Chapter 4

Properdin and factor H production by human dendritic cells is differentially regulated by IFNγ and has a functional role in the T cell stimulatory capacity of dendritic cells.

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Abstract
Dendritic cells (DCs) and complement are both key members of the innate immune system, and are critical for immunogenic as well as regulatory functions of adaptive immunity. Recent experimental mouse models have shown that production of alternative pathway (AP) components by DCs strongly affects their functional capacity to activate and regulate T cell responses. In this study we investigated the production and regulation of properdin (fP) and factor H (fH) both integral regulators of the AP, by both human monocyte-derived DCs and tolerogenic DCs (tolDCs). Both fP and fH were expressed and produced by DCs, as shown by Q-PCR, intracellular staining, Western blotting and ELISA, with significantly higher levels of both AP components produced by tolDCs. Upon activation with IFNγ both cells increased fH production, while simultaneously decreasing production of fP. This was unique for IFNγ as LPS and IFNα or IFNβ did not demonstrate this dual regulation. IL-27, a member of the IL-12 family which elicits features of IFNγ stimulation, did increase fH, but production of fP remained unaffected. The functional capacity of fP and fH produced by DCs and tolDCs was confirmed by their ability to bind C3b and the capacity of fP to bind to necrotic cells. Inhibition of fH production by DCs through siRNA, resulted in a greater ability to induce allogenic $CD4^+$ T cell proliferation. In contrast, inhibition of fP production by DCs^- led to a significantly reduced allostimulatory capacity. In summary this study shows that production of fP and fH by DCs, differentially regulates the T cell stimulatory capacity of these cells, and that the local cytokine environment can profoundly affect the production of fP and fH by DCs.

Introduction
Dendritic cells (DCs) and the complement system are both integral members of the innate immune system[1,2]. The complement system is composed of three distinct pathways-classical, lectin and alternative. Together these pathways serve as key mediators of the innate immune response, involved in defending the host from pathogens, removing immune complexes and facilitating efficient phagocytosis of apoptotic cells[2,3]. A key role of complement in the humoral arm of the adaptive immune response has been demonstrated through experimental depletion of complement resulting in poorer antibody responses, and opsonisation with complement fragments which profoundly lowers the amount of antigen needed to induce antibody production[4,5]. Recently, there has been an increasing body of evidence demonstrating that complement may function more broadly as a link between innate and adaptive immunity. This includes murine studies which have demonstrated a role for the AP in the DC: T cell synapse[6-8]. Both APC and T cells have been shown to increase C3a and C5a receptor on their surface, while a surface regulator, decay accelerating factor, has been shown to be decreased,

allowing for further complement activation[9,10].
The AP is unique in that it can become auto activated via C3 hydrolysis generating a C3b like molecule which can bind covalently to an activating surface recruiting factor B, which is then cleaved by factor D generating a C3 convertase (C3bBb). This C3 convertase can convert further C3 to C3b, with stabilisation of the enzyme by properdin (fP) allowing for propagation of the AP activation[11]. Regulation of the AP is key, with fP promoting AP activation and negative regulators such as factor H (fH), limiting the availability of C3b, or acting as a co-factor for factor I, which degrades C3b. This delicate balance between activators and inhibitors of the AP is crucial for controlling complement activation[12]. The majority of complement proteins are produced by the liver, although white blood cells including DCs can also act as a local source of certain complement proteins[13]. The source of fP is largely restricted to white cells, including neutrophils[14,15]. Factor H is most abundantly expressed in the liver, but also here extra hepatic production has been described, which includes mesangial cells, endothelial cells and fibroblasts[16-19]. This suggests that local cell populations, either resident or infiltrating, can significantly contribute to the overall complement activation at a particular site of inflammation.
DCs are distinct among other cells in the immune system as they are uniquely equipped to respond to a host of innate stimuli while also adapting and tailoring their functions in response to the local cytokine environment. This had led to the understanding that DCs are not just immunogenic but that tolerogenic DCs also exist. These tolDCs are characterised by differences in cytokine production and expression of co-stimulatory molecules, which has a direct impact on the strength and quality of their T cell stimulatory capacities[20]. Previous studies have demonstrated by RT-PCR that DCs can express many components of the complement system[21,22], however protein data to support this has been limited. Furthermore there is only limited data available on the regulation of AP component production by DCs or particularly tolDC populations. Local production of complement components such as C3 by APCs at the site of inflammation or immunological synapse can be instrumental in the immune response[8]. With the demonstration that local AP activation at the interface of APC and T cells plays a key role in the regulation of T cell responses[23], regulation of the AP in the local environment will be key in controlling the strength of the T cell response. Therefore, we investigated the regulation of two integral modulating factors of the AP, fP and fH. Combined both molecules have the ability to influence the balance of the AP towards activation or regulation.
In this study, we demonstrate that DCs are a source of fP and fH and that tolDCs are superior producers of both components. We observed a differential regulation of fP and fH, whereby IFNγ profoundly inhibited fP in both cell types, while

simultaneously increasing production of fH. This distinct dual effect was found to be unique for type II IFN. IL-27 did significantly enhance fH levels in DC supernatants but not fP. Importantly, RNA interference of fH in DCs increased their allostimulatory capacity, while siRNA targeting of fP decreased T cell proliferation. Taken together, our data suggests that the local cellular and cytokine microenvironment are crucial for overall complement regulation and thereby T cell immunity.

