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The α7β0 Isoform of the Complement Regulator C4b-Binding Protein Induces a Semimature, Anti-Inflammatory State in Dendritic Cells

Rut Olivar,* Ana Luque,* Mar Naranjo-Gómez, † Josep Quer, † Pablo García de Frutos,§ Francesc E. Borràs, ‡,† Santiago Rodríguez de Córdoba, †,‖ Anna M. Blom, †,‖ and Josep M. Aran*

The classical pathway complement regulator C4b-binding protein (C4BP) is composed of two polypeptides (α- and β-chains), which form three plasma oligomers with different subunit compositions (αβ1, αβ0, and α6β1). We show in this article that the C4BP α7β0 isoform (hereafter called C4BP[β0]) activity on these cells. Immature DCs (iDCs) treated with C4BP(β0) retained high endocytic activity, but, upon LPS treatment, they did not upregulate surface expression of CD83, CD80, and CD86. Transcriptional profiling of these semimature DCs revealed that treatment with C4BP(β0) prevented the induction of IDO and BIC-1, whereas TGF-β1 expression was maintained to the level of iDCs. C4BP(β0)-treated DCs were also unable to release proinflammatory Th1 cytokines (IL-12, TNF-α, IFN-γ, IL-6, IL-8) and, conversely, increased IL-10 secretion. They prevented surface CCR7 overexpression and, accordingly, displayed reduced chemotaxis, being morphologically indistinguishable from iDCs. Moreover, C4BP(β0)-treated DCs failed to enhance allogeneic T cell proliferation, impairing IFN-γ production in these cells and, conversely, promoting CD4+CD127low/neg CD25highFoxp3+ T cells. Deletion mutant analysis revealed that the complement control protein-6 domain of the α-chain is necessary for the tolerogenic activity of C4BP(β0). Our data demonstrate a novel anti-inflammatory and immunomodulatory function of the complement regulator C4BP, suggesting a relevant role of the acute-phase C4BP(β0) isoform in a number of pathophysiological conditions and potential applications in autoimmunity and transplantation. The Journal of Immunology, 2013, 190: 2857–2872.

Complement is a major component of innate immunity with crucial roles in pathogen and apoptotic cell clearance, immune complex handling, and modulation of adaptive immune responses (1). The complement cascade is triggered by three activation pathways, the classical pathway, the lectin pathway, and the alternative pathway, which converge in the formation of unstable protease complexes, named C3 convertases that cleave C3 to generate the activated fragment, C3b. Because a disproportionate complement response may lead to organ damage and pathology, complement activation is strictly controlled by a number of soluble or membrane-associated regulatory proteins that dissociate the C3/C5 convertases and function as cofactors for the proteolysis of the activated proteins C3b and C4b (2, 3). C4b-binding protein (C4BP) is the major soluble inhibitor of the classical and lectin pathways of complement activation. This large (570-kDa) oligomeric plasma glycoprotein is primarily synthesized in the liver and secreted in three main isoforms to the circulation (4). The major C4BP isoform (75–80% in plasma) is composed of seven identical 70-kDa α-chains and a single 40-kDa β-chain (α7β1) joined in their C termini by disulphide bonds, resulting in a spider-like structure (5–7). The α- and β-chains of C4BP are composed of, respectively, eight and three 60-aa residue domains termed “short consensus repeats,” “Sushi,” or “complement control protein” (CCP) domains (8). The C4BP containing

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Abbreviations used in this article: C4BP, C4b-binding protein; C4BP[β0], C4BP lacking the β-chain; C4BP[β1], C4BP containing the β-chain; CCP, complement control protein; DC, dendritic cell; iDC, immature DC; mDC, mature DC; PtoS, protein S; RT-qPCR, quantitative RT-PCR; Treg, regulatory T cell.

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the β-chain (C4BP[β⁺]) isoforms (αββ₁ and ααβ₂) function as a cofactor in factor I-mediated proteolysis of activated complement factors C4b and C3b, and accelerate the decay of classical C3/C5-convertases (9, 10). In addition, the presence of the β-chain enables them to limit necrotic and apoptotic cell-mediated complement activation (11, 12). No other functions, apart from its complement regulatory activity, have been reported for the C4BP lacking the β-chain (C4BP[β⁻]) (αββ₂) isoform. Interestingly, however, during acute-phase conditions, this C4BP[β⁻] isoform becomes the majority as a consequence of the differential regulation of the genes encoding the α-chain and the β-chain by proinflammatory cytokines (13, 14). It has been recently reported that i.p. administration of human C4BP was able to inhibit the development of autoimmune arthritis in mice, although it was only moderately affecting complement activity (15). This suggests that other mechanisms might be involved in the anti-inflammatory activity of C4BP.

Dendritic cells (DCs) are key components of innate immunity. Located in most tissues, they have a unique ability to sense pathogens (infectious agents, foreign tissues, and inflammatory products), process them while migrating into the lymph nodes, and further act as professional APCs critical in regulating the adaptive immune response by stimulating the activation and polarization of T cells (16, 17). Nevertheless, the role of DCs as mobile sentinels is dichotomous; they may both present Ags and the appropriate costimulatory molecules to initiate an adaptive immune response, or they may induce tolerance and release anti-inflammatory signals (18, 19). Indeed, tolerogenesis is associated with altered DC behavior (immature phenotype, altered cytokine expression pattern), playing a protective function in autoimmunity and graft acceptance (20).

The α-chains of C4BP bind to several ligands, including the complement proteins C3b and C4b, heparin, pentraxins, CD91, C4BP([2]), which sustains a fundamental role in DC biology. Thus, C4BP might affect DC activation by interacting with CD40 and/or with another as yet unidentified ligand. In this study, we suggest a novel function of the acute-phase–induced C4BP([2]) isoform regulating DC activation during inflammation. Indeed, tolerogenesis is associated with altered DC behavior (immature phenotype, altered cytokine expression pattern), playing a protective function in autoimmunity and graft acceptance (20).

