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Complement components, regulators and receptors are produced by human monocyte-derived dendritic cells

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Abstract

Complement and dendritic cells (DCs) are essential components of innate immunity. Both participate in local inflammation and moreover have roles in the initiation of the acquired immunity response and in the maintenance of tolerance. Recent studies have demonstrated the ability of DCs to synthesize C1q, C3, Factor I, Factor B and complement receptors 3 and 4. In this study, we demonstrate that human DCs are a source of other soluble complement proteins including C1q, C4b binding protein (C4BP), C7 and C8. Complement receptors (CR)1 and the CD18 chain (common for CR3 and CR4) were also present on DCs while CR2 was not detected.
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Introduction

Complement and dendritic cells (DCs) are essential components of innate immunity (Walport, 2001; Janeway and Medzhitov, 2002). Both participate in local inflammation and moreover have a role in the initiation of acquired immunity and in the maintenance of tolerance (Banchereau et al., 2000; Carroll, 2004).

The complement system is made up of approximately 30 proteins that participate in its activation or act as receptors and regulators of activation (Walport, 2001). The plasma complement proteins are mainly produced by hepatocytes but some are produced principally by extra-hepatic tissues; for example C1q and properdin are mainly produced by monocytes and macrophages, C7 by polymorphonuclear cells and Factor D by adipocytes (Morgan and Gasque, 1997). Complement activation leads to the release of biologically active fragments with inflammatory and chemotactic properties. They also participate in immune complex and apoptotic cell clearance, microbial lysis and B and T cell activation (Walport, 2001; Dempsey et al., 1996; Kopf et al., 2002).

DCs are present in most tissues and migrate to secondary lymphoid organs after maturation in response to inflammatory signals such as cytokines, prostaglandins or bacterial products. During maturation, DCs lose their ability to efficiently take up and process antigens, down-regulate the expression of receptors for inflammatory chemokines (Sallusto and Lanzavecchia, 2000) and up-regulate the expression of costimulatory molecules (e.g. CD40, CD80, CD86) and others correlated to antigen presentation (Caux et al., 1997).

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Our group (Reis et al., 2006) and others have investigated DC secretion of complement proteins (Castellano et al., 2004; Peng et al., 2006) and expression of CRs (Sozzani et al., 1995; Verbovetski et al., 2002; Gutzmer et al., 2004). We recently showed that human monocyte-derived DCs express C3, C5, C9, Factor I, Factor H, Factor B, Factor D and properdin mRNAs in a stimuli-specific manner independent of DC maturation. We also demonstrated that these cells are able to secrete the proteins C3, Factor I and Factor B (Reis et al., 2006). Peng et al. (2006) also demonstrated that C3 secretion by mouse bone-marrow-derived DCs is required for DC stimulation of the alloreactive T cell response. The production of C1q by human monocyte-derived DCs was first demonstrated by Castellano et al. (2004) who showed that C1q produced by these cells is functionally active in sheep erythrocyte lysis and apoptotic cell binding assays. CRs were also detected on DC surfaces and their binding can influence the state of DC activation. CR3, CR4 and C1qR are involved in the phagocytosis of apoptotic cells by macrophages and C3aR and C5aR are involved in DC maturation and migration (Verbovetski et al., 2002; Morelli et al., 2003; Castellano et al., 2004; Soruri et al., 2003a, b).

Considering that both DCs and complement are present at inflammatory sites and contribute to the immune response as well as the previous observation that DCs are able to secrete several complement proteins and express CRs, we investigated the production of other complement proteins and CRs whose production by DCs has not been previously demonstrated.

Materials and methods

Generation of monocyte-derived DCs and macrophages

Monocytes were isolated from buffy coats obtained from healthy donors using Ficoll (GE Health Care) density gradient centrifugation. After 2 h of culture in six-well plates (2×10^7 cells/well) the non-adherent cells were removed by washing with phosphate-buffered saline (PBS). DCs and macrophages were generated in vitro from the adherent fraction of human mononuclear cells cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen). Granulocyte macrophage-colony stimulating factor (GM-CSF) [(50 ng/mL, R&D] and interleukin (IL)-4 [(50 ng/mL, R&D)] were added only to DC cultures (not to macrophages cultures) at days 1, 3 and 5. The purity of the DC cultures was considered adequate when 98% of cells were $CD14^-/CD1a^+$ and $CD209^{high}$, a well known DC phenotype, while macrophages are $CD14^+/CD1a^{low}$ and $CD209^{low}$ (Sallusto and Lanzavecchia, 1994). Lipopolysaccharide (LPS) from *E. coli* (1 μg/mL; SIGMA) was used where indicated on day 5–7 to induce DC maturation and macrophage activation.