Materials and Methods

Cell culture and Reagents

Human monocytes were isolated from buffy coats obtained from healthy donors using Ficoll density gradient centrifugation followed by positive selection using anti-CD14 MACS microbeads (Miltenyi Biotech GmBH, Bergisch Gladbach, Germany). DCs were generated and cultured in RPMI 1640 supplemented with 10% heat inactivated foetal calf serum (FCS), 90 U/mL penicillin, and 90 µg/mL streptomycin (Gibco/Life technologies, Breda, The Netherlands), 5 ng/mL GM-CSF and 10 ng/mL IL-4 (Biosource Europe, Belgium), as described before. Tolerogenic DC (tolDCs) were generated by addition of Dexamethasone (10^{-6}M Dex) (Pharmacy, L.U.M.C., Leiden, the Netherlands) only at the start of culture (day 0)[24]. Cultures were refreshed with medium containing cytokines on day 3. For stimulation experiments, immature DCs were harvested on day 6, washed and seeded accordingly, followed by addition of 200 ng/ml LPS (E.Coli EH100 Enzo, Belgium), 100 ng/ml each of IFNγ, IFNα, IFNβ (Peprotech) or 100 ng/ml IL-27 (R&D). Neutrophils were isolated as previously described[25], briefly blood from healthy donors was collected using ACD tubes (BD Vacutainer). Neutrophils were isolated by Ficoll-Paque and Dextran T-500 gradients (Sigma Aldrich). The preparation contained greater than 90% neutrophils as confirmed by flow cytometry using CD16 (R&D Systems), CD11b (BD Biosciences), and CD66b (AbD Serotec) antibodies.

Flow Cytometry

For intracellular staining, cells were stimulated overnight, with the addition of Brefeldin-A (10µg/ml) for the last 5 hrs of culture. The cells were then harvested, washed and fixed in PBS containing 4% formaldehyde and 1% heat inactivated FCS, washed with PBS containing 1% BSA and permeabilised with perm buffer (PBS, 0.5 % saponin, 0.1% BSA) for 10 mins. Cells were stained with primary antibodies including anti-fP (Quidel) and anti-fH (Hycult Biotech, Uden, The Netherlands) for 30 mins at 4°C in perm buffer followed by goat anti mouse Ig APC conjugated secondary antibody. Isotype matched control antibodies were used to determine the level of background staining. The fluorescence was measured on a FACS Calibur flow cytometer, and data were analyzed with Flow Jo and Cell Quest Software.

mRNA isolation, cDNA synthesis, and RT-PCR

Cells were harvested and mRNA was isolated from DCs using an Rneasy kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). DNA was digested using the on-column RNase-free DNase set. cDNA was synthesised using a reverse transcription system kit (Promega) following the manufacturer's guidelines and stored at −20°C until analysis. Specific primers for human Properdin, Factor H, IFNγR1, IFNγR2, gp130 and WSX-1 (Table I) were designed using the computer software Oligo explorer and synthesised at Biolegio. Primer specificity was tested by homology search with the human genome (basic local alignment search tool or BLAST; National Center for Biotechnology Information) and later confirmed by electrophoresis through 2% agarose gels containing ethidium bromide followed by visualisation under UV light. *GAPDH* was used as an

endogenous reference gene. For each sample, the relative abundance of target mRNA was calculated from the obtained Ct values for the target gene and expressed relative to the endogenous reference gene *GAPDH*.

Table I: **Real Time PCR Oligonucleotide sequences**

Gene	NCBI ID	Protein ID	Forward Sequence (5`-3`)	Reverse Sequence (5`-3`)
CFP	5199	Properdin	GTAATCACCCTGCTCCCAAG	TTGCGGCTTCGTGTCTCC
CFH	3075	fH	GCACACAAGATGGATGGTCG	GGTCTGCGCTTTTGGAAGAG
IL27RA	9466	WSX-1	AAGTTCTGATCTGCCAGTTCCACTA	GCTCCAAATCTTGGATCTCAACA
IL6ST	3572	gp130	GTGTTTAