particles. First we tested the binding and uptake of iC3b opsonized yeast (Saccharomyces cerevisiae) and iC3b opsonized bacteria (Staphylococcus aureus). These two types of microbes are known to fix C3-fragments on their surface mainly as iC3b (Turner et al., 1986). Figure 2D shows the phagocytosis of opsonized

yeast by imMDCs at 4°C and 37°C, and Figure 2E demonstrates the binding of iC3b opsonized Staphylococcus aureus to imMDCs under the same conditions.

3. CR3 has a dominant role over CR4 in the uptake of iC3b opsonized yeast and bacteria

Although it has not been clearly demonstrated so far, it is generally thought, that the functions of CR3 and CR4 are very similar, since both take part in the binding and uptake of iC3b opsonized particles. To clarify whether the role of these receptors is indeed identical, we analyzed the process of phagocytosis of iC3b opsonized yeast particles and Staphylococcus aureus using imMDCs pretreated with anti-CD11b and anti-CD11c antibody, respectively. Our results show, that treatment with anti-CD11b significantly decreased the phagocytic capacity of imMDC compared to the control sample, where isotype-matched antibody was used. This result was independent of the particle used, since the uptake of both the iC3b-opsonized yeast and bacteria was inhibited in a similar manner (Figure 3A, 3B). Interestingly, incubation with anti-CD11c did not affect the uptake of the opsonized microorganisms by imMDCs (Figure 3A, 3B), in contrast to anti-CD11b.

It is known that Fc receptors also mediate phagocytosis and work in cooperation with complement receptors. To test whether blocking CD11b affects the phagocytosis mediated by FcRs or pattern recognition receptors we also assessed the uptake of non-opsonized bacteria and bacteria treated by heat inactivated (HI) serum. In this latter case IgG may bind to the bacteria, thus FcR.mediated phagocytosis can take place As shown in Figure 3C only 12% of imMDCs phagocytosed non-opsonized bacteria and opsonization with HI serum enhanced the percentage of bacteria positive cells only slightly (up to 17%). In contrast to this, when S. aureus was opsonized by normal serum where complement activation and iC3b deposition occurs, 66% of the cells took up the bacteria (Figure 3C). Although these data show that the possible opsonization by IgG alone does not cause a strong enhancement, we analyzed

whether CD11b or CD11c plays a role in the uptake of non-opsonized or HI serum opsonized microorganisms. To this end we blocked the complement receptors by antibodies. Our results show that neither the possible pattern recognition receptor mediated uptake (Figure 3D) nor the IgG mediated phagocytosis (Figure 3E) is influenced by CD11b or CD11c. To further prove that under our experimental conditions the Fc receptors do not play a major role in the uptake of iC3b opsonized bacteria, we assessed phagocytosis in the presence or absence of FcR blocking, and found no difference (Figure 3F).

These results were further strengthened by our experiments where we employed CD11b and CD11c silenced MDCs. We treated MDCs during their he differentiation with CD11b or CD11c specific siRNA fragments and thus obtained imMDCs expressing significantly lower level of CR3 (CD11b, Figure 4A) or CR4(CD11c, Figure 4B) than the control siRNA treated cells. Silencing efficiency was approximately 70%. Using the CD11c and CD11b silenced cells for phagocytosis we confirmed our findings obtained by blocking the receptors with antibodies; namely CD11c downregulation had no effect on phagocytosis. (Figure 4C, 4D), while CD11b silenced MDCs exhibited significantly lower capacity to uptake the iC3b opsonized yeast (Figure 4E) or bacteria (Figure 4F). To exclude that CD11b silencing might change the general phagocytic capacity of the MDCs we also determined how the control and the CD11b silenced MDCs internalize non-opsonized yeast (Figure 4G) and bacteria (Figure 4H), and found no difference.

4. The fate of CR3 and CR4 during uptake of iC3b opsonized yeast and bacteria

To analyze the iC3b-mediated phagocytosis in more detail, and to determine the participation of CR3 and CR4 in the process, we monitored the route of iC3b opsonized Staphylococcus aureus by confocal microscopy. Figure 5A-5D shows the distribution of CD11b (red) and CD11c (blue) on imMDCs, when determined separately, in the absent of the

ligand. The yellow lines that crosses two adjacent cells are used as markers, along which the fluorescence intensity of the two dyes has been calculated and is represented in the histogram (Figure 5D). These data clearly demonstrate that the two integrins, CR3 and CR4 colocalize in the cell membrane of imMDCs. Figure 5E-5H shows the results of a similar measurement, when imMDCs were incubated with 200µg/ml iC3b opsonized Staphylococcus aureus after labeling of the receptors. In this case CD11b is almost completely transferred into the cytoplasm, thus it is absent from the cell membrane, whereas CD11c remains mainly there. This is clearly seen in Figure 5H where CD11b intensity is high only inside the cells and in the region of the cell membrane where two cells are in contact.

To confirm our data obtained by confocal microscopy we measured the cell surface expression of CD11b and CD11c before and after phagocytosis of opsonized S. aureus also by flowcytometry. We stained the cells for CD11b and CD11c in the absence of the ligand and also after iC3b opsonized bacteria were phagocytosed. Figure 5I shows that the expression of CD11b on the cell membrane is significantly reduced after phagocytosis, but that of CD11c is not, supporting our results obtained by confocal microscopy. The virtual contradiction between data on Figure 5E where CD11b is almost completely missing from the cell surface and data on Figure 5I where CD11b is present still in substantial amounts even after phagocytosis can be explained by the fact, that CD11b recycles very quickly between intracellular pools and the cell membrane (Bretscher, 1992). Therefore in cytofluorymetric measuerements we can only stain those receptors that come to the cell surface during phagocytosis (as seen in Figure 5I), while in the microscopic measurements CD11b molecules disappear, (Fig. 5E) since we can label them only prior to phagocytosis. These data suggest that although CD11c may also participate in the process of the complement-dependent phagocytosis (Figure 5H), its role is not essential.

To further analyze the function of CR3 (CD11b/CD18), we used the monoclonal antibody TMG6-5, which interacts specifically with the ligand binding site of CD11b. First, we analyzed whether the antibody behaves similarly to the natural ligand. We treated the cells with TMG6-5 at 10µg/ml, and followed its internalization by cytofluorimetry at the indicated periods of time. As shown in Figure 5J, CD11b molecules crosslinked by the TMG6-5 mAb were internalized similarly to the natural ligand. Based on these data, in our next experiments we used the TMG6-5 antibody to analyze the function of CD11b on imMDCs.