Materials and Methods

Proteins

We used throughout the study both C4BP(β⁺) and C4BP(β⁻) isoforms. C4BP(αββ₁) isoforms refer to the major C4BP αββ₁ (Fig. 1A, left) plus the minor C4BP αββ₁ isoforms (both in complex with ProS) purified from pooled human plasma, as previously described (6). In this study, the C4BP (β⁻) isoform refers to both: 1) the polymeric recombiant full-length C4BP ααβ₂, expressed in HEK293 cells (CRL-1573; American Type Culture Collection, Manassas, VA) and purified by affinity chromatography after FastGel column (Bio-Rad, Hercules, CA) coupled with mAb MK104 against the CCP1 domain of the C4BP α-chain (23); and 2) human plasma C4BP ααβ₂, analogously purified by affinity chromatography after C4BP[β⁻]-ProS removal by BaCl₂ precipitation (6, 15) (Fig. 1A, center and right, respectively). The corresponding C4BP ααβ₂ mutants lacking individual α-chain CCPs (ΔCCP1-5) were also expressed in eukaryotic cells and purified by affinity chromatography (23).

Cell culture and C4BP treatment

RPMI 1640 was supplemented with 100 μg/ml streptomycin, 100 IU/ml penicillin, 2 mM l-glutamine (all from Invitrogen, Carlsbad, CA) and 10% heat-inactivated FBS (Linus, Culek, Spain) (complete medium), unless otherwise stated. PBMCs were obtained from buffy coat preparations collected from healthy donors from the Blood and Tissue Bank (Barcelona, Spain) after Ficoll-Paque density centrifugation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). For surface phenotype determination, monocytes were plated at 1 × 10⁵ cells/ml in 60-mm culture plates (Cultek, Madrid, Spain), in RPMI 1640 medium without serum, and allowed to adhere for 2 h at 37°C in 5% CO₂. The nonadherent cells were removed by washing in PBS. The final population contained >75% of monocytes, as demonstrated by flow cytometry of anti-CD14–stained isolated. For all functional assays, including the DC:T cell cocultures, monocytes were purified using colloid superparamagnetic microbeads conjugated with monoclonal mouse anti-human CD14 Abs (MACS [Miltenyi Biotec, Auburn, CA] or EasySep Human CD14 Positive Selection Kit [Stem Cell Technologies, Grenoble, France]), and counted using Perfect Count microspheres (CytoSens LS, Salamanca, Spain). The purity of CD14+ cells was tested by CD14 staining and flow cytometry analysis (>90% CD14+ cells).

Monocyte-derived DCs were generated supplementing the monocyte cultures with complete RPMI 1640 medium plus GM-CSF (800 IU/ml) and IL-4 (500 IU/ml; both from Gentaur, Kampenhout, Belgium) at days 0 and 3 of culture. For DC maturation, at day 5, iDCs were further stimulated for 48 h with either 5 μg/ml LPS (Escherichia coli 055.B5, Sigma L2880; Copenhagen, Denmark) or 2 μg/ml recombinant human sCD40 Ligand/ TRAP (ProSpec, Rehovot, Israel).

CD3+ T cells were isolated from PBMCs by negative selection using EasySep Human T Cell Enrichment Kit (Stem Cell Technologies). CD3+ T cells were >90% pure, as assessed by CD3 staining through flow cytometry.

Both C4BP(β⁺) and C4BP(β⁻) isoforms were added at 2, 5, or 10 μg/ml, at days 0, 3, and 5 (at the last time point, combined with LPS or CD40L; differentiation and maturation), or only at day 5, either alone or combined with LPS (maturation). For control and comparison purposes, in some assays, we included a parallel, analogous treatment with the immunomodulator vitamin D3 (calcitriol [Calcijex]; Abbott Laboratories) at 1 nM.

Double staining using the fluorescent dyes Annexin V (Annexin V-PE Apoptosis Detection Kit I; BD Pharmingen, San Diego, CA) and 7-amino-actinomycin D (BD Pharmingen), and flow cytometry analysis were used to assess the viability/apoptosis status of C4BP-treated, vitamin D3–treated, and untreated DCs.

Abs and flow cytometry

Cell-surface phenotypes were analyzed using the following mAbs: FITC-conjugated anti–HLA-DR (IgM-357), FITC-conjugated anti–CD38 (HI108), FITC-conjugated anti–CD14 (RMO52), PE-conjugated anti–CD4 (I208), PE-conjugated anti–CD8 (I202), PE-conjugated anti–CD45RA (I241), PE-conjugated anti–CD45RO (I255), PE-conjugated anti–CD1a (C1A1), PE-conjugated anti–CD86 (HA5.2B7; all from Becton Dickinson). Subsequent analyses used FlowJo software (Treestar, Ashland, OR).

Quantitative RT-PCR

DCs (10⁶/condition) were harvested at day 7, and the mRNA was extracted using the RNeasy RNA Isolation kit (Qiagen) and incubated with RNase-free DNase I (Ambion, Austin, TX) according to the manufacturer’s protocol. A two-step real-time RT-PCR technique was used to determine the relative mRNA levels of the human C-C chemokine receptor type 7 (CCR7), the precursor of mir-155 (BIC-7), and IDO (IDO). Reverse transcription reactions were performed with 500 ng total RNA using the Omniscript RT kit (Qiagen). Quantification of mRNA levels was performed by real-time PCR with the use of the LightCycler technology.
(Roche Molecular Biochemicals, Indianapolis, IN). The following primers were used: CCR7-f (5'-TGGGCATCTGAGATCTAGC-3'), CCR7-r (5'-AA-GAAAGGTGACGCACG-3'), IDO-f (5'-GGTCATGGAGATGTCCGAA-3'), IDO-r (5'-CAAATAGAGACAGACAAAGA-3'); BIC-1-f (5'-AACCTACCAGAGACCTTACC-3'), BIC-1-r (5'-ATGCTTCTTTGTCCATCCTCC-3'), yielding products of 435, 227, and 296 bp, respectively. These gene-specific primer pairs were designed using Oligo 4.0 and Primer 3 softwares (MBI, Cascade, CO) and selected to prevent primer-dimer formation.