Flow cytometry analysis

Cells were harvested and washed in PBS containing 0.5% bovine serum albumin and 0.02% NaN_3. The presence of DCs and macrophage surface markers and CRs was evaluated by flow cytometry using a panel of phycoerythrin- or fluorescein isothiocyanate-conjugated monoclonal antibodies specific for CD14, CD1a, HLA-DR, CD86, CD209, isotype-controls (Pharmingen), CD18, CD21 and CD35 (DAKO). Fluorescence intensity was monitored with a FACScalibur flow cytometer using the CellQuest software.

Reverse transcriptase-polymerase chain reaction

At day 7 total RNA was extracted from DC and macrophage cultures using "Total RNA Isolation System" (Promega) according to the manufacturer's instructions. In some experiments we also included RNA from Epstein-Barr virus-transformed B lymphocytes (Ly). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed in two steps: (1) cDNA was produced by incubating 200 ng of total RNA with 500 μM oligo d(*T*) for 5 min at 70 °C followed by addition of 100 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega), 0.4 mM dNTPs and buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiotreitol) at 42 °C for 1 h, (2) 2.5 μL of cDNA were amplified by PCR (40 cycles) in the presence of 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, 0.4 mM dNTPs and 0.8 pmol of each specific primer (all sequences are 5′–3′): *CR1-forward*: CTA CCA ACC TAA CTG ATG AG, *reverse:* ATC GTC ATT GCT GGT GAG T; *CR2-forward:* GAG GAA GGA GTG AAC TTC ATG, *reverse:* CGT ATT TCC ACC TGT ATG ATG and *human glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*)*-forward:* TCT CTG CTC CTC CTG TTC GAC, *reverse:* GGA TCT CGC TCC TGG AAG ATG. No amplification was observed in reactions without cDNA and no amplification of genomic DNA was observed in the reactions. Levels of human mRNA were evaluated after amplification of the constitutively expressed *GAPDH* gene.

Western blot

Supernatants from DC and macrophage cultures were analyzed by SDS-PAGE (10% acrylamide) in reduced (DTT 40 mM) or non reduced conditions, after transferring to nitrocellulose membranes. Normal human serum was diluted (1:20) and 1 μL was used in the gel as

positive control. Membranes were blocked by incubation for 12 h with TBST buffer (5 mM Tris, 75 mM NaCl, 0.028% Tween 20) containing 5% non-fat milk. Primary antibodies (Calbiochem–Novabiochem) monospecific to human C1q (goat serum anti-human C1q), C4b binding protein (rabbit serum anti-human C4BP), C7 (goat serum anti-human C7) and C8 (goat serum anti-human C8) were diluted 1:1000 in TBST containing non-fat milk and incubated with the membranes at 4 °C for 12 h. After washing, the blots were incubated with monospecific alkaline phosphatase-conjugated rabbit anti-goat IgG or goat anti-rabbit IgG (affinity purified, dilution 1:10 000, Calbiochem–Novabiochem) for 2 h at room temperature. Immunoreactive bands were visualized after incubating membranes with 5-bromo, 4-chloro, 3-indolylphosphate and nitroblue tetrazolium (Calbiochem–Novabiochem).

Results

DC and macrophage phenotype

DCs were phenotypically characterized by flow cytometry and exhibited the phenotype $CD14^{-}/CD1a^{+}$, clearly distinguishing them from macrophages which are $CD14^{+}/CD1a^{low}$. DCs expressed high levels of the DC-specific intercellular adhesion molecule (ICAM)-3 grabbing non-integrin (DC-SIGN, CD209), which has a residual expression in macrophages (Fig. 1A). As expected, immature DCs presented lower expression levels of co-stimulatory protein CD86 and MHC class II molecule (HLA-DR) when compared to mature DCs (Fig. 1B).

Complement protein production

We evaluated the production of some complement proteins in DC and macrophage supernatants by Western blot under reducing conditions. The presence of C1q, C4BP, C7 and C8 was analyzed independently on the same cell supernatant using specific antibodies. In each experiment blood cells from different healthy adult donors were used to cultivate DCs and macrophages. Three independent experiments gave similar results, one of which is shown in Fig. 2. Culture medium was included as a negative control and no specific bands were visualized.