5. CR3 crosslinking does not induce the maturation of MDC

We have shown that both CR3 and CR4 are present in an active conformation on the surface of imMDCs ready to bind iC3b, their natural ligand,. As we found that CD11b is the dominant receptor in the iC3b mediated phagocytosis, we wanted to clarify whether this interaction transduces activating signals to the MDCs. To this end we treated imMDCs either with iC3b or the TMG6-5 antibody, which is specific to the ligand binding site of CD11b The advantage of using iC3b is that the complement receptors are triggered by their physiological ligand, but the disadvantage is that both CD11b and CD11c are stimulated simultaneously. ImMDCs were treated with the different stimuli for 30 minutes at 37°C, then washed and cultured for additional two days. The activation state of MDCs was estimated by flowcytometry, by assessing the expression of MHCII, MR, CD40, CD83 and CD86 molecules. Data summarized in Table1 demonstrate that stimulation with iC3b, anti-CD11b (TMG6-5) or the isotype matched control did not cause significant alteration in the phenotype of imMDCs. This indicates that the previously reported tolerogenic effect (Birdsall et al., 2005; Ehirchiou et al., 2007; Morelli et al., 2003; Schmidt et al., 2006; Veldhoen, 2007; Verbovetski et al., 2002) is not mediated by CD11b. As positive control, LPS stimulated

maMDCs were used, which highly expressed MHCII, CD40, CD83 and CD86 while the level of MR was decreased (Table1.).

6. Engagement of CR3 has no effect on the capacity of MDCs to trigger allogenic T cell activation and cytokine production

To find out whether certain functions of MDCs can be regulated via CD11b, we analyzed the allogenic T cell stimulatory capacity and cytokine production of iC3b and TMG6-5 treated cells. ImMDCs were treated with iC3b or with the binding-site specific anti-CD11b (TMG6-5) mAb for two days, then cocultured with allogenic T cells at DC:T=1:5 ratio for 4 days. Proliferation of CFSE and CD4 labeled T cells was measured by flowcytometry. As it is shown in Figure 6A neither iC3b, nor anti-CD11b affected the T cell stimulatory capacity of MDCs. We also measured the amount of IL-8, IL-6 and TNF-α from the supernatants of differently treated MDCs (Figure 6B, 6C, 6D). However, as shown in Figure 6B, 6C, and 6D, engagement of CR3 did not increase significantly the production of none of these cytokines. In both set of experiments LPS-matured maMDCs were used as positive control, which are known to stimulate allogeneic T cells and to produce high amounts of the measured cytokines. (Figure 6B, 6C, 6D).

Discussion

Constantly increasing data provide evidence that certain complement activation products strongly influence adaptive immune responses (Carroll, 2004; Kemper and Atkinson, 2007, Török et al., 2012). It has been also suggested that the uptake of complement opsonized particles via complement receptors C1qR, CR3, CR4 might play an important role in the maintenance of peripheral tolerance (Morelli et al., 2003; Nauta et al., 2004; Nauta et al., 2002; Schmidt et al., 2006; Skoberne et al., 2006; Sohn et al., 2003; Verbovetski et al., 2002). In this process DCs are of prominent importance. Regarding receptors for various C3-derived fragments, MDCs are reported to express iC3b-binding CR3 and CR4, while CR1 (CD35) and CR2 (CD21), interacting with C3b and C3d fragments can not be detected on the surface of these cell types. (Pickl et al., 1998,Li et al., 2003,Bajtay et al. 2004). Integrins – including CR3 and CR4 - are known to be involved in various cellular processes associated with cytoskeletal remodeling, which are necessary for adherence and phagocytosis. It has been demonstrated that ligation of CR3 on mouse DCs increases the appearance of surface MHC and costimulatory molecules, and inhibits the release of inflammatory cytokines. This suggests that CR3 may provide a non-danger signal that suppresses the stimulatory capacity of DCs (Behrens et al., 2007).

β_2 integrins are also known to mediate phagocytosis, which is crucial for the removal of antigens and apoptotic cells. The importance of these molecules in anti-microbial defense is confirmed by the fact that patients with leukocyte adhesion deficiency type I (LAD I) syndrome lacking functional β_2 integrins are highly susceptible to bacterial infections (Bunting et al., 2002; Gresham et al., 1991). Recently MacPherson and colleagues found that a mutation in the CD11b gene results in the lack of cell adhesion via ICAMs or iC3b. These deficiencies may ultimately lead to detrimental effects on the immune system and contribute

to the development of certain diseases, such as systemic lupus erythematosus (MacPherson et al., 2011).

While CD11c is a widely accepted marker of myeloid cells, the proper function of CD11c/CD18 is not well characterized yet, in contrast to CD11b/CD18, which is known for long as a potent phagocytic receptor. Dissection of the function of CR3 and CR4 is hampered by the fact that their ligand specificity is identical and the extracellular domains of these molecules share 87% homology. Many data suggest however, that beside their overlapping functions, CR3 and CR4 may also have distinct functions. Ligand binding to integrins induces conformational changes and is involved in *outside-in* signaling, which can affect a variety of cellular functions such as apoptosis, cytotoxicity, proliferation, cytokine production, antigen presentation, and gene activation (Abram and Lowell, 2009; Berton and Lowell, 1999).

In our present study we show that ligation of CD11b with its natural ligand, iC3b or by specific antibody induce receptor internalization within 10 minutes (Figure 1). This interaction however does not result in any significant change in the phenotype of the MDCs (Table1). This is an interesting finding, since according to available data, CR3 can be considered rather an inhibitory complement receptor, that mediates the induction of tolerance (Behrens et al., 2007; Ehirchiou et al., 2007; Sohn et al., 2003; Varga et al., 2007; Veldhoen, 2007; Wagner et al., 2001). In our system we found that the inhibition of CD11b significantly decreases the uptake of iC3b opsonized yeast and Staphylococcus aureus particles by imMDCs, while the inhibition of CD11c had no significant effect on this process (Figure 3). This is in a good agreement with the results of Georgakopoulos et al, who demonstrated that CD11c/CD18 was less susceptible to activation than the other β2-integrins, suggesting that CR4 is more tightly regulated. Although in these experiments CD11c was also shown to be involved in the adhesion of cultured monocytes, the role of CD11b proved to be more important to induce cytoskeletal changes leading to increased spreading and formation of

actin foci, suggesting a pivotal role of CD11b in the cells' responses (Georgakopoulos et al., 2008).