All samples were normalized with the use of the following primer set for the constitutively expressed human cyclophilin A gene (CypA; PPIA): CypA-f (5'-CTCCTTTGAGCTGTTTGCAG-3') and CypA-r (5'-CACACATGCTTGCCATCC-3'). All primers were purchased from Bonsai Technologies (Copenhagen, Denmark).

PCR amplifications were performed in a 20 μl volume containing 2 μl ready-to-use reaction mix, 10× DNA Master SYBR Green I (Roche Molecular Biochemicals); MgCl₂ (3 mM for CCR7, 4 mM for BIC-1, and 5 mM for IDO); 0.15 μM of each primer; 5% DMSO; and 75 ng cDNA as template. The amplification program used an initial denaturation at 95°C for 10 min, followed by 45 cycles: 95°C for 1 s; 58°C (CCR7)/60°C (BIC-1 and IDO) for 5 s; 72°C for 10 s.

The relative levels of TGF-β1 were assessed using TaqMan technology. In brief, total RNA was converted to single-stranded cDNA using the high-capacity cDNA archive kit according to the manufacturer’s instructions (Applied Biosystems, Carlsbad, CA). Each TaqMan gene expression assay contained a forward and reverse primer both for TGF-β1 (Hs00998133_M1) and for PPIA (Hs99999904_M19; Applied Biosystems) chosen for normalization. The real-time RT-PCR amplifications were run on an ABI Prism 7900Ht sequence Detection System (Applied Biosystems). Thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. The reproducibility of the assays was verified, and the expression of the four genes was shown to be within the linear range at the chosen cell concentration.

Scanning electron microscopy

Monocytes were seeded on glass slides covered with either poly-L-lysine (25 μg/ml) or fibronectin (42 μg/ml), cultured for 5 d in complete RPMI 1640 medium supplemented with 800 U/ml GM-CSF, 500 U/ml IL-4, and the C4BP(b1) or C4BP(b2) isoforms, or the C4BP α6β0 deletion mutants, and further stimulated with LPS for 48 h in the same medium. The resulting DCs were fixed in 1% paraformaldehyde and 1.25% glutaraldehyde in cacodylate buffer for 2 h. Finally, the cells were postfixed in 1% osmium tetroxide and dehydrated with graded series of ethanol followed by acetone. After dehydration, the cells were dried in a critical-point dryer and coated with gold before observation by scanning electron microscopy.

![Figure 1](http://www.jimmunol.org/)"
(Zeiss DSM940A). To quantify the percentage of iDCs or mature DCs (mDCs) under a given condition, 100 cell profiles were recorded by a blinded scorer using random scans at a fixed magnification (×1750).

Endocytic activity
Endocytosis of iDCs and mDCs was assessed using DQ-OVA (1 mg/ml; Molecular Probes, Leiden, The Netherlands) and Lucifer Yellow CH (10 mg/ml; Sigma). DQ-OVA is a mannose receptor ligand consisting of naturally mannosylated OVA extensively labeled with the fluorochrome BODIPY. Because of oversaturation with BODIPY, self-quenching occurs. Thus, fluorescent detection will occur only if DQ-OVA degrades within an endolysosomal compartment. Conversely, loading of the polar tracer Lucifer Yellow is usually accomplished by pinocytosis. In brief, 2 × 10^5 cells/ml were resuspended in 100 μl PBS and incubated either with 4 μl BODIPY FL-conjugated DQ-OVA at 37°C or at 0°C for 15 min (receptor-dependent endocytosis), or with 6 μl Lucifer Yellow CH at 37°C or at 0°C.

FIGURE 2. The C4BP(β−) isoform modulates the overall activation phenotype of human DCs. Human DCs were incubated throughout their differentiation and maturation process with 2 μg/ml of the appropriate C4BP isoforms, unless otherwise indicated. DC maturation was achieved by LPS treatment. Cells were then collected, washed, and analyzed by flow cytometry for cell-surface expression of HLA-DR, CD14, CD40, CD80, CD83, CD86, and CD1a. (A) Each histogram is representative of three to eight independent experiments. Isotype controls are shown in gray. The median fluorescence intensities (MFIs) for the different cell surface markers are indicated. (B) Relative MFI for the different cell surface markers. C4BP(β−), recC4BP(β−); C4BP(β−), C4BP isoform-treated, LPS-matured DCs; iDC, untreated iDCs; mDC, untreated, LPS-matured DCs; mo, peripheral blood monocytes. Results shown are the mean ± SD from three to eight independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 compared with mDC.
for 2 h (fluid-phase endocytosis). The incubations were stopped by adding 1 ml cold FACS buffer. The cells were washed two times with cold FACS buffer, and their fluorescence was analyzed using flow cytometry.

**DC cytokine secretion**

Concentrations of IL-12p70, TNF-α, IFN-γ, IL-10, IL-6, and IL-8 were determined from DC supernatants treated with the C4BP(β⁺) or C4BP(β⁻) isoforms using the Th1/Th2 Flow cytometry Multiplex kit (Bender-Medystems, Vienna, Austria), according to the manufacturer’s instructions. Alternatively, secretion of IL-12p70, TNF-α, and IFN-γ from C4BP ΔCCP6- and C4BP ΔCCP8-treated DCs was assessed with the Human Inflammatory Cytokines Multi-Analyte ELISArray Kit (SA Biosciences, Frederick, MD).

**Chemotaxis assays**

DCs differentiated and matured (LPS for 48 h) in the presence of the C4BP(β⁺) or C4BP(β⁻) isoforms were tested for migration toward the CCL21 chemokine using transwell assays. In brief, the lower chambers of transwell plates (polycarbonate filters of 8.0-μm pore size; Costar, Corning, NY) were filled with 400 μl complete RPMI 1640 medium with or without CCL21 (200 ng/ml). A total of 1 × 10⁵ DCs in 100 μl complete RPMI 1640 medium was added into the upper chamber, and cells were incubated at 37°C for 2 h. We verified the absence of filter-associated DCs by removing nonmigratory cells from the upper chamber and upper side of the membrane and examining the underside by crystal violet staining. Cells that migrated into the lower chambers were harvested and counted with a flow cytometer, acquiring events for a fixed time period of 2 min. The migration assays for all stimulation conditions were performed in triplicate wells. Values are given as percentage of migrated cells relative to the untreated mDCs (100%).

**Intracellular cytokine staining**

T cells were labeled with the intracellular fluorescent dye CFSE (Molecular Probes), plated in 96-well round-bottom plates at a density of 1 × 10⁵ cells/well, and stimulated for 5 d with allogeneic DCs (2.5 × 10⁵ cells/well). Cell proliferation was determined by the sequential loss of CFSE fluorescence of cells, as detected by flow cytometry. Then total cells were stimulated with 50 ng/ml PMA plus 500 ng/ml ionomycin for 5 h in the presence of 10 ng/ml brefeldin A (all from Sigma). After stimulation, cells were washed with PBS, fixed and permeabilized using an IntraStain kit (Dako Cytomation, Glostrup, Denmark), and incubated for 30 min at room temperature with anti-human IFN-γ allophycocyanin-conjugated mAb (Miltenyi). Cells were washed and analyzed by flow cytometry.

**Mixed leucocyte reaction**

Allogeneic CD3⁺ T cells (10⁵/well) were stimulated in vitro with C4BP(β⁻)- or C4BP(β⁺)-treated, or vitamin D3-treated, and LPS-activated DCs in a 96-well round-bottom plate at various DC:T cell ratios (1:40, 1:80, and 1:160) and cultured in X-VIVO 15 medium (Biowhittaker, Walkersville, MD) supplemented with 2% Human AB serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 2 mM L-glutamine. Alloproliferation was measured after 5 d of incubation. At day 4, the cells were pulsed with [³H] thymidine (1 μCi/well; Amersham, Freiburg, Germany), followed by incubation for another 16 h. Labeled cells were then harvested onto glassfiber filters (Harvester 96; Tomtec, Hamden, CT), and the T cell proliferation rate was determined by the amount of [³H]thymidine incorporation using a scintillation counter (1450 MicroBeta Trilux plate reader; Wallac, Turku, Finland). Results are reported as the mean cpm ± SD of thymidine incorporation in quadruplicate culture wells.

**FIGURE 3.** The C4BP(β⁺) isoforms do not interfere with the activity of the C4BP(β⁻) isoform on human DCs. Human DCs were incubated throughout their differentiation and maturation process with 5 μg/ml of the individual C4BP isoforms or with a C4BP(β⁻)/C4BP(β⁺) isoform mix at the indicated concentration ratios. DC maturation was achieved by LPS treatment. Cells were then collected, washed, and analyzed by flow cytometry for cell-surface expression of CD83 and CD86. (A) Each histogram is representative of five independent experiments. Isotype controls are shown in gray. The median fluorescence intensities (MFIs) for the different cell-surface markers are indicated. (B) Relative MFI for the different cell-surface markers. C4BP(β⁺) and C4BP(β⁻), C4BP isoform-treated, LPS-matured DCs; iDC, untreated iDCs; mDC, untreated, LPS-matured DCs. Results shown are the mean ± SD from five independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 compared with mDC.
Determination of CD4+ CD127low/neg CD25high and Foxp3+

T cells

Negatively selected CD3+ T lymphocytes were plated (10^5 cells/well) in 96-well round-bottom plates and cocultured at a 1:40 DC:T cell ratio. After 6 d of coculture without restimulation and any supplemental cytokines, we used flow cytometry to determine the percentage of regulatory T cells (Tregs) defined as CD4+, CD127 low/neg, CD25 high, and intracellular Foxp3+ (Human Regulatory T Cell Staining Kit; eBioscience, San Diego, CA).

Statistical analysis

Unless otherwise indicated, three technical replicates were performed from each independent experiment. Results are presented as means ± SD. DC variables under different experimental conditions with respect to a reference condition (usually mDCs or iDCs) were compared using the ANOVA, considering p < 0.05 as significant.

Results

The C4BP(β−) isoform affects the activation phenotype of DCs

We first assessed whether the main natural C4BP isoforms, C4BP (β+) (complexed with ProS) or C4BP(β−), influenced the expression of different monocyte and DC surface markers, including CD14, HLA-DR, CD40, CD80, CD83, CD86, and CD1a, when added concomitantly with the DC differentiation factors GM-CSF and IL-4. Interestingly, although the C4BP(β+) isoforms had no effect on the expression of any of the above markers on LPS-matured DCs, the C4BP(β−) isoform (both C4BP αβ0 and recombinant C4BP αβ0) significantly prevented CD83, CD86, and CD80 overexpression in a dose-dependent manner (Figs. 1, 2). Conversely, expression of HLA-DR, CD40, CD14, and CD1a on DCs was not significantly altered by C4BP(β−) isoform treatment.
Moreover, an analogous cell-surface marker behavior was observed when C4BP-treated DCs where matured by CD40L exposure (Supplemental Fig. 1). Together, these data are evidence that the C4BP(β−) isoform has the potential to modify proinflammatory DC differentiation/maturation as judged by the expression pattern of various cell-surface markers. In contrast, neither the C4BP(β+) isoform alone (data not shown) nor C4BP(β−) plus LPS (Supplemental Fig. 2) incubated from days 5–7 (maturation) had any effect on DC surface marker expression. Furthermore, a mixing assay confirmed that the C4BP(β+) isoforms were unable to interfere with the modulatory activity of the C4BP(β−) isoform (Fig. 3).