A band of approximately 25 kDa corresponding to the C1q chains was detected in DC and macrophage supernatants (Fig. 2). C1q is made up of three individual polypeptide chains: a chain (27.5 kDa), B chain (25.2 kDa) and C chain (23.8 kDa), which migrated together in the polyacrylamide gels. The presence of C4BP was observed as a band of 45 kDa in the supernatants of both DC and macrophage cultures (Fig. 2). C4BP is an oligomeric protein and is composed of two chains: α (70 kDa) and β (45 kDa). Even though we used polyclonal anti-C4BP, only C4BP α chain was observed in our experiments.

C7 is a single-chain protein of approximately 95 kDa that could be detected in the DC and macrophage supernatants (Fig. 2). We also observed a 151 kDa band corresponding to C8 (Fig. 2). C8 individual chains were not observed in reduced conditions. No significant differences in the amounts of C1q, C4BP, C7 and C8 produced by DC or macrophages were observed when using cultures with or without previous treatment with LPS (Fig. 2).

Complement receptor expression

Besides secretion of soluble proteins, we also evaluated if DCs could express CRs. We analyzed the expression of CR1 and CR2 mRNAs from immature and mature DCs and macrophages by RT-PCR. The results shown in Fig. 3A demonstrate that DCs and macrophages express CR1 but not CR2 mRNA. However we detected a small increase in CR2 cDNA in DCs when stimulated with LPS. We considered this result insignificant since no CR2 was present on the surface of mature DCs (Fig. 3B).

The results of FACS analysis of CR1 expression (Fig. 3B) agree with what was shown in Fig. 3A when we observed that CR1 is expressed at the surface of immature DCs, but not at the surface of mature DCs. CR2 was not observed at the surface of immature or mature DCs. The expression of CD18, a common chain for LFA-1, CR3 and CR4 was also found to be present at the surface of both immature and mature DCs (Fig. 3B).

Discussion

Complement is commonly present at sites of inflammation, including immune-mediated kidney diseases, rheumatoid arthritis, inflammatory bowel disease, acute lung injury and cardiac injury (Laufer et al., 2001; Niederbichler et al., 2006). The liver is considered the main origin of complement but extra-hepatic synthesis of complement proteins has been now extensively demonstrated and plays an important role in local inflammatory processes (Laufer et al., 2001). In some organs like kidneys (Passwell et al., 1990) and brain (Gasque et al., 1996), the synthesis of complement proteins by resident or infiltrating cells is essential for the maintenance of inflammatory processes and local