Regarding the function of CR3 an other notable finding has also been published earlier, namely that functionally distinct populations of CD11b/CD18 molecules are present on monocytes and neuotrophil granulocytes; one of them is involved in the binding of iC3b opsonized particles while the other takes part in the process of phagocytosis mediated via several different receptors (Graham et al., 1989). In the present study we demonstrate that while both CR3 and CR4 are involved in the uptake of complement opsonized particles, binding to CD11c alone is not sufficient to initiate phagocytosis. We clearly show that CD11b has a primary role in this process (Figure 3, 4, 5).

Complement component C3 is an acute phase protein, the activation of which is an indicator of the actual physiological state of the body. It is well-known that inflammatory stimuli cause an increase in the amounts of C3 activation products in various body fluids. Earlier we have shown that MDCs – in contrast to macrophages - do not secrete C3 protein, but are able to fix freshly generated C3b fragments covalently (Sandor et al., 2009).This interaction directs MDCs to differentiate into mature DCs and to stimulate allogenic T cell proliferation. Our present work demonstrates that the pathogen bound iC3b interacting with CR3 and CR4 on MDCs act in a different manner. We show that in contrast to the covalently fixed C3-fragments, iC3b bound to CR3 and CR4 stimulate neither the differentiation of MDCs, nor the production of inflammatory cytokines, furthermore the capacity of MDCs to stimulate allogeneic T-cells is also not influenced by iC3b. These results together with our previous ones point to the importance of the actual microenvironment of the maturing DCs. Namely, the functional consequences are completely different if the cells have the possibility to fix nascent C3b via their C3b-acceptor sites, or if they interact with opsonized antigens bearing iC3b. We assume that in the former case complement producing macrophages deliver

native C3 in the close vicinity of DCs (e.g. in the lymph nodes) where it is cleaved by cell membrane proteases (Erdei et al., 1992; Fabry et al., 1985; Gergely et al., 1985; Kerekes et al., 1998; Maison et al., 1989). In the case however, when maturing DCs get into contact with iC3b-opsonized particles, complement receptors CR3 and CR4 take action and the outcome might be profoundly different. Activation via CR3 does not induce differentiation of DCs but the contribution of these receptors is crucial in the uptake of complement opsonized pathogens. On the other hand CR4 alone is unable to initiate phagocytosis. Thus it seems that there is a complex interplay between the various complement binding structures of DCs, which decisively influences the actual response of the cells. Further studies revealing the intricacy of these interactions will help to clarify the exact molecular mechanisms and the specific signaling processes mediated by CD11b and CD11c.

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Figure legends

Figure 1. Expression of CD35, CD21, CD11b and CD11c on the surface of im- and maMDCs

Expression of CD35 (CR1) (**A**), CD21 (CR2) (**B**), CD11b (CR3) (**C**) and CD11c (CR4) (**D**) was measured on imMDCs and maMDCs using specific antibodies. MaMDCs were generated by stimulating the imMDCs with 1 μg/ml LPS for 2 days. Black lined histogram shows receptor expression on imMDCs, gray lined histogram on maMDCs, while the filled grey histograms represent the isotype control. One representative experiment out of five indepent is shown. **E:** Maturation downregulates CD11b expression while upregulates CD11c expression on MDCs, this is expressed as ΔMFI CD11b on imMDCs/ ΔMFI CD11b on maMDCs and ΔMFI CD11c on imMDCs/ ΔMFI CD11c on maMDCs. Results are mean+/-SD of five independent expreiments.

Figure 2. Binding of iC3b and iC3b opsonized particles to imMDCs

A: ImMDCs were treated with 50 μg/ml iC3b at 4^oC for 30 minutes, washed and further incubated at 37^oC. Cells were analyzed for surface bound iC3b using anti-iC3b+anti-mouse IgG-Alexa488 at the indicated timepoints using cytofluorimetry. Mean ΔMFI+/-SD of iC3b staining of three indepent experiments is shown, ***p=0,0004. **B**: Confocal microscopic picture of imMDCs treated with 50 μg/ml iC3b at 37^oC for 2 hours, fixed, permeabilized and stained with anti-iC3b+anti-mouse IgG-Alexa488. 60x magnification with further digital magnification was used. One representative experiment out of five indepent is shown. **C**: ImMDCs were treated with 50 μg/ml iC3b at 4^oC for 30 minutes, washed and double stained for iC3b and CD11b (upper panel) or CD11c (lower panel) using anti-iC3b+anti-mouse IgG-Alexa488 and biotin conjugated anti-CD11b (upper panel) or biotin conjugated anti-CD11c (lower panel) antibody + streptavidine-Alexa647. Upper panel shows iC3b in green and CD11b in blue and merged. Lower panel shows iC3b in green and CD11c in blue and merged.

60x magnification with further digital magnification was used. One representative experiment out of three is shown. **D**: imMDCs were coincubated with opsonized yeast at DC:yeast=1:5 ratio for 2 hours at 4°C or 37°C and phagocytic index was determined. Phagocytic index using non-opsonized yeast is substracted in each case. Values are calculated as mean+/-SD of three independent experiments. **E**: ImMDCs were incubated with FITC labeled 200 μg/ml opsonized Staphylococcus aureus at 4°C or 37°C for 2 hours and analyzed for phagocytosed bacteria using cytofluorimetry. Results using non-opsonized bacteria are substracted in each case. Values are calculated as mean+/-SD of three independent experiments.