DCs treated with the different C4BP isoforms remained highly viable throughout the differentiation/maturation process, as assessed by Annexin V/7-amino-actinomycin D staining, with <10% of apoptotic cells evidenced at 48 h after LPS-mediated DC maturation. Conversely, an analogous treatment with the reference immunomodulator vitamin D3 slightly reduced DC viability and yield (Supplemental Fig. 3).

**LPS-induced DCs exposed to the C4BP(β−) isoform fail to reduce TGF-β1 expression and prevent IDO and BIC-1 overexpression**

To further assess the effect of the C4BP isoforms on key transcripts conforming the molecular signature of maturing DCs (24), we analyzed by quantitative RT-PCR (RT-qPCR) the expression of the genes coding for the immunomodulatory factor TGF-β1, involved in Treg generation, for the immunoregulatory enzyme IDO, involved in tryptophan metabolism, and for miR-155 (BIC-1), an important microRNA involved in immune function. Expression of both IDO and BIC-1 were upregulated, whereas TGF-β1 was downregulated, on LPS-mediated DC maturation. In contrast, DC treatment with the C4BP(β−) isoform, but not with the C4BP(β+) isoforms, suppressed the subsequent induction of IDO and BIC-1 by LPS, whereas maintaining TGF-β1 expression, which is normally reduced by LPS (Fig. 4). Thus, all three molecular biomarkers analyzed reached transcript levels comparable with those from iDCs.

**The C4BP(β−) isoform affects the morphology of LPS-induced DCs**

Efficient sampling of the microenvironment for microbes and other potential danger signals by dendrite formation is a distinctive feature of DC development. Scanning electron microscopy was used to assess the detailed surface morphology of DCs (Fig. 5). Before LPS exposure, 91% of the untreated iDCs were essentially round, whereas after 48 h of LPS-mediated maturation, the dendritic morphology became evident in 71% of the analyzed cells, with numerous long projections protruding from the cell surface. Again, C4BP(β−)-treated DCs had an analogous appearance to untreated DCs upon LPS stimulation (66% of the analyzed cells). Conversely, DC treatment with both the native (Fig. 5) and the recombinant C4BP(β−) isoform (not shown) prevented the “por-
cupine-like" DC morphology resulting upon LPS induction (76% of the analyzed cells had iDC appearance). These percentages are comparable with those obtained by flow cytometry quantification using the DC maturation marker CD83 (see, e.g., Fig. 1B).

The endocytic activity of DCs remains unaltered after C4BP isoform treatment

The Ag internalization capacity of DCs was assessed by flow cytometry of both self-quenching DQ-OVA (mannose receptor-mediated endocytosis marker) and Lucifer Yellow CH (macropinocytosis marker). The high endocytic activity of iDCs was unaltered by treatment with either C4BP(β+) or C4BP(β−) isoforms (Fig. 6A). Uptake of both DQ-OVA and Lucifer Yellow CH was significantly inhibited by incubation at 4°C, confirming that the fluorescence shift was due to active internalization rather than from membrane association alone (data not shown). Likewise, C4BP-treated DCs behaved analogously to untreated mDCs after LPS induction, showing a reduced endocytic capacity for soluble

![Flow cytometry histograms](image)

**FIGURE 6.** C4BP treatment does not perturb the endocytic capacity of human DCs. The endocytic activity of DCs was assessed by flow cytometry, measuring both the fluorescent DQ-OVA internalization and processing (receptor-mediated endocytosis), and Lucifer Yellow CH uptake (fluid-phase endocytosis) at the differentiation and maturation stages. (A) Monocytes were differentiated either untreated (iDC; white bar) or treated with C4BP(β+) or C4BP(β−) (gray bars), both at 5 μg/ml. (B) iDCs (white bar) were LPS-matured either untreated (mDC; black bar) or treated with the different C4BP isoforms at 5 μg/ml (gray bars). Representative histograms for each condition are shown. Dye uptake controls are displayed in gray. The median fluorescence intensities (MFIs) for the different fluorescent cell populations is indicated in each histogram. In both cases, data shown are the mean MFI ± SD from eight (DQ-OVA) or three (Lucifer Yellow) independent experiments. *p < 0.05, **p < 0.01 compared with iDC.
proteins (Fig. 6B). Furthermore, C4BP treatment of iDCs did not modify the surface levels of the mannose receptor CD206 (data not shown).

The C4BP(β−) isoform inhibits the release of inflammatory cytokines by LPS-induced DCs

We next assessed whether the effect of the different C4BP isoforms on DC phenotype was accompanied by changes in their release of cytokines (IL-12p70, IL-10, IL-8, IL-6, TNF-α, and IFN-γ). Compared with untreated iDCs, secretion of each of the proinflammatory cytokines was upregulated when iDCs were matured with LPS. DCs pretreated with the C4BP(β+) isoforms secreted the same cytokine levels as untreated DCs upon maturation. In contrast, pretreatment with the C4BP(β−) isoform prevented the release of IL-12p70 and IFN-γ, significantly decreased the release of TNF-α, reduced the secretion of IL-8 and IL-6, and increased the production of the anti-inflammatory cytokine IL-10 (Fig. 7). Thus, Th1 proinflammatory cytokine production upon LPS-mediated DC stimulation was significantly diminished in C4BP(β+)–treated DCs.

The C4BP(β−) isoform alters the chemotaxis of DCs

Maturation signals determine the expression of distinct DC functions, such as migration toward lymph node–directing chemokines. C4BP(β+) isoform treatment suppressed the induction of the chemokine receptor CCR7 at both the transcriptional and the translational level upon LPS-mediated activation of DCs (Fig. 8A, 8B). Reduced surface CCR7 expression, in turn, significantly decreased the migration of LPS-activated DCs toward the chemokine CCL21 (Fig. 8C). In contrast, LPS-mediated activation of both untreated and C4BP(β−)–treated DCs induced maximal migration in response to CCL21.

DCs exposed to the C4BP(β−) isoform fail to enhance allogeneic T cell proliferation

Given that the C4BP(β−) isoform was found to affect on phenotypic maturation and the amount of inflammatory cytokines released by DCs, we next examined the immunostimulatory capacity of DCs exposed to the major C4BP isoforms. When DCs were preincubated with the C4BP(β+) isoforms and matured with LPS, maximal allogeneic T cell proliferation was observed, similar to that obtained using untreated, LPS-matured DCs. In contrast, mDCs preincubated with the C4BP(β−) isoform failed to enhance CD3+ T cell proliferation, approaching the levels observed using iDCs (Fig. 9).

DCs exposed to the C4BP(β−) isoform avoid promoting a Th1 profile and instead induce Treg generation from allogeneic T cells

To assess the functional outcome of T cells allostimulated with C4BP(β−)–treated DCs, we restimulated the resulting T lymphocytes with PMA + iomycin and measured IFN-γ production by intracellular staining. A significant reduction in IFN-γ production (∼50%) was evident from T cells exposed to C4BP(β−)–treated DCs relative to T cells exposed to untreated mDCs, approaching the IFN-γ levels achieved by T cells allostimulated with iDCs (Fig. 10A, upper row, Fig. 10B). Conversely, T cells allostimulated with C4BP(β+)–treated DCs experienced only a slight, non-significant reduction of IFN-γ production. When only CSFElow proliferating T cells were analyzed, C4BP(β−) DC-allostimulated T cells showed again a significant decrease in IFN-γ production relative to untreated DC-allostimulated T cells (Fig. 10A, lower row, Fig. 10C). In contrast, although vitamin D3–treated DCs also suppressed IFN-γ production in cocultures with allogeneic T cells, they were unable to reduce the capability of responding T cells to produce IFN-γ.

Finally, we further investigated the presence of Treg cells, defined as CD4+CD127low/highCD25highFoxp3+, under the earlier DC: T cell coculture conditions. Only blast T cells exposed to C4BP (β−)–treated DCs, but not to C4BP(β+)–treated DCs, showed a significant increase in the percentage of CD4+CD127low/high CD25highFoxp3+ cells, analogously to that achieved by blast T cells allostimulated with iDCs (Fig. 11). Taken together, these
results support the C4BP(β−)-mediated induction of a tolerogenic, anti-inflammatory phenotype in DCs.

The CCP6 domain of the C4BP α-chain is necessary for the tolerogenic activity of the C4BP(β−) isoform over DCs

We then aimed to further characterize the structural requirements of the C4BP(β−) isoform for its immunomodulatory or “tolerogenic” activity over DCs. Thus, recombinant C4BP (α6β0) mutants that lack individual CCP domains (Fig. 12A) were tested for their ability to affect the activation phenotype of DCs. All individual deletion mutants but one, ΔCCP6, were able not only to significantly prevent the upregulation of the CD83 maturation marker (Fig. 12B, 12C), but also to preclude IL-12p70 and IFN-γ production, to reduce TNF-α release, and to avert dendrite network development upon LPS induction (as an example, see ΔCCP6 behavior in Supplemental Fig. 4). Conversely, C4BP ΔCCP6-treated DCs behaved like untreated or C4BP(β+)-treated DCs, and did not affect: 1) CD83 surface expression (Fig. 12B, 12C); 2) other molecular biomarkers such as IDO (data not shown); 3) proinflammatory cytokine secretion; or 4) surface morphology, upon LPS induction (see ΔCCP6 behavior in Supplemental Fig. 4). Thus, the C4BP α-chain CCP6 domain is necessary for the immunomodulatory activity of the C4BP(β−) isoform in DCs.

Discussion

C4BP is a multifunctional protein. Each α-chain has a binding site for C4b/C3b, and this interaction regulates complement activation by reducing the formation and stability of C3/C5 convertases. The β-chain binds and inactivates ProS, interfering with the anti-coagulatory protein C–dependent pathway. C4BP-bound ProS mediates binding of C4BP to apoptotic/necrotic cells. In addition, a number of bacterial and fungal pathogens express different outer-membrane proteins with binding sites in the α-chain of C4BP, which mediate C4BP incorporation to the pathogen surface and contribute to their pathogenic potential (25). Not surprisingly,
FIGURE 9. Human DCs exposed to the C4BP(β–) isoform fail to enhance allogeneic T cell proliferation. Untreated iDCs (iDC), LPS-matured untreated DCs (mDC), LPS-matured, and C4BP isoform–treated (2 µg/ml) (C4BP[β+] and C4BP[β–]), or vitamin D3–treated (VitD3) DCs were cultured in triplicate with allogeneic, purified CD3+ T cells (10^5/well) at 1:40 DC:T cell ratio for 5 d (n = 10). [3H]Thymidine (1 µCi/well) was added for the last 16 h of culture, and incorporation was measured in a beta-plate counter. ***p < 0.001 compared with mDC.

the unique multidomain oligomeric structure of C4BP could accommodate additional functional properties.