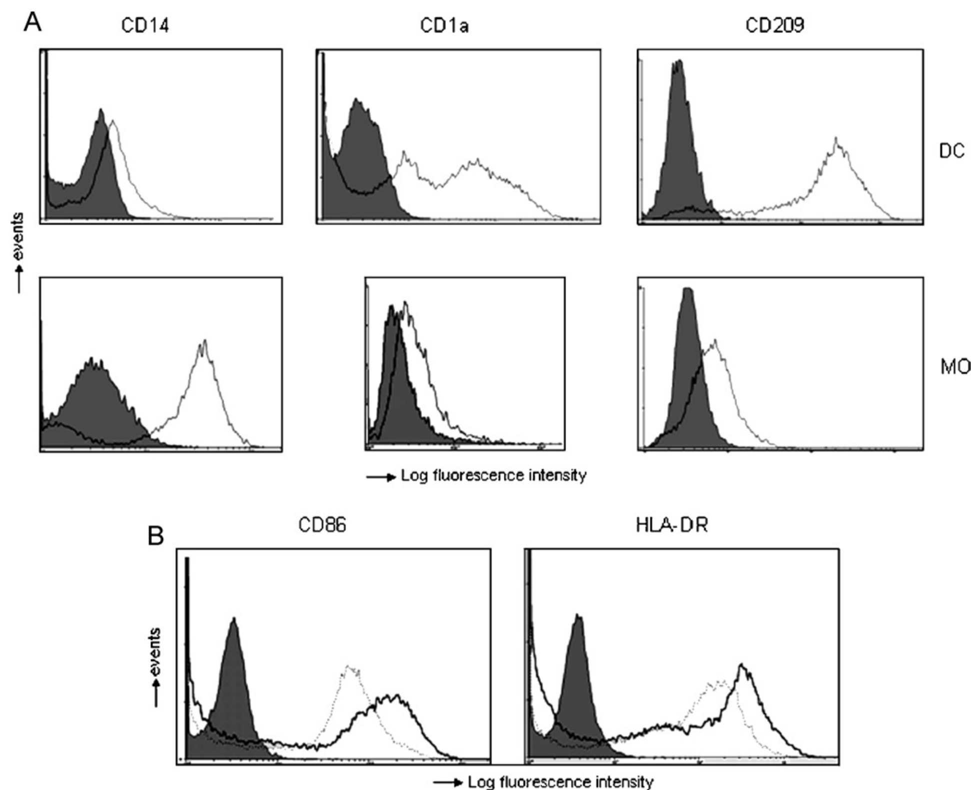

Fig. 1. Analysis of membrane markers of dendritic cells and macrophages. Dendritic cells (DC) and macrophages (MO) were cultured for 7 days and analyzed by FACS. (A) CD14, CD1a and CD209 expression by DC and MO. (B) CD86 and HLA-DR expression by immature DC (dashed line) and mature DC (full line) obtained after treatment with LPS (1 μg/mL) for 48 h. Similar results were obtained in three independent experiments using blood cells from three different healthy adult donors.

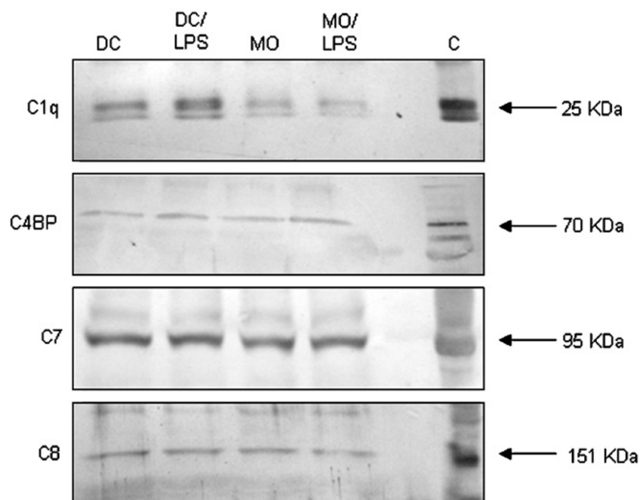

Fig. 2. Secretion of C1q, C4BP, C7 and C8 by immature and mature dendritic cells. Day-7 supernatants from macrophages (MO) and dendritic cells (DCs) were analyzed by Western blot for complement protein secretion. Diluted normal human serum (C) was used as positive control. All samples were used in reduced conditions with the exception for C8 detection. All data refer to a single supernatant. Similar results were obtained in three independent experiments using blood cells from three different healthy adult donors.

synthesis of C3 is implicated in experimental acute renal allograft rejection (Pratt et al., 2002).

Complement component C1q is responsible for classical pathway initiation by binding to immune complexes (Augener et al., 1971) and participates in the clearance of apoptotic cells (Navratil et al., 2001). In contrast to other complement proteins, C1q is mainly produced by monocytes and macrophages (Petry et al., 2001). Our findings agree with Castellano et al. (2004) who demonstrated that monocyte-derived and $CD34^+$-derived interstitial DCs are a rich source of C1q that is functionally active in complement activation and binding to apoptotic cells. However, we did not observe any significant down-regulation of C1q production after DC activation that these authors described.

C4BP is a regulatory component of the classical pathway that is synthesized by liver cells (Kusada-Funakoshi et al., 1991) and activated monocytes (Lappin and Whaley, 1990). C4BP circulates in the plasma as three isoforms, each derived from a different combination of α (70 kDa) and β (45 kDa) chains. It is a cofactor for Factor I-dependent degradation of C3b and C4b (Dahlback and Hildebrand, 1983; Nagasawa and Stroud, 1977) and accelerates the decay of classical pathway C3/C5-convertases (Gigli et al., 1979). Trouw et al. (2005) showed that C4BP has other roles besides regulation of