Figure 3. CR3 has a dominant role over CR4 in the uptake of iC3b opsonized yeast and bacteria

ImMDCs were pretreated with anti-CD11b, anti-CD11c or isotype matched control antibody at 100μg/ml, washed and were incubated with iC3b opsonized yeast particles for 1 hour at 37°C (**A**). Phagocytic index was determined. Data shown are mean values calculated from four independent experiments +/- standard deviation. *p=0,0329. **B**: ImMDCs were pretreated the same way as in A and were incubated with 200 μg/ml iC3b opsonized FITC labeled Staphylococcus aureus and analyzed by cytofluorimetry. Data shown are mean values calculated from four independent experiments +/- standard deviation. *p=0,0387. **C**: Phagocytosis of 200 μg/ml non-opsonized, heat-inactivated serum opsonized and normal human serum opsonized FITC labeled Staphylococcus aureus by imMDCs was aalyzed by flow cytometry. Cells were incubated with the bacteria for 1 hour, washed and analyzed for FITC positivity. In the case of non-opsonized and heat-inactivated serum opsonized bacteria only 12% and 17% of MDCs were able to phagocytose. When bacteria was opsnoized by normal human sera, 66% of MDCs phagocytosed. One representative measurement out of five is shown. Black lined histogram represents MDCs that were coincubated with bacteria, filled gray histogram represents autofluorescence. **D-E:** ImMDCs were pretreaed as in A and were

incubated with 200 μg/ml non-opsonized FITC labeled Staphylococcus aureus (D) or 200 μg/ml heat-inactivated serum opsonized FITC labeled Staphylococcus aureus (E) and analyzed by cytofluorimetry. Data shown are mean values calculated from four independent experiments +/- standard deviation. **F**: ImMDCs were left untreated (control) or pretreated with Fc receptor blocking reagent and their capacity to phagocytose 200 μg/ml iC3b opsonized FITC labeled Staphylococcus aureus was analyzed by +/- standard deviation.

Figure 4. The dominant role of CD11b in the uptake of iC3b opsonized yeast and bacteria is supported by RNA silencing.

To analyze the role of CD11c in the phagocytosis of iC3b opsonized particles, CD11c and CD11b expression of imMDCs was downregulated using RNA silencing method. **A**: Cytofluorimetric analysis of a representative CD11b silencing experiment is shown. Filled grey histogram represents isotype control, black line shows CD11b expression on negative control siRNA transfected cells, grey line shows CD11b expression on CD11b targeted siRNA transfected cells. The average value of receptor downregulation was 70%. **B**: Cytofluorimetric analysis of a representative CD11c silencing experiment is shown. Filled grey histogram represents isotype control, black line shows CD11c expression on negative control siRNA transfected cells, grey line shows CD11c expression on CD11c targeted siRNA transfected cells. The average value of receptor downregulation was 70%. **C-D**: Phagocytosis of iC3b opsonized yeast (C) and iC3b opsonized bacteria (D) by control siRNA transfected and CD11c silenced imMDCs. Phagocytosis was measured as mentioned in Figure 2. Represented data are mean values calculated from five independent experiments +/- standard deviation. **E-F**: Phagocytosis of iC3b opsonized yeast (E) and iC3b opsonized bacteria (F) by control siRNA transfected and CD11b silenced imMDCs. Phagocytosis was measured as mentioned in Figure 2. Represented data are mean values calculated from five independent experiments +/- standard deviation, p=0,0065 for phagocytosis of yeast (E) and p=0,0012 for

phagocytosis of bacteria (F). **G-H**: Phagocytosis of non-opsonized yeast (G) and non-opsonized bacteria (H) by control siRNA transfected and CD11b silenced imMDCs. Phagocytosis was measured as mentioned in Figure 2. Represented data are mean values calculated from five independent experiments +/- standard deviation.

Figure 5. Route of CR3 and CR4 during uptake of iC3b opsonized yeast and bacteria

A-D: ImMDCs were labeled for CD11b (red) and CD11c (blue) using anti-CD11b biotin + streptavidin-Alexa546 and anti-CD11c-Alexa647. **A** shows CD11b staining, **B** shows CD11c staining, **C** shows the two pictures merged. As seen on **D** that represents the fluorescence intensity of the two labeled receptors along the yellow line that crosses two cells, CD11b (red) and CD11c (blue) are both present in the cell membrane. Yellow arrows are pointing to the cell membrane on the microscopic picture and on the histogram. **E-H**: ImMDCs were labeled the same as in A-D but after staining of the receptors cells were incubated with 200 µg/ml iC3b opsonized Staphylococcus aureus. As seen on the pictures (**E,G**) and the intensity histogram (**H**) CD11b enters the cells, and almost completely disappears from the cell membrane, while CD11c mostly stays in the membrane, although some molecule enters the cell. Yellow arrows are pointing to the cell membrane on the microscopic picture and on the histogram. It is also shown that colocalization of CD11b and CD11c is reduced during phagocytosis (**G**) in contrast to untreated cells (**C**). **I**: ImMDCs were analyzed for CD11b and CD11c expression before and after phagocytosis of iC3b opsonized Staphylococcus aureus to analyze the internalization of CR3 and CR4. Mean ΔMFI of three independent experiments +/-SD is shown, **p=0,0052. **J:** ImMDCs were treated with TMG6-5 antibody that recognizes the iC3b ligand binding site of CD11b at 10 µg/ml concentration for 30 minutes on ice (■) to allow binding to CD11b but preventing internalization. Than the cells were washed to remove unbound antibodies and transferred to 37°C. Samples were analyzed for the

remaining CD11b on cell surface at the indicated timepoints by flow cytometry. Data are means of three independent experiments +/-SD.

Figure 6. Functional effect of CR3 triggering on MDCs.

A: The differently treated MDCs at day 7 were washed and cocultured for additional 4 days with CFSE loaded allogeneic T cells at DC:T cell ratio 1:5. Cell proliferation was assesed by determining the CFSE MFI of the dividing anti-CD4-Alexa647 labeled cells. Data represented are mean values calculated from five independent experiments +/- standard deviation. *p=0,0271. **B-D**: IL-6, IL-8 and TNF-α concentrations were measured from the differently treated cells 24 hour supernatant by ELISA. Data represented are mean values calculated from five independent experiments +/- standard deviation. ***p<0,0001.

	imMDC	maMDC	isotype	CD11b	iC3b
MHCII	293.95 +/- 61.08	456.61 +/- 51.35 ***	317.46 +/- 34.53	265.65 +/- 61.2	288.73 +/- 40.23
MR	44.97 +/- 2.87	32.26 +/- 1.6 ***	44.57 +/- 3.83	45.02 +/- 5.11	45.84 +/- 3.46
CD40	204.42 +/- 14.18	294.42 +/- 24.84 ***	217.42 +/- 15.79	226.45 +/- 23.18	224.6 +/- 18.61
CD83	8.10 +/- 0.45	25.93 +/- 2.33 ***	7.53 +/- 0.76	7.25 +/- 0.74	7.08 +/- 0.75
CD86	50.44 +/- 3.03	138.38 +/- 58.32 **	49.65 +/- 12.08	47.22 +/- 10.23	39.21 +/- 7.98

Table1. Triggering via CR3 does not affect the phenotype of human MDCs

ImMDCs on day 5 were treated with iC3b, the iC3b ligand binding site specific anti-CD11b antibody and an isotype-matched control antibody, or left untreated. After incubation for 30 min at 37°C cells were washed and cultured for additional two days. MaMDCs were generated by stimulating the imMDCs with LPS. MDCs were analyzed for the expression of the indicated markers by cytofluorimetry on day 7. Data presented are mean ΔMFI values calculated from five independent experiments, +/- standard deviation. Students' paired t-test was used to compare the effect of the different treatments to untreated control cells (imMDCs), Individual p values are as follows: maMDC MHCII: 0,0019; maMDC MR:<0,0001; maMDC CD40: 0,0001, maMDC CD83: <0,0001; maMDC CD86: 0,0098.