In this study, we unveil the capacity of the C4BP(β–) isoform to modulate the behavior of DCs, key innate immune cells influencing the development of the adaptive immunity, and map this novel functionality to CCP6 of the α-chain. We show that both plasma-purified and recombinant C4BP(β–) abrogated the proinflammatory phenotype of LPS-stimulated, monocyte-derived DCs in a dose-dependent manner and instead induced a semimature, tolerogenic phenotype on these cells. Likewise, DC maturation induced by CD40L was also prevented by the C4BP(β–) isoform. Furthermore, considering the physiological activity of C4BP as soluble complement modulator, its interaction with other agents such as mesenchymal stem cells (32), and pharmacological agents (corticoids, immunosuppressors) (33, 34), different cell types such as mesenchymal stem cells (32), and pharmacological agents (corticoids, immunosuppressors) (33, 34), have all been involved in impeding DC maturation, which was associated with the retention of a tailored “immature” or “semimature” phenotype, although it has not yet been possible to correlate surface marker phenotype with tolerogenic DC potency. A focused comparative transcriptional profiling between iDCs, LPS-matured, and C4BP(β–)-treated and LPS-matured DCs has been reported (41). Conversely, other agents such as the hormones activin A and inhibin A (26) or certain oligonucleotides (27) induce a semimature DC state characterized by inhibition of HLA-DR expression, whereas the expression of costimulatory molecules (CD40, CD83, CD86) remains unaffected. Indeed, a variety of mediators, notably cytokines and growth factors (IL-10, vascular endothelial growth factor) (28, 29), vasoactive peptides and vitamins (vasoactive intestinal peptide, vitamin D3) (30, 31), different cell types such as mesenchymal stem cells (32), and pharmacological agents (corticoids, immunosuppressors) (33, 34), have all been involved in impeding DC maturation, which was associated with the retention of a tailored “immature” or “semimature” phenotype, although it has not yet been possible to correlate surface marker phenotype with tolerogenic DC potency. A focused comparative transcriptional profiling between iDCs, LPS-matured, and C4BP(β–)-treated and LPS-matured DCs has been reported (41). 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A focused comparative transcriptional profiling between iDCs, LPS-matured, and C4BP(β–)-treated and LPS-matured DCs has further revealed particular traits associated with regulatory DCs. Thus, mDCs generated in the presence of the C4BP(β–) isoform transcriptionally prevented the induction of the chemokine receptor CCR7, involved in DC migration to lymph nodes (35), BIC-1, coding for miR-155, an important modulator of the immune function in DCs (36), and the immunosuppressive enzyme IDO (37), whose expression has been shown to be strongly dependent on the maturation status of the cells (38). All of them were significantly suppressed to the levels present in iDCs. In contrast, the anti-inflammatory and immunoregulatory cytokine TGF-β (39) was maintained at levels approaching those from iDCs. These results are in agreement with the recently reported molecular signature of LPS/IFN-γ–matured monocyte-derived DCs (24). Our ongoing work comprehensively assessing the genome-wide comparative transcriptional profile of proinflammatory DCs either untreated or in the presence of C4BP(β+) or C4BP(β–) isoforms will delineate the complete molecular signature of C4BP(β–)-induced tolerogenic DCs.

Another differential trait of C4BP(β–)-treated DCs involved their cell-surface topography. Thus, untreated DCs displayed an increased number and length of dendrites upon LPS maturation, conferring a high surface-to-volume ratio. This prominent and dense dendritic network characteristic of mDCs was mostly abrogated by C4BP(β–) pretreatment, limiting DC to T cell contact and preventing the full development of the adaptive immune response (31).

At the functional level, the uptake of pathogenic agents is a key initial step in iDC maturation, followed by intracellular processing and presentation of the corresponding antigenic fragments on the surface of the simultaneously matured DCs (40). C4BP(β–) treatment modified neither the high endocytic activity characteristic of monocyte-derived iDCs nor the surface levels of HLA-DR and, consequently, did not appear to influence Ag capture, processing, and peptide load. Inconsistencies between endocytosis and maturation of DCs have already been reported (41). Conversely, vitamin D3 treatment impaired the capacity to take up soluble Ag and prevented surface HLA-DR overexpression on DCs (42). Thus, the endocytic capacity seems independent of the maturation status of DCs. Importantly, a comparative analysis of the cytokine expression profile of the LPS-stimulated, C4BP(β–)-treated DCs revealed a significant reduction of Th1 proinflammatory cytokines (IL-12, TNF-α, IFN-γ, IL-6, IL-8) and an increase of IL-10, an anti-inflammatory cytokine that inhibits Th1 immune responses (43). Additional functional attributes of C4BP(β–)-treated mDCs involved their reduced ability to migrate toward CCL21, a strong chemotactic stimulus. Indeed, the CCL21/C-CR7 axis plays a central role not only in DC migration to the draining lymph nodes, but also in the control of the cytoarchitecture and the maturation of DCs (44). We verified a significant reduction of CCR7 expression at both transcriptional and translational levels in C4BP(β–)-treated DCs, as a major determinant of this effect. Finally, it is well-known that DC maturation is directly linked to T cell stimulatory capacities (45). Thus, we demonstrate that C4BP(β–)-treated DCs fail to enhance T cell alloproliferation in MLRs, behaving similarly to iDCs.
Several effector molecules in the complement cascade, such as C1q, iC3b, and C3, have also been proposed to be directly involved in DC biology. Opposite functions have recently been reported for C1q on monocyte-derived DCs. For example, C1q is able to induce maturation of iDCs cultured on immobilized C1q (50). On one hand, a recent report suggests that C1q is able to induce tolerogenic properties in developing DCs (51). On the other hand, absence of C3 has been shown to impair DC differentiation and maturation inducing, among other features, decreased CD1a expression and IL-12 secretion (52). Finally, the C3 product iC3b was also able to induce a hyporesponsive phenotype on DCs, characterized by inhibition of CD80 and CD40 expression and of IL-12 production (53). Nevertheless, to our knowledge, this is the first time that a classical complement modulator has been shown to influence DC function.

We have demonstrated the modulatory effects of the C4BP(β−) isoform using both plasma-purified and recombinant proteins. Surprisingly, neither phenotypic nor functional alterations have been observed on treatment of proinflammatory DCs with the C4BP(β−) isoforms. Circulating C4BP(β−) isoforms are invariably associated with ProS under physiological conditions. Moreover, in competition assays, we have confirmed that the C4BP(β−)–ProS complex does not inhibit the C4BP(β2) activity in DCs. The α-chains of C4BP bind to several ligands; most of them (C3b, C4b, heparin, low-density lipoprotein receptor–related protein, bacterial pathogens) interact with the outer CCP domains (CCP1-4), whereas the pentraxin serum amyloid P binds to the central core. Conversely, ProS binds to the CCP1 of the single C4BP β-chain (54). We have found that the CCP6 domain of C4BP

**FIGURE 10.** C4BP(β−)-treated DCs induce decreased production and secretion of IFN-γ from allogeneic T cells. Proliferating T lymphocytes were obtained from allostimulatory cocultures with untreated iDCs (iDC), LPS-matured untreated DCs (mDC), LPS-matured and C4BP isoform-treated (2 μg/ml) (C4BP[β−] and C4BP[β+]) or vitamin D3–treated (VitD3) DCs. The production of IFN-γ was measured by intracellular staining after restimulating the cells with PMA + ionomycin in the presence of brefeldin for 5 h. (A) The first row plots indicate the proportion of total IFN-γ–producing cells. The second row shows the percentages of cells that responded to allostimulation (CFSElow) and produced IFN-γ. The numbers inside the dot plots indicate the percentage of cells in each quadrant or box (a representative experiment is shown). (B) Relative percentages of total IFN-γ production [(A), upper row; n = 4]. (C) Percentage of IFN-γ–producing T cells that responded to allostimulation [CSFElow CD3+ cells; (A), lower row]. Each symbol represents an individual sample. *p < 0.05, **p < 0.01 compared with mDC.
α-chain is necessary for the C4BP(β−) activity over DCs, although the contribution of other CCPs directing and stabilizing CCP6 is not discarded. No other function has been ascribed to CCP6, in contrast with most other CCP domains. Because ProS binds with high affinity to C4BP(β+) isoforms (55), structural arrangements in the polymeric C4BP spatial conformation because of the presence of ProS either by an allosteric mechanism or by direct steric hindrance might: 1) preclude a direct interaction of the CCP6 domain from the α-chains with the putative “tolerogenesis” receptor on DCs, or 2) reduce the overall avidity of polymeric C4BP binding to the receptor through the CCP6 domain. In this respect, although the C4BP(β+)–ProS complex attached to apoptotic cells through ProS is still able to bind C4b through domains CCP1-3 of the α-chains (23), serum amyloid P

FIGURE 11. C4BP(β−)-treated DCs promote CD4+ CD127low/CD25high Foxp3+ induction from blast T cells. Untreated iDCs (iDC), LPS-matured untreated (mDC), and LPS-matured and C4BP-treated (2 μg/ml) (C4BP(β−)) and C4BP(β+) or vitamin D3-treated (VitD3) DCs were cultured in triplicate with allogeneic, purified CD3+ T cells (10^5/well) at 1:40 DC:T cell ratio. After 6 d of coculture without restimulation and any supplemental cytokines, cell sizes were evaluated by flow cytometry (FSC versus SSC parameters) to differentiate blast from nonblast cells. (A) Phenotype of T cells as CD4+, Foxp3+, and CD25+ with low CD127 expression. One of 10 representative experiments is shown. (B) Summary of percentages of T cells from nonblast (left) and blast (right) origin. n = 10. *p < 0.05, **p < 0.01.
binding to the central core linking the seven or eight chains impairs its factor I cofactor activity (56). Nevertheless, further structure-activity studies will be necessary to dissect the relationship between structure and function of polymeric C4BP on DCs. Moreover, ongoing research will enable assessment of the putative monocyte/DC surface receptor responsible for the tolerogenic activity of C4BP(β<sup>2</sup>) isoforms. In that sense, it has been proposed that the CD40 receptor could bind directly C4BP on B cells inducing proliferation, overexpression of CD54 and CD86, and IL-4–dependent IgE isotype switching (22), or might bind indirectly C4BP, through interaction with CD40L, on cholangiocytes preventing apoptosis by interference with CD40-CD40L signaling in these cells (57).

Our data extend the regulatory role of C4BP beyond preventing runaway complement activation and complement-mediated inflammation, because the activity of the C4BP(β<sup>2</sup>) isoform on DCs
could represent a regulatory feedback loop on adaptive immunity, avoiding excessive T cell activation to circumvent the consequent host tissue damage under acute proinflammatory conditions. In this respect, it is very interesting that under proinflammatory conditions, the C4BP(b) isoform is specifically upregulated (13, 14), and that the level of expression of the α-chain is a polymorphic trait in humans that results in important differences in the levels of the C4BP(β) isoform between individuals (4). In addition, it has been reported that a reduction of C4BP α-chain expression could be a key determinant in the immune dysfunction and chronic inflammation associated with aging (58). Conversely, the fact that some pathogens, in addition to hijacking the host’s C4BP to downregulate complement activation (21), might also exploit the C4BP(β) isoform to avoid DC activation and mount a specific acquired immune response against the pathogen is also a tantalizing possibility.

Regarding the potential therapeutic utility of C4BP(β) isoform treatment in an in vivo proinflammatory scenario, a recent study indicated that although i.p. injection of human C4BP induced a low level of complement inhibition, it was able to significantly inhibit the development of experimental arthritis in mice (15). Thus, our study could provide a possible mechanism explaining the in vivo effect of C4BP(β) regulating autoimmunity.

In summary, according to a distinctive tolerogenic DC profile, C4BP(β)-treated DCs display a immature phenotype when challenged by a proinflammatory stimulus, mainly characterized by altered cytoarchitecture; lower expression of the maturation marker CD83 and the costimulatory molecules CD80 and CD86; decreased production of key proinflammatory cytokines such as IL-12, TNF-α, IFN-γ, IL-6, and IL-8; and preferential production of immunomodulatory mediators such as IL-10 and TGF-β. Moreover, C4BP(β)-treated DCs show reduced CCR7 expression and migration capacity, impaired CD4+ T cell alloproliferation, inhibition of IFN-γ secretion by the allostimulated T cells, and, conversely, induction of CD4+CD122high/CD25high/Foxp3+ cells. Thus, both C4BP(β)-treated DCs and the C4BP(β) isoform on its own might have therapeutic potential as anti-inflammatory and immunomodulatory agents in hypersensitivity, transplantation, and autoimmunity, although future studies will need to determine the full molecular mechanisms of C4BP(β)-induced, tolerogenic DC generation and the efficacy of their application to appropriate animal models of inflammation.

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Disclosures
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