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Impaired dendritic cell differentiation and maturation in the absence of C3

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Abstract

Human monocytes can be differentiated into immature dendritic cells (DCs) in the presence of serum and cytokines. One of the main functions of immature DCs is to capture and process antigens. Following maturation, they differentiate into antigen presenting cells. The role of complement in the differentiation process from monocytes towards immature DCs remains elusive. Here we demonstrate that complement 3 (C3) has a regulatory impact on the expression of specific DC surface molecules and DC-derived cytokine production during DC differentiation. We isolated human adherent peripheral blood mononuclear cells, which were cultured in the presence of GM-CSF plus IL-4 in medium supplemented with normal human serum or C3 deficient serum. The lack of C3 during DC differentiation negatively impacted the expression of C-type lectin receptor DC-SIGN, the antigen presenting molecules HLA-DR and CD1a, and the costimulatory molecules CD80 and CD86. Further, the spontaneous production of IL-6 and IL-12 was reduced in the absence of C3. Moreover, the maturation of immature DCs in response to LPS challenge was impaired in the absence of C3 as evidenced by reduced MHC-II, co-stimulatory molecule expression as well as modulated IL-12 and TNF-α production. Collectively, our results provide evidence for a novel role of C3 as a critical cofactor in human DC differentiation and maturation.

Keywords: Dendritic cells; Complement; C3; C3 deficiency; Innate immunity

1. Introduction

Complement and dendritic cells (DCs) are essential components of innate immunity (Banchereau and Steinman, 1998; Köhl, 2006) that regulate the development of adaptive immune responses (Köhl and Wills-Karp, 2007; Carroll, 2004; Banchereau et al., 2000). Moreover, recent reports provide evidence that complement components and DCs cooperate to modulate T cell responses (Köhl et al., 2006; Zhou et al., 2007; Chen et al., 2007). In contrast, the impact of complement on DC differentiation is ill-defined.

Human DCs evolve either from pluripotent precursor cells of the bone marrow or from blood-derived monocytes (Shortman and Liu, 2002). Human adherent blood mononuclear cells can be differentiated into DCs in medium supplemented with serum, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (Sallusto and Lanzavecchia, 1994). These cells develop a typical immature DC phenotype. Maturation can be induced by stimulation with pro-inflammatory mediators such as lipopolysaccharide (LPS) or TNF-α. Immature and mature DCs differ with regard to their phagocytic activity, cytokine production and surface expression of co-stimulatory molecules (Banchereau et al., 2000). While immature DCs are in an "antigen-capture mode", characterized by low expression of MHC-II, co-stimulatory molecules and low CD40, mature DCs adopt an "antigen-presenting mode" expressing high numbers of MHC-II, co-stimulatory molecules and CD40.

Immature DCs capture antigens by several pathways including macropinocytosis, phagocytosis involving CD36 and integrins as well as receptor-mediated endocytosis via Fcγ and C-type lectin receptors (Banchereau et al., 2000). DEC-205 and dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) are important C-type lectin receptors that direct subcellular destinations of antigens and activation of antigen

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processing pathways (Jiang et al., 1995; Geijtenbeek et al., 2000).

Mature DCs present processed antigens in association with MHC and CD1 molecules. They provide unique properties allowing them to activate *naïve* T helper (Th) cells (Banchereau et al., 2000). Appropriate Th cell activation requires the recognition of MHC-peptide complexes on DCs by antigen-specific TCR together with the interaction of co-stimulatory molecules such as CD80 and CD86 present on the DC surface and CD28 on T cells. T cells can also activate DCs by the interaction of CD40 and CD40 ligand, which up-regulates CD80 and CD86 expression and cytokine release (Banchereau et al., 2000). The different types and levels of cytokines produced by DCs generate the microenvironment which regulates the quality and the quantity of the immune response (Steinman, 2003; Sporri and Reis e Sousa, 2005; de Jong et al., 2005). Important cytokines produced by DCs are those of the IL-12 family, IL-10, IL-6 and TNF-α (Moser and Murphy, 2000; Pasare and Medzhitov, 2003). IL-12 is a heterodimeric cytokine composed of the p40 and the p30 subunits forming biologically active IL-12p70, both of which are transcriptionally regulated (Liu et al., 2003). IL-12 drives naïve Th cell differentiation towards a Th1 phenotype. IL-10 is a regulatory cytokine with pleiotropic functions that is a strong inhibitor of IL-12-mediated immunoregulation. IL-6 was recently found to suppress the function of T cells with a regulatory phenotype (T_{reg}), which makes this cytokine an important regulator of adaptive immune responses (Pasare and Medzhitov, 2003).

We have recently demonstrated that human monocyte-derived DCs are able to express several complement proteins such as C3, C5, C9, Factor I (fI), fH, fB, fD and properdin. The expression levels equal those of macrophages, are stimulus-specific and independent of DC maturation (Reis et al., 2006a). In addition, we found human immature and mature DCs to express C1q (Castellano et al., 2004; Reis et al., 2007), C7, C8, C4b binding protein and complement receptors (CR)-3 and CR4 (Reis et al., 2007).

As the microenvironment has a strong impact on DC differentiation and function and since C3 can be locally produced by many cell types we sought to determine the impact of complement C3 on the differentiation of human monocytes into immature DCs and the LPS-induced maturation process towards mature DCs.

2. Materials and methods

2.1. DC culture

Monocytes were isolated from buffy coat obtained from healthy blood donors using Ficoll PREMIUM (GE Healthcare) density gradient centrifugation. After 2 h of culture in 6-well plates (2×10^7 cells/well), non-adherent cells were removed by washing with phosphate-buffered saline (PBS). DCs were generated *in vitro* from the adherent fraction of human mononuclear cells and cultured for 7 days in RPMI 1640 (Invitrogen) supplemented with 10% normal human serum (NHS) pooled from 130 healthy donors volunteers or C3 deficient serum (C3DS), GM-CSF (50 ng/ml, R&D) and IL-4 (50 ng/ml, R&D). Cells received fresh medium supplemented with serum and cytokines on days 3 and 5. The purity of the DC cultures was considered appropriate when more than 95% of cells were $CD14^-/CD1a^+/CD209^{high}$ as determined by flow cytometry. LPS from *E. coli* (1 μg/ml, Sigma) was used where indicated on days 6–7 to induce DC maturation. DCs activated by LPS were referred to as NHS/LPS or C3DS/LPS. Where indicated, purified C3 (50, 100 or 150 μg/ml, Calbiochem, EMD biosciences) was added to the culture on days 1, 3 and 5. The viability in all cell cultures was assessed using Trypan blue dye. The absence of C3 did not affect cell viability (>95%).

2.2. C3 deficient serum

C3 deficient serum was obtained from a patient with complete homozygous C3 deficiency described by Ulbrich et al. (2001) and Reis et al. (2004). This C3 deficiency was due to the presence of a stop codon (R848Ter) in the C3 mRNA. This serum does not activate either the classical or alternative pathway but reconstitution with purified C3 (1 mg/ml) restored the hemolytic activity of the serum. ELISA analysis showed very low levels of C3 antigen: 0.15 μg/ml [normal levels: 0.8–1.9 mg/ml (Ritchie et al., 2004)]. Blood samples were kept on ice for 60 min and centrifuged at 600 × *g* for 15 min at 4 °C. Aliquoted serum samples were kept at −80 °C and thawed immediately prior to use.

2.3. Flow cytometry

Cell surface molecules expression was detected using 2×10^5 DCs and FITC-conjugated anti-human CD14 (Pharmingen), PE-conjugated anti-human CD1a (Pharmingen), FITC-conjugated anti-human CD11c (Pharmingen), PE-conjugated anti-human HLA-DR (Pharmingen), FITC or PE-conjugated anti-human CD40 (Pharmingen), FITC-conjugated anti-human CD80 (Pharmingen), PE-conjugated anti-human CD86 (Pharmingen), FITC-conjugated anti-human CD205 (Serotec), FITC-conjugated anti-human CD209 (Pharmingen), FITC-conjugated anti-human CD35 (CR1) (Dako), PE-conjugated anti-human CD18 (Dako) or the appropriate isotype control antibody, at 4 °C for 20 min followed by washing with PBS/0.5% of bovine serum albumin. The cells were then fixed in PBS/0.5% of bovine serum albumin/2% paraformaldehyde. Fluorescence was monitored using FACSCalibur and CellQuest software (BD Biosciences).

2.4. ELISA

Cytokine production was detected by ELISA using Human ELISA Set (BD OptEIA™ BD Biosciences) specific for human IL-6, IL-10, IL-12p40, IL-12p70 and TNF-α according to the manufacturer's instructions. Supernatants were used without dilution (IL-12p70), or diluted 1:2 (IL-12p40, TNF-α), 1:10 (IL-10) or 1:200 (IL-6).

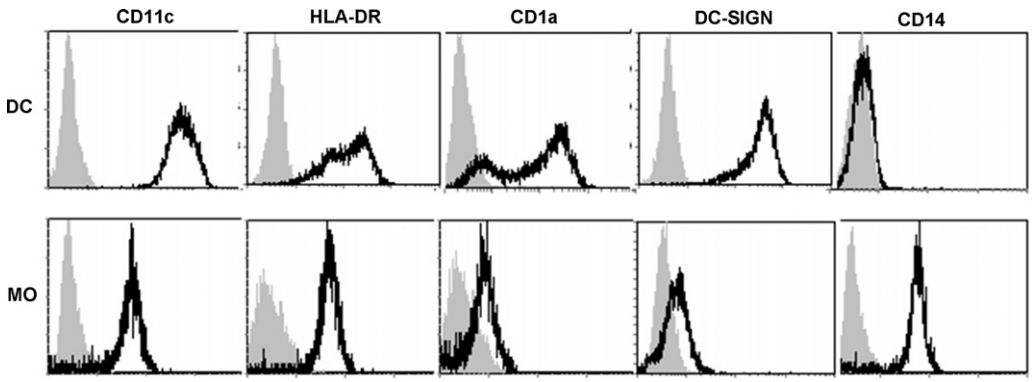

Fig. 1. Dendritic cell and macrophage phenotype. Dendritic cells (DC) or macrophages (MO) were differentiated for 7 days in the presence of GM-CSF, IL-4 and 10% normal human serum and the expression of surface molecules was analyzed by FACS. Similar results were obtained in three independent experiments using blood cells from three different donors. Filled gray corresponds to isotype control.

2.5. Statistical analysis

ELISA was performed in duplicates. Data obtained by flow cytometry or ELISA were from 5 to 12 individuals and were subjected to Student's paired *t*-test using SigmaStat 3.5 software. A normal distribution was assumed for small sample sizes. We considered the data significant when $P < 0.05$.

3. Results

3.1. Serum and cytokines promote monocyte differentiation into DCs

Differentiation of human monocytes into DCs is characterized by increased expression of CD11c, MHC-II, CD1a and DC-SIGN molecules and down-regulation of CD14 (Sallusto and Lanzavecchia, 1994; Geijtenbeek et al., 2000). Here, DCs differentiated for 7 days in medium supplemented with GM-CSF, IL-4 and normal human serum (NHS) showed the characteristic $CD11c^+$, $HLA\text{-}DR^+$, $CD1a^+$, $DC\text{-}SIGN^+$, $CD14^-$ human DC phenotype (Fig. 1). In contrast, monocytes kept in culture for 7 days in the absence of cytokines resulted in characteristic macrophages with lower expression levels of CD11c, HLA-DR, CD1a and DC-SIGN molecules and high expression of CD14 (Fig. 1). We always observed lower expression of DC-SIGN, HLA-DR and CD86 in monocytes when compared to differentiated DCs (Table 1).

Table 1
Differential expression of DC-SIGN, HLA-DR and CD86 in monocytes and differentiated dendritic cells

	DC-SIGN		HLA-DR		CD86	
	%	MFI	%	MFI	%	MFI
Monocytes	84.75	104.13	95	214.8	71.26	22.27
NHS	100	749.89	100	661.17	100	129.8

Dendritic cells were differentiated for 7 days in the presence of GM-CSF, IL-4 and 10% normal human serum (NHS). The expression levels of DC-SIGN, HLA-DR and CD86 were analyzed by FACS in fresh isolated monocytes and differentiated DCs. MFI: mean fluorescence intensity. Similar results were obtained in other experiments using blood cells from different healthy adult donors.

3.2. Lack of C3 during DC differentiation negatively impacts DC-SIGN expression

The C-type lectin proteins DEC-205 and DC-SIGN mediate antigen uptake. We found low surface expression of DEC-205 (MFI range 11.55–15.96) (Fig. 2A) and high surface expression of DC-SIGN (MFI range 245.82–465.55) (Fig. 2B) following DC differentiation. The lack of C3 during differentiation resulted in decreased expression of DC-SIGN but had no effect on DEC-205 expression (Fig. 2A and B). DC maturation by LPS treatment induced a marked down-regulation of DC-SIGN expression that was independent of the presence of C3 (Fig. 2C). DEC-205 expression was not affected by LPS exposure (data not shown). The lower expression of DC-SIGN in the absence of C3 was maintained after LPS challenge (Fig. 2B, lower panel). Further, we found no impact of C3 on the percentage of cells expressing DEC-205 or DC-SIGN (data not shown).

3.3. Lack of C3 during DC differentiation negatively impacts CD1a and MHC-II expression

Differentiation of human monocytes into DCs is characterized by increased expression of surface molecules critical for antigen presentation and costimulation. We determined whether the absence of C3 would interfere in the surface expression levels of such molecules during differentiation.

CD1a and MHC-II are molecules that present antigens to Th cells. We found low CD1a (MFI range 12.3–105.54) (Fig. 3A) and moderate HLA-DR (MFI range 57.25–196.32) (Fig. 3B) expression levels following monocyte differentiation into immature DCs in NHS. In the absence of C3, CD1a as well as HLA-DR expression levels were consistently lower than in the presence of C3 (Fig. 3A and B, upper panel). These data suggest that C3 may be an important co-factor for CD1a and HLA-DR expression during DC differentiation. LPS stimulation increased expression levels of HLA-DR in DCs cultivated with NHS or C3DS (Fig. 3C). In contrast, LPS did not affect CD1a expression levels (data not shown). Importantly, although LPS treatment increased HLA-DR expression levels in the absence of C3 (Fig. 3C), HLA-DR expression levels in the absence of C3 were constantly lower

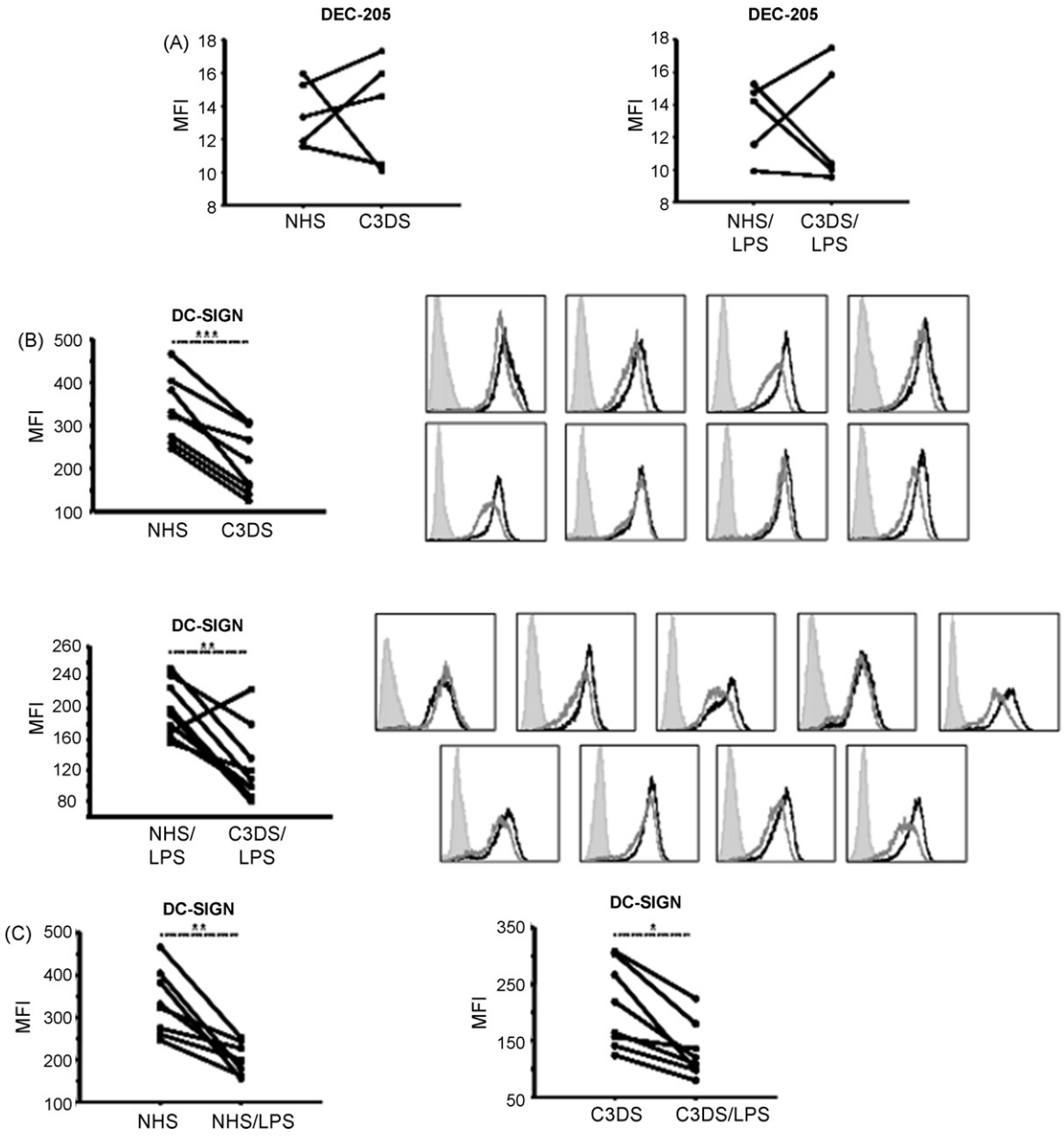

Fig. 2. C3 promotes DC-SIGN expression during DC differentiation. DCs were differentiated in the presence of GM-CSF, IL-4 and 10% normal human serum (NHS) or C3-deficient serum (C3DS). Where indicated cells were challenged with LPS (1 μg/ml) for 24 h. After 7 days of culture, the expression levels of DEC-205 (A) and DC-SIGN (B and C) were analyzed by FACS. MFI: mean fluorescence intensity. Each histogram represents data from an individual donor. Filled gray: isotype; solid black: NHS treated cells; solid gray: C3DS treated cells. *P* values were compared between groups using paired Student's *t*-test. $*P<0.05$, $**P<0.01$, $***P<0.001$.

than in the presence of C3 (Fig. 3B, lower panel). These data demonstrate that LPS challenge is not able to rescue the low HLA-DR expression following DC differentiation in the absence of C3, suggesting that C3 and LPS promote HLA-DR expression through activation of distinct pathways. The presence of C3 did not modulate the percentage of cells expressing CD1a or HLA-DR (data not shown).

3.4. Absence of C3 differentially regulates CD80 and CD86 expression during DC differentiation and maturation

Next, we assessed whether the absence of C3 during DC differentiation affects CD80, CD86 and CD40 molecule expression. Culturing the cells in NHS resulted in low to moderate CD80 (MFI range 44.51–210.97) (Fig. 4A), CD86 (MFI range 36.03–184.34) (Fig. 4B) and CD40 (MFI range 14.59–71.05, data not shown) surface expression in most donors. Intriguingly, two donors showed substantially higher surface expression of CD86 (MFI 261.8 and 296.93) (Fig. 4B, upper panel). When monocytes were differentiated in C3DS, surface expression levels of CD80 and CD86 were reduced (Fig. 4A and B, upper panel). Importantly, the reduction of CD86 expression in the absence of C3 was more profound in the two donors that exhibited high CD86 surface expression (Fig. 4B, upper panel). In contrast, the lack of C3 did not affect CD40 expression (data not shown). These data suggest that C3 may serve as a critical

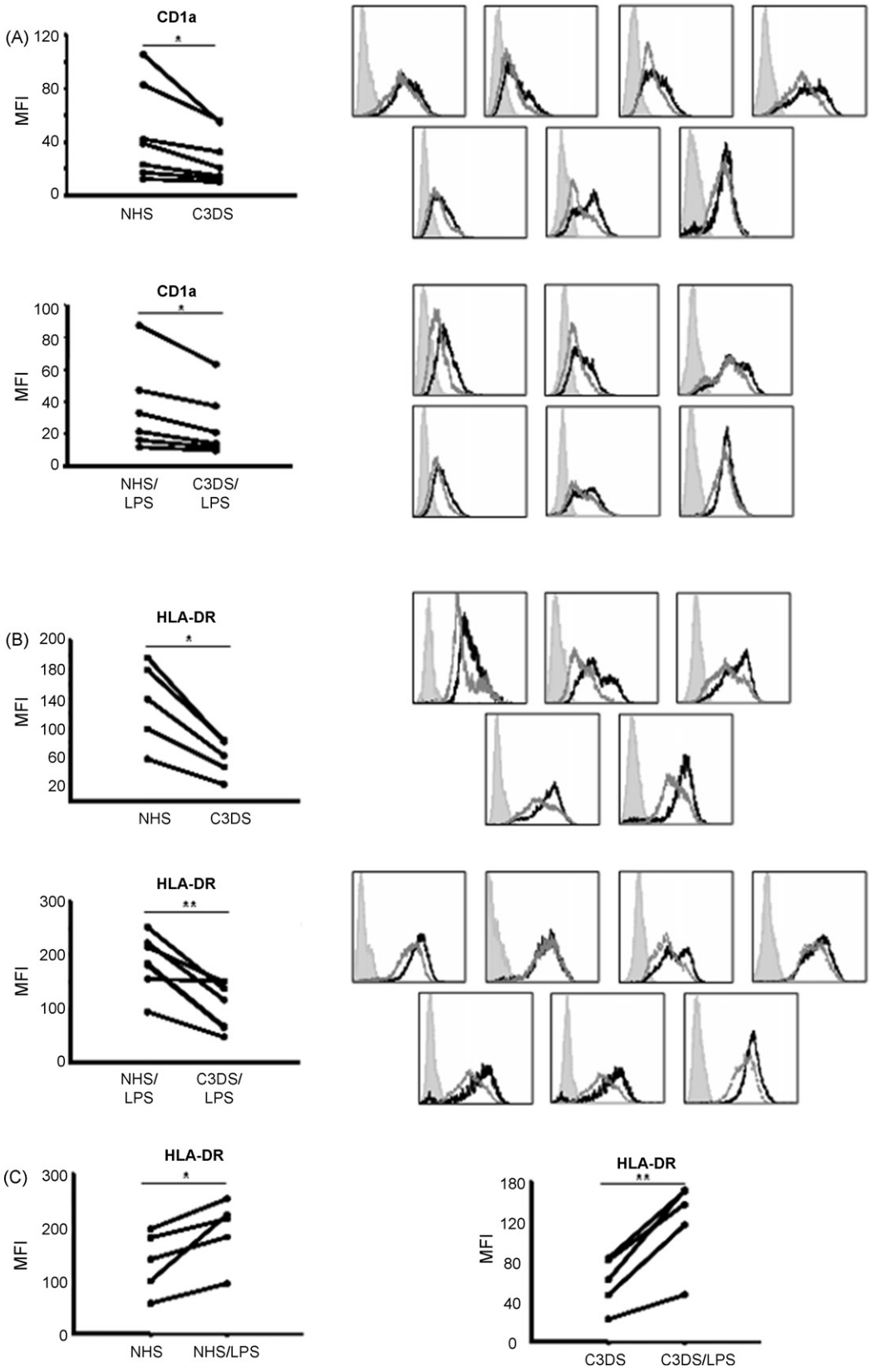

Fig. 3. C3 promotes CD1a and MHC-II expression during DC differentiation. DCs were differentiated in the presence of GM-CSF, IL-4 and 10% normal human serum (NHS) or C3-deficient serum (C3DS). Where indicated cells were challenged with LPS (1 μg/ml) for 24 h. After 7 days of culture, the expression levels of CD1a (A) and HLA-DR (B and C) were analyzed by FACS. MFI: mean fluorescence intensity. Each histogram represents data from an individual donor. Filled gray: isotype; solid black: NHS treated cells; solid gray: C3DS treated cells. *P* values were compared between groups using paired Student's *t*-test. $*P < 0.05$, $**P < 0.01$.

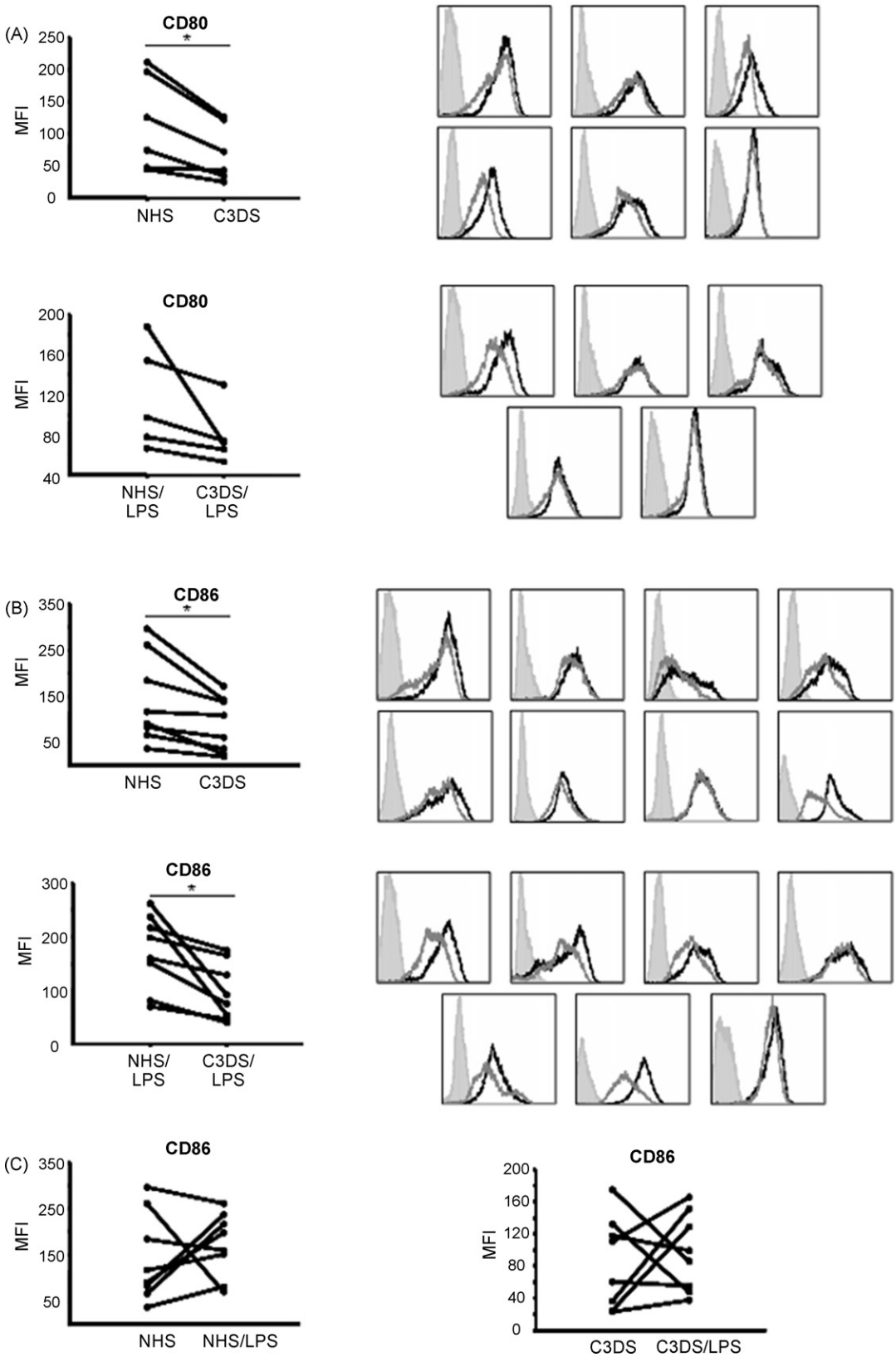

Fig. 4. C3 regulates CD80 and CD86 expression during DC differentiation. DCs were differentiated in the presence of GM-CSF, IL-4 and 10% normal human serum (NHS) or C3-deficient serum (C3DS). Where indicated cells were challenged with LPS (1 μg/ml) for 24 h. After 7 days of culture, the expression levels of CD80 (A) and CD86 (B and C) were analyzed by FACS. MFI: mean fluorescence intensity. Each histogram represents data from an individual donor. Filled gray: isotype; solid black: NHS treated cells; solid gray: C3DS treated cells. *P* values were compared between groups using paired Student's *t*-test. $^{*}P < 0.05$.

co-factor for up-regulation of co-stimulatory molecules during DC differentiation.

DC challenge with LPS resulted in up-regulation of CD86 surface expression on DCs from five out of six donors with low to moderate CD86 expression. In contrast, CD86 expression in response to LPS stimulation was reduced in the two donors with high CD86 expression (Fig. 4C, left panel). LPS challenge in the absence of C3 led to a significant reduction of CD86 expression as compared with DC cultured in NHS (Fig. 4B, lower panel). LPS stimulation did not impact CD40 or CD80 expression neither in the presence or absence of C3 (data not shown). These findings support the well-appreciated view that LPS challenge up-regulates CD86, however, they also demonstrates that LPS can reduce CD86 expression in some individuals. As described for DC-SIGN and HLA-DR expression LPS stimulation is not able to counterbalance the low CD86 expression resulting from the lack of C3 during DC differentiation. The percentage of cells expressing the different co-stimulatory molecules was not affected by the absence of C3 (data not shown).

3.5. C3 has no regulatory impact on CD11c and CD83 expression

Monocyte differentiation into a DC phenotype is associated with the increase of the CD11c marker. We found a moderate to strong surface expression of CD11c in the monocyte-derived DCs (MFI range 39.95–777.37) which was independent of the presence or absence of C3 (Fig. 5A). The CD83 maturation marker was scarcely expressed following DC differentiation independent of C3 (Fig. 5B). LPS challenge had only a modest impact in the CD83 expression levels (MFI range 9.82–14.72 in NHS treated DCs and 9.31–27.38 in NHS/LPS treated DCs) (data not shown).

3.6. C3 does not modulate complement receptor expression

Our data suggest that C3 regulates several DCs surface molecules and our previous findings that DCs express several complement receptors (Reis et al., 2007) prompted us to assess the impact of C3 on the expression of complement C3 receptors CR1 and CD18 (the common chain for CR3 and CR4) during DC differentiation. We found low expression of CR1 (MFI range 12.63–27.88) and CD18 (MFI range 50.94–83.54) on monocytes-differentiated DCs which was independent of the presence of C3 during the differentiation process (Fig. 5C and D).

Collectively, our data demonstrate that complement C3 plays important roles in the regulation of DC surface molecule expression during DC differentiation that are well appreciated for their important roles in antigen uptake, presentation and activation of Th cells. Further, our findings provide evidence that C3 has a regulatory impact on LPS-induced maturation of DCs through the modulation of DC-SIGN, HLA-DR and CD86 expression.

3.7. Lack of C3 impacts cytokine production during DC differentiation and LPS-induced maturation

Next, we determined whether C3 might also regulate the ability of DCs to produce cytokines important for Th cell activation and differentiation such as IL-6, IL-10, IL-12 and TNF-α. We found minor production of TNF-α by immature DCs, which was independent of the presence of C3 (Fig. 6A). In contrast, DCs differentiated in NHS produced considerable amounts of IL-6, which were significantly lower in the absence of C3 (Fig. 6B). Further, we found that immature DCs produce IL-12p40 and some IL-12p70 (Figs. 6C and D). Importantly, we observed a clear trend towards reduced production of IL-12p40 and IL-

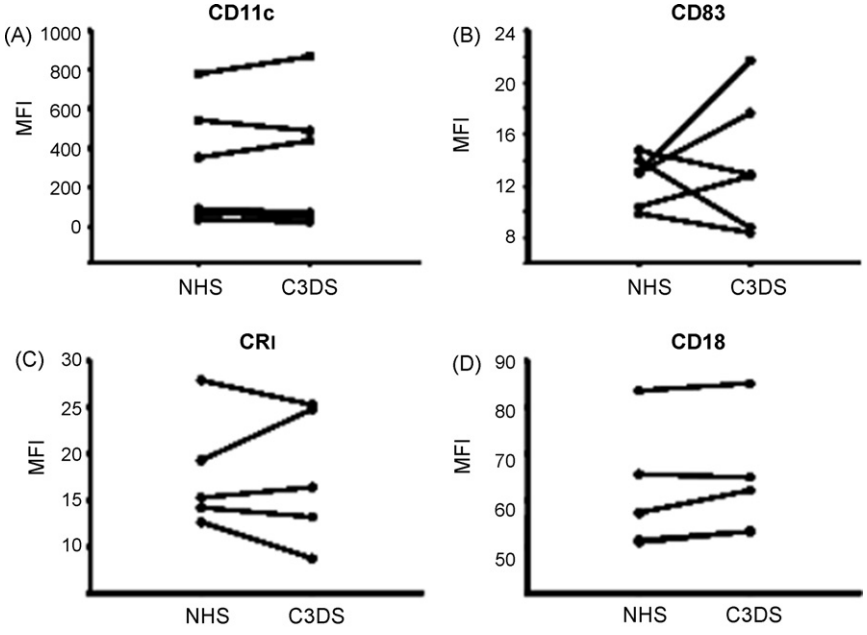

Fig. 5. C3 does not modulate CD11c, CD83 and complement receptors expression. DCs were differentiated in the presence of GM-CSF, IL-4 and 10% normal human serum (NHS) or C3-deficient serum (C3DS). After 7 days of culture, the expression levels of CD11c (A), CD83 (B) and complement receptors (C and D) were analyzed by FACS. MFI: mean fluorescence intensity. *P* values were compared between groups using paired Student's *t*-test.

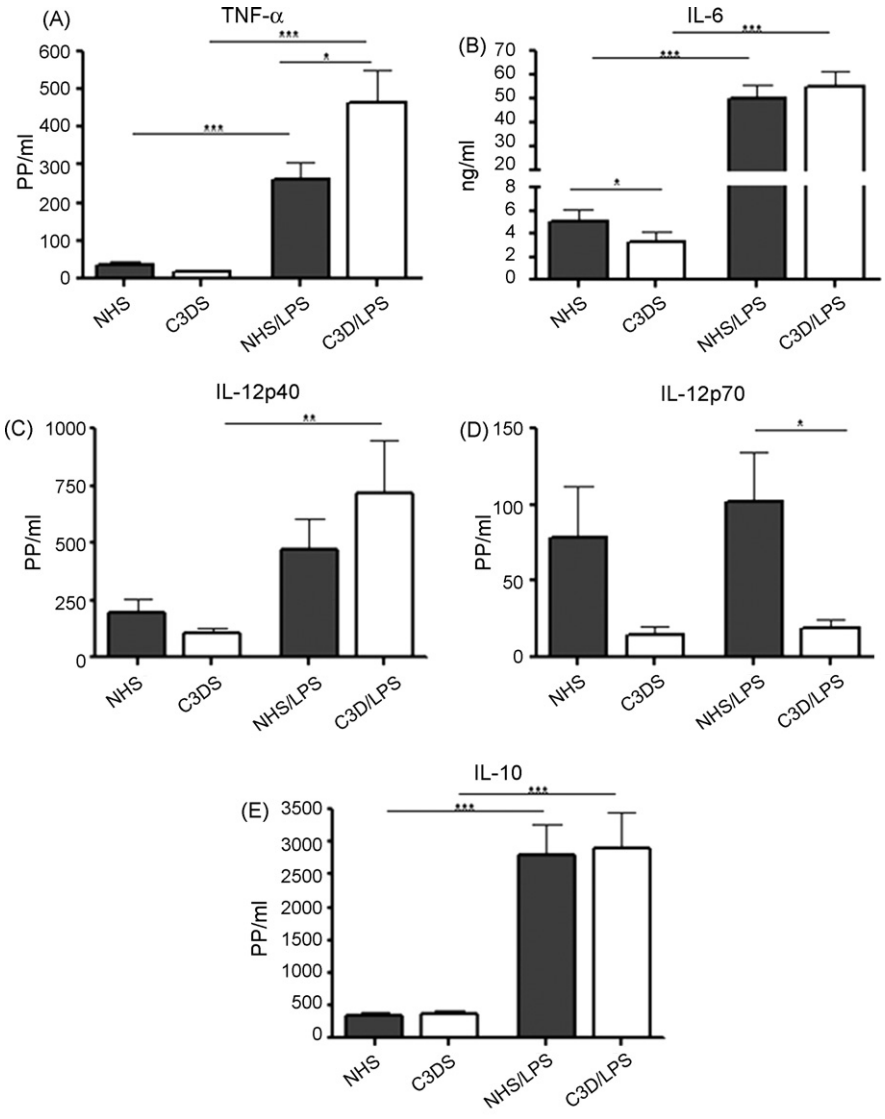

Fig. 6. C3 regulates cytokine production during DC differentiation. Dendritic cells were differentiated in the presence of GM-CSF, IL-4 and 10% normal human serum (NHS) or C3-deficient serum (C3DS). Where indicated cells were challenged with LPS (1 μg/ml) for 24 h. After 7 days of culture, the cytokine production was measured by ELISA. *P* values were compared between groups using paired Student's *t*-test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

12p70 in the absence of C3, however, due to the large inter-donor variability these values did not reach the level of statistical significance (Figs. 6C and D). In addition to IL-12, we found substantial C3-independnet production of IL-10 (Fig. 6E).

LPS challenge of differentiated DCs resulted in increased production of TNF-α, IL-6, IL-10 and IL-12p40 (Figs. 6A–C and E) in the presence or absence of C3. In contrast, IL-12p70 production was not affected by LPS stimulation with or without C3 (Fig. 6D). Of note, differentiated DCs stimulated with LPS produced significantly lower concentrations of IL-12p70 in the absence than in the presence of C3 (Fig. 6D), suggesting that C3 contributes to IL-12p70 production from mature monocyte-derived DCs.

In contrast to the positive regulatory impact of C3 on IL-12p70 production, we found significantly increased DC-derived TNF-α production in response to LPS in C3DS as compared with NHS cultured DCs (Fig. 6A). These data provide evidence that C3 acts as a negative regulator of LPS-induced production of TNF-α from human DCs.

3.8. Addition of C3 restores impaired expression of DC surface molecules

The regulatory effects of C3DS on the DC phenoytpe and DC function may not only result from the lack of C3 but from other primary or secondary immune defects of the patient that could impact on composition of serum factors. To address this issue, we added different concentrations of purified C3 to the culture. As shown in Table 2, DCs from C3-reconstituted cultures have then been used to determine the expression levels of HLA-DR, CD80 and C86. We found that increasing concentrations of C3 (50–150 μg/ml) lead to a dose-dependent increase in the expression levels of HLA-DR, CD80 and CD86. Further, we found increased expression levels of DC-SIGN after C3DS

Table 2
Expression of HLA-DR, CD80 and CD86 (mean fluorescence intensity) after dendritic cell differentiation

Purified C3 (μg/ml)	Experiment 1			Experiment 2		
	HLA-DR	CD80	CD86	HLA-DR	CD80	CD86
0	125.21	11.04	31.06	103.66	11.14	20.35
50	171.54	21.58	34.6	110.4	29.43	35.23
100	203.51	29.69	34.6	164	33.98	50.03
150	399.54	34.6	53.28	294.27	35.55	67.32

Dendritic cells were differentiated in the presence of GM-CSF, IL-4 and different concentrations of purified C3. After 7 days of culture, the expression levels of HLA-DR, CD80 and CD86 was analyzed by FACS. Two independent experiments are shown.

was reconstituted with 100 μg/ml of purified C3 (Table 3). In contrast, down-regulation of CD1 in C3DS was not affected by C3 reconstitution (data not shown). These data strongly suggest that the absence of C3 accounts for most of the negative regulatory effects on DC surface molecule expression that we observed following DC differentiation in C3DS.

4. Discussion

Recent awareness of the key immunoregulatory role of complement in infectious and inflammatory disease models has fueled interest in its role in DC function (Köhl and Wills-Karp, 2007; Zhou et al., 2007). Several recent reports suggest that complement proteins regulate differentiation of precursor cells; however, the role of C3 in DC differentiation from human monocytes and their maturation in response to bacterial compounds such as LPS has not been assessed. For example, C3a potentiates CXCL12-dependent chemotaxis of human $CD34^+$ cells (Reca et al., 2003). Further, C3-defcient mice show increased mobilization of hematopoietic stem cells in response to G-CSF, suggesting that the signaling through C3aR pathways prevent the egress of hematopoietic stem cells from the bone marrow into the circulation (Ratajczak et al., 2004). Moreover, it was shown that fragments C3a and C5a regulate IL-6 and TNF-α production and nuclear factor kappa B and STAT-3 activation, essential for full liver regeneration (Strey et al., 2003). In addition to the anaphylatoxins, CR2 has been demonstrated to regulate cell differentiation. More specifically, recent data show that CR2 mediates plasma cell differentiation through the induction of Blimp-1 and XBP-1 (Gatto et al., 2005).

Table 3
Expression of DC-SIGN after dendritic cell differentiation

	DC-SIGN (MFI)	
	Experiment 1	Experiment 2
NHS	78.44	313.4
C3DS	44.11	67.93
C3DS + C3 (100 μg/ml)	113.42	88.96

Dendritic cells were differentiated in the presence of GM-CSF, IL-4 and 10% normal human serum (NHS) or C3-deficient serum (C3DS) or C3DS + 100 μg/ml purified C3. After 7 days of culture, the expression levels of DC-SIGN were analyzed by FACS. Two independent experiments are shown. MFI: mean fluorescence intensity.

Here, were provide evidence that the absence of C3 during human monocyte differentiation into DCs and/or their maturation in response to LPS impacts the expression of the C-type lectin receptor DC-SIGN, the antigen presenting molecules CD1a and HLA-DR, the costimulatory molecules CD80 and CD86 and the production of IL-6, TNF-α and IL-12p70. C3 is the most abundant complement protein, reaching plasma concentrations from 0.8 to 1.9 mg/ml in healthy adults (Ritchie et al., 2004). C3 is mainly produced by hepatocytes (Alper et al., 1969), although extra-hepatic sources may substantially contribute to C3 production in tissues (Reis et al., 2006b). C3 activation is the critical step of complement activation in response to classical, alternative and lectin pathway activation. C3 cleavage by specific proteolytic enzymes, the so-called C3 convertases, leads to generation of C3a, C3b, iC3b, C3dg cleavage fragments that regulate innate and adaptive immune responses through activation of distinct complement receptors on a variety of distinct cell types including phagocytes, DCs and B cells (Walport, 2001; Köhl, 2006; Kemper and Atkinson, 2007; Carroll, 2004).

To assess the role of C3 in the differentiation process of human monocytes into immature DCs, we used C3 deficient serum from a patient suffering from C3 deficiency (Ulbrich et al., 2001; Reis et al., 2004). Thus, monocytes were almost devoid of C3 during their differentiation into immature DCs. However, we cannot rule out that the differentiating cells may have produced minor amounts of C3 (Reis et al., 2006a; Peng et al., 2006). In fact, the inter-donor variations of the C3-dependent effects may be in part explained by different endogenous C3 production from donor DCs. C5a serum concentrations were indistinguishable between NHS (60.35 ng/ml) and C3DS (62.32 ng/ml) suggesting that C5a does not contribute to the effects of C3DS on DC differentiation. The generation of C5a in C3DS is likely to result from thrombin-dependent C5a generation (Huber-Lang et al., 2006).

Human monocyte differentiation into DCs is characterized by the up-regulation of CD1a. Our data provide evidence that C3DS regulates this process; however, it does not depend on C3, as C3 reconstitution of C3DS failed to upregulate CD1a expression levels. Importantly, we further found that C3 is required for the up-regulation of MHC-II and co-stimulatory molecules which provide two critical signals for *naïve* T cell activation (Banchereau and Steinman, 1998). The fact that C3 also appears to be required for IL-12p70 production highlights the importance of C3 during the differentiation and the maturation process and

suggests a role for C3 in Th1 skewing by IL-12 (de Jong et al., 2005). The reduced expression of co-stimulatory molecules and the impaired IL-12 production is likely to affect the development of adaptive immune responses in C3-deficient patients. In support of this view, of the 27 reported cases of homozygous C3 deficiency, seven patients presented autoimmune manifestations and seven had glomerulonephritis, proteinuria or IgA nephropathy and higher susceptibility to severe infections (Reis et al., 2006b).

In addition to IL-12p70, we found that C3 is regulating the LPS-induced production of TNF-α. In contrast to the decreased IL-12p70 production in the absence of C3, TNF-α production was significantly enhanced. The negative regulatory impact of C3 on TNF-α production may result from lack of C3a generation which has been described to decrease the production of cAMP (Sayah et al., 2003). One important function of cAMP is to inhibit the production of TNF-α in various cell types including human peripheral blood mononuclear cells (Zidek, 1999).

At this point it remains open which downstream products of C3 contribute to the observed regulatory effects. A recently work (Chen et al., 2007) showed that human monocyte-derived DCs differentiated in the presence of sublytic C5b-9 up-regulate HLA-DR and several co-stimulatory molecules including CD80 and CD86. However, in contrast to our findings C5b-9 did also affect CD83. Further, C5b-9 treated DCs produced more IL-12 and TNF-α than untreated controls, while we found opposing effects of C3 in IL-12 and TNF-α production. These authors did not investigate the impact of C5b-9 in the expression of C-type lectin receptors, IL-6 and IL-10. Thus while some of the effects of C3-deficiency may be explained by the effects of sublytic concentrations of C5b-9, the different effects on CD83, CD1a surface expression, cytokine production and our findings that C3 regulates C-lectin receptor expression suggest that different pathways downstream of C3 mediate the profound effect of C3 on DC differentiation and maturation. Candidates include C3a which was found to increase the expression of CD54, CD83 and CD86 but not of CD40, CD80 and HLA-DR in human monocyte-derived DCs treated with a combination of IFN-α and IFN-γ (Gutzmer et al., 2004). This treatment further increased the production of IL-10 and IL-12p70. In addition, these authors showed that C3a has a direct migratory effect on DCs. A C3a- and C5a-dependent migratory effect of monocytes was also reported by (Soruri et al., 2003a) who found increased migration of intravenously injected monocytes into the peritoneal cavity of severe combined immune deficient (SCID) mice. Importantly, C5a not only recruited the monocytes into the peritoneum but induced their differentiation into DCs by a TNF-α and PGE_2-dependent mechanism (Soruri et al., 2003b).

In summary, we describe a novel role for C3 as an important factor for DC differentiation in concert with growth factors and cytokines. Further, our findings suggest cross-talk between pattern recognition pathways activated by LPS and complement receptors that regulate the expression of MHC-II, co-stimulatory molecules and cytokine production. Such cross-talk has been already been demonstrated in animal models to critically impact the development of adaptive immune responses *in vitro* and *in vivo* (Hawlisch et al., 2005; Zhang et al., 2007) and is likely to affect the development of maladaptive immune responses in humans leading to allergy and autoimmunity.

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