Figure1

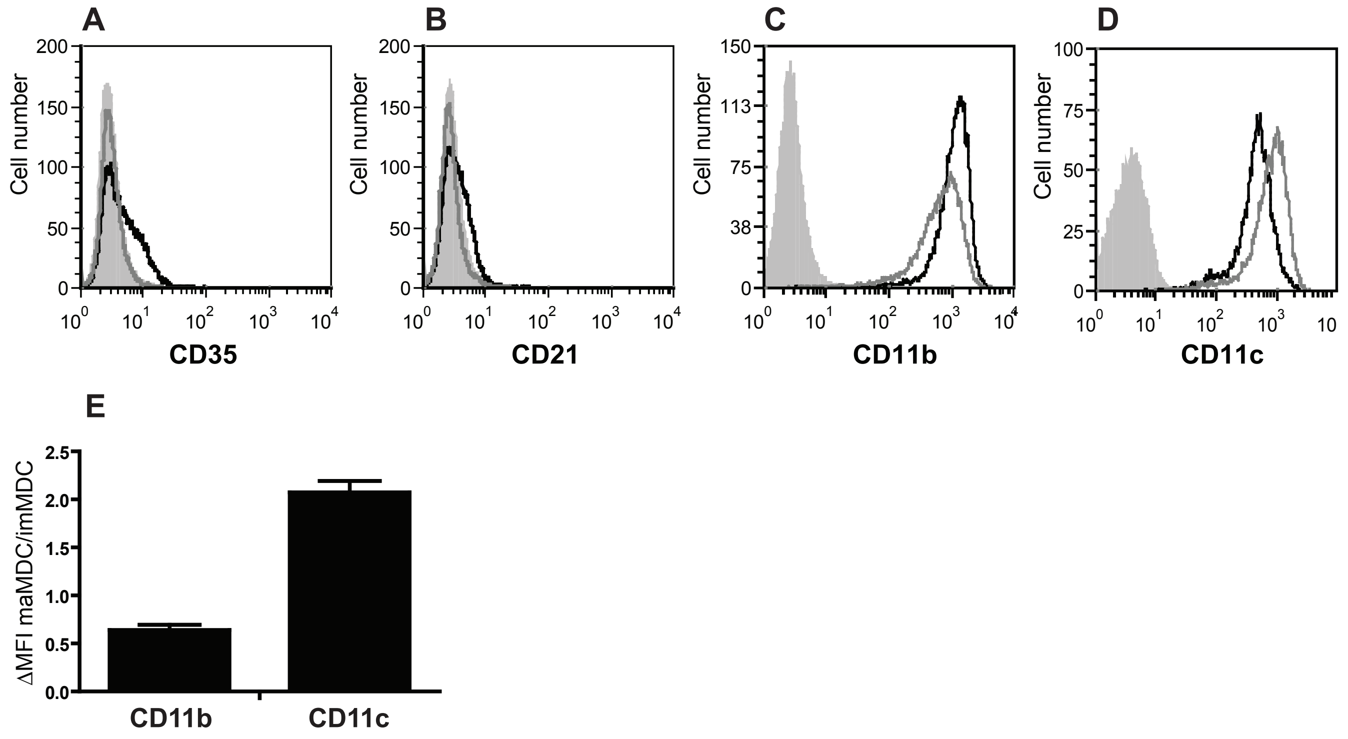
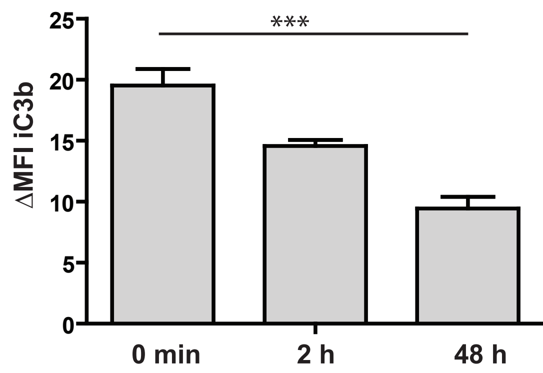
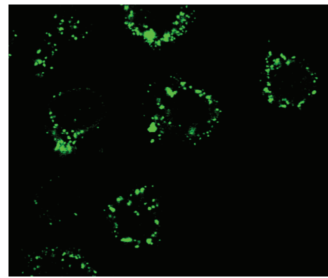
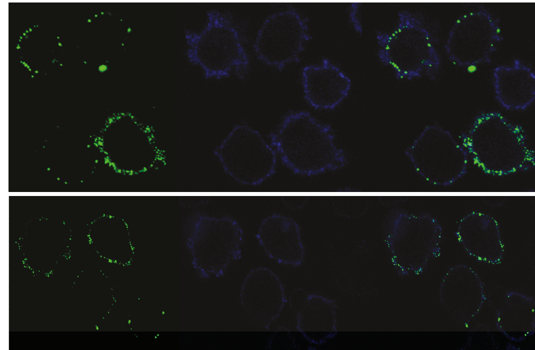
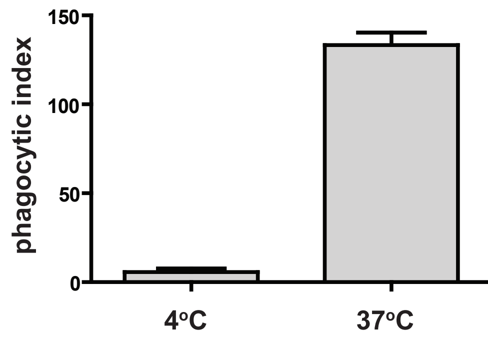