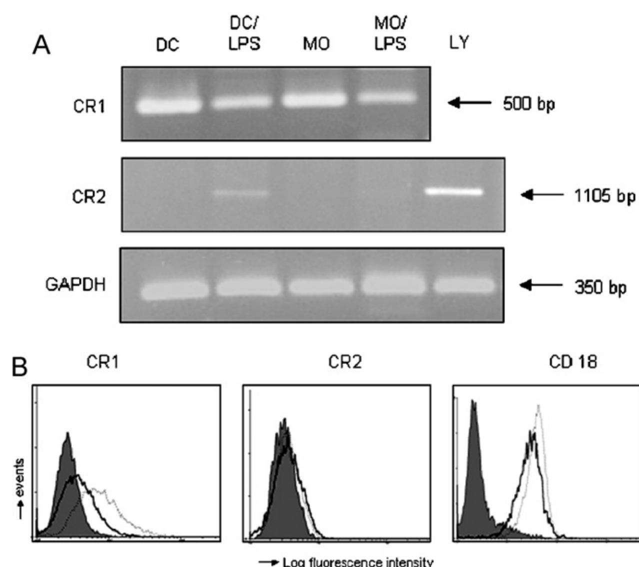

Fig. 3. CR1, CR2 and CD18 expression by immature and mature dendritic cells. Dendritic cells (DCs) maturation was induced by LPS treatment. (A) Total RNA was extracted from DC or macrophage (MO) cultures with or without treatment with LPS (1 μg/ml) for 48 h or from Epstein-Barr virus-transformed B lymphocytes (Ly). GAPDH was used as a control. (B) CR1, CR2 and CD18 expression by immature DC (dashed line) and mature DC (full line) were analyzed by FACS. Similar results were obtained in three independent experiments using blood cells from three different healthy adult donors.

complement: it can activate B cells after binding to CD40 (Brodeur et al., 2003) and can bind to necrotic cells and DNA, limiting DNA release and inhibiting complement activation (Trouw et al., 2005). The present report is the first to show that DCs can secrete C4BP but the role of this DC-derived protein in the local immuno-inflammatory reaction still needs to be determined.

Complement C7 is primarily synthesized by monocytes (Hetland et al., 1986) and granulocytes (Hogasen et al., 1995) and its binding to the C5b6 complex allows the binding of C8 and subsequently of several C9 molecules to form the membrane attack complex (C5b-9_n) responsible for cell lysis. Complement C8 exists in the circulation as an oligomeric protein of 151 kDa. It is composed of three non-identical subunits (α, β and γ) arranged as a disulfide-linked α–γ dimer and a non-covalently associated β chain. It is primarily produced by hepatocytes (Ng and Sodetz, 1987) but other cells like monocytes, macrophages, fibroblasts, astrocytes and endothelial cells also produce this protein. The formation of the C5b-9 is limited by C7 concentration near the site of complement activation and sub-lytic doses of MAC were shown to activate endothelial cells and polymorphonuclear leukocytes (Würzner, 2000). In this way, C7 and C8 production by DCs could influence local inflammation.

Besides the expression of soluble complement proteins, we found that DCs also express CR1 but not CR2. CR1 is a receptor for C3b, C4b, C1q (Klickstein et al., 1997) and also to mannose binding lectin (Ghiran et al., 2000). CR1 present in neutrophils and monocytes can mediate phagocytosis if the cells are primed or activated (Ahearn and Fearon, 1989). It also acts as a cofactor in Factor I-mediated cleavage of C3b and C4b. CR1 present on B cells and follicular DCs participates in localization of the antigen for presentation to T cells (Carroll, 1998). CR1 also mediates inhibitory signals in human B and T lymphocytes (Jozsi et al., 2002; Wagner et al., 2006). Until now, no information is available regarding CR1 function in DCs. Nevertheless, our observation of its down-regulation upon DC maturation agrees with the general phenomenon of decrease in the number of phagocytic receptors after the maturation process (Caux et al., 1997). In addition, CD18 (which participates in the formation of both CR3 and CR4) was observed in immature and mature DCs, as has been previously reported by other groups (Verbovetski et al., 2002; Morelli et al., 2003). These CRs may be involved with the capture of antigens for further processing and latter presentation to T lymphocytes.

The present study provides evidence that human monocyte-derived immature and mature DCs are able to synthesize complement components C1q, C4BP, C7, C8 and also CR1 and CD18, but not CR2. These cells expressed most of the complement proteins tested in amounts similar to those observed in macrophages – an important local source of complement proteins. These results, together with our previously reported observation that these DCs can express C3, C5, C9, Factor H, Factor I, Factor B and Factor D and properdin suggest that DC, like macrophages can be a relevant source of complement proteins within tissues. Considering the unique role DCs have in antigen presentation to T cells, their ability to secrete complement proteins within the microenvironment where these cell interactions occur, could affect significantly the establishment and pattern of immune responses. This is a particularly important observation, especially if one considers that DCs are present in the vast majority of tissues strategically located at critical sites to capture, process and present antigens to T lymphocytes where complement might not be available at high concentrations. The production of complement proteins at the site of inflammation could perhaps modulate the state of DC activation and consequently influence the acquired immune response.

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