Figure2









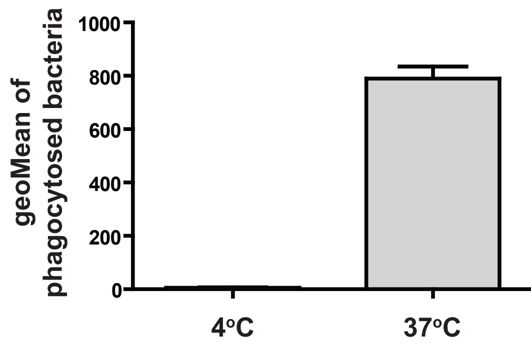


Figure3

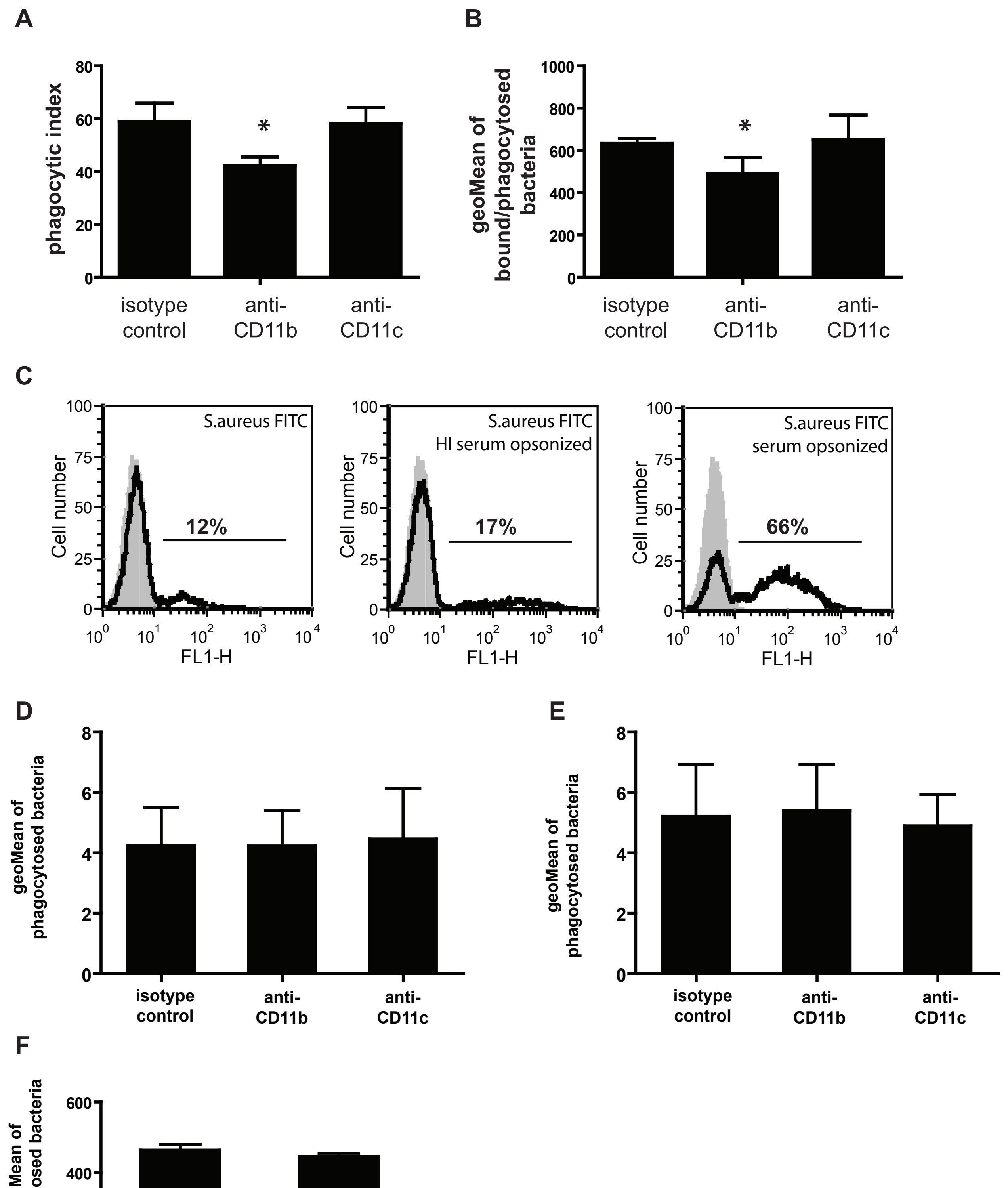
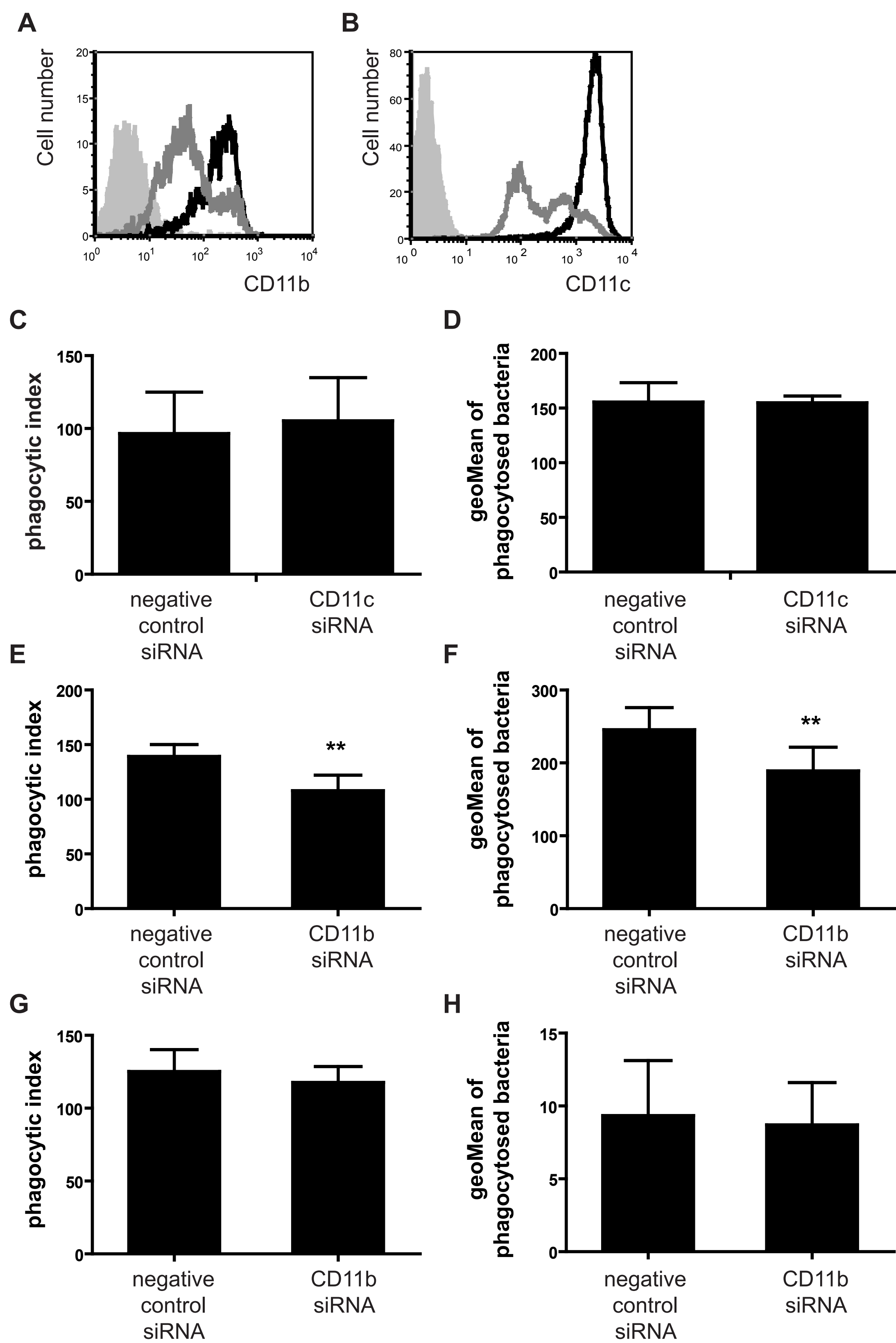


Figure4



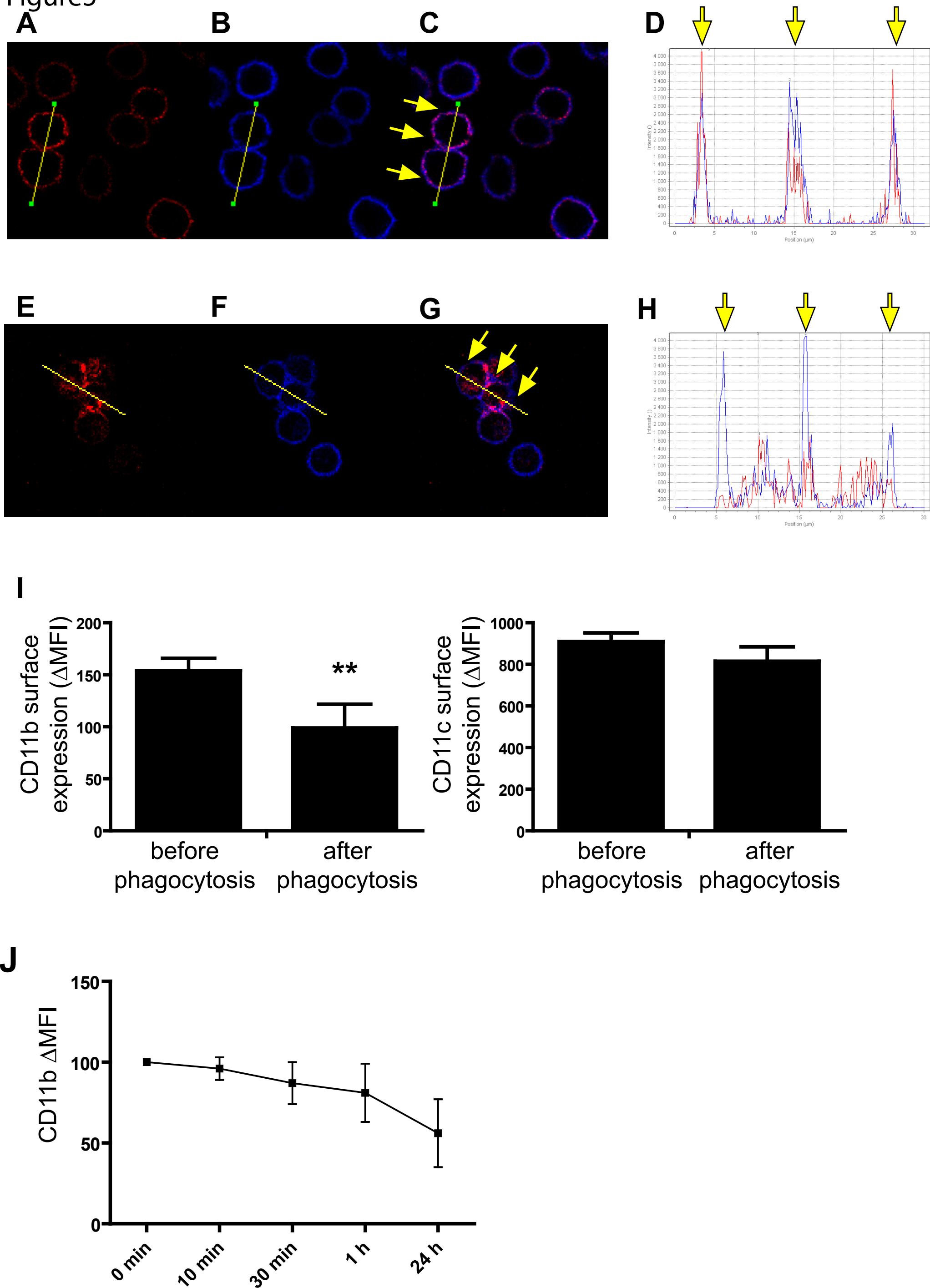
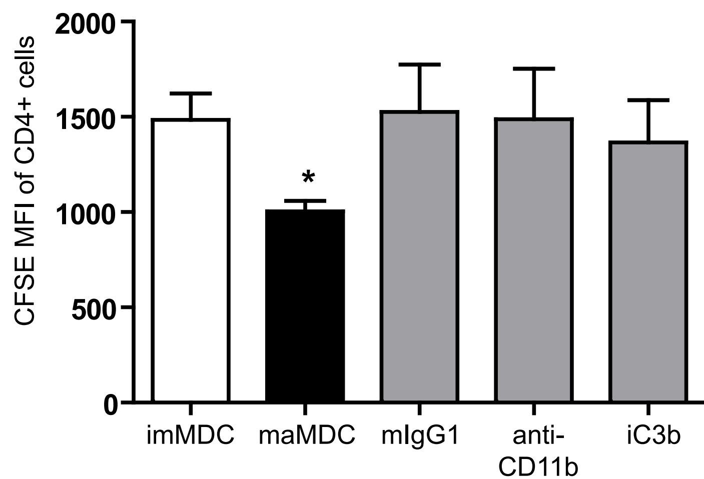
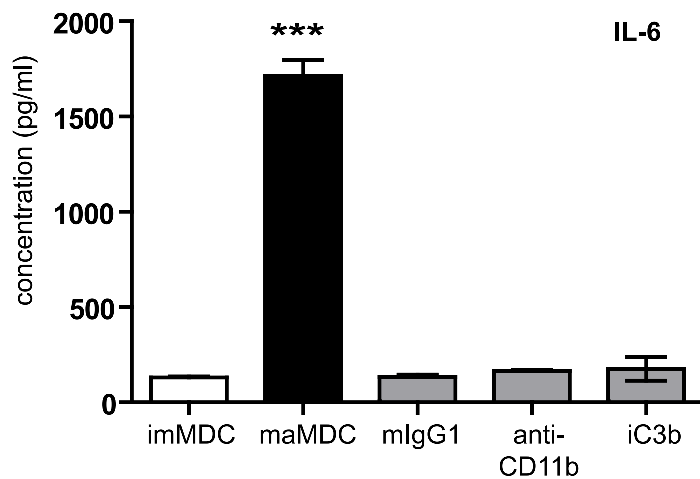
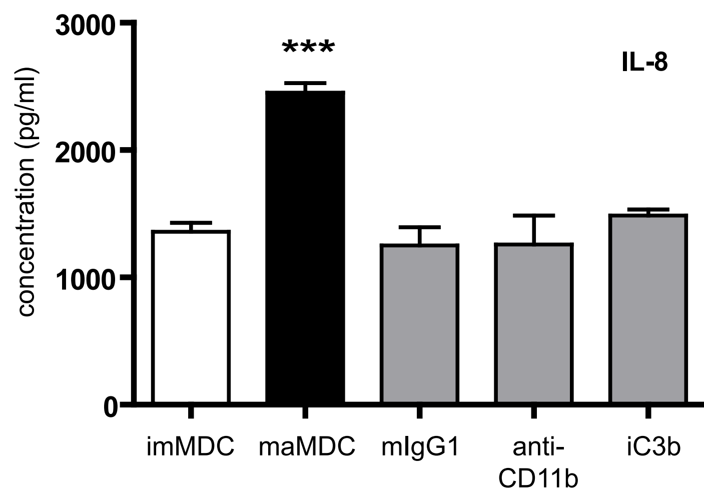


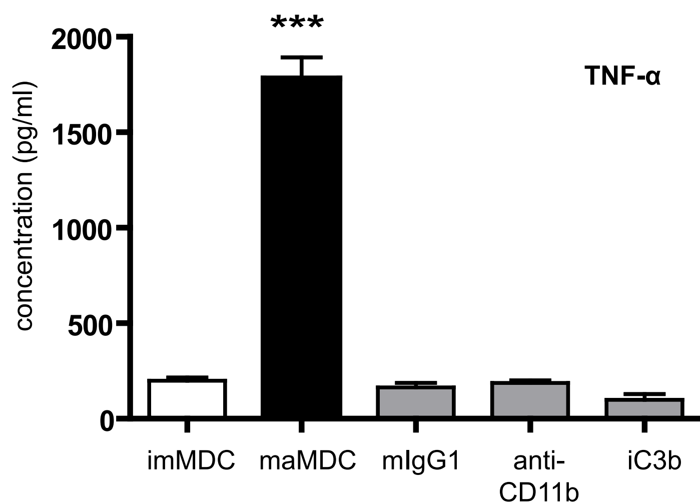
Figure5
A
B
C
D
E
F
G
H
I
CD11b surface expression (ΔMFI)
200
150
100
50
0
**
before phagocytosis
after phagocytosis
CD11c surface expression (ΔMFI)
1000
800
600
400
200
0
before phagocytosis
after phagocytosis
J
CD11b ΔMFI
150
100
50
0
0 min
10 min
30 min
1 h
24 h

Figure6









	imMDC	maMDC	isotype	CD11b	iC3b
MHCII	293.95 +/- 61.08	456.61 +/- 51.35 ***	317.46 +/- 34.53	265.65 +/- 61.2	288.73 +/- 40.23
MR	44.97 +/- 2.87	32.26 +/- 1.6 ***	44.57 +/- 3.83	45.02 +/- 5.11	45.84 +/- 3.46
CD40	204.42 +/- 14.18	294.42 +/- 24.84 ***	217.42 +/- 15.79	226.45 +/- 23.18	224.6 +/- 18.61
CD83	8.10 +/- 0.45	25.93 +/- 2.33 ***	7.53 +/- 0.76	7.25 +/- 0.74	7.08 +/- 0.75
CD86	50.44 +/- 3.03	138.38 +/- 58.32 **	49.65 +/- 12.08	47.22 +/- 10.23	39.21 +/- 7.98

Table1. Triggering via CR3 does not affect the phenotype of human MDCs

ImMDCs on day 5 were treated with iC3b, the iC3b ligand binding site specific anti-CD11b antibody and an isotype-matched control antibody, or left untreated. After incubation for 30 min at 37°C cells were washed and cultured for additional two days. MaMDCs were generated by stimulating the imMDCs with LPS. MDCs were analyzed for the expression of the indicated markers by cytofluorimetry on day 7. Data presented are mean ΔMFI values calculated from five independent experiments, +/- standard deviation. Students' paired t-test was used to compare the effect of the different treatments to untreated control cells (imMDCs), Individual p values are as follows: maMDC MHCII: 0,0019; maMDC MR:<0,0001; maMDC CD40: 0,0001, maMDC CD83: <0,0001; maMDC CD86: 0,0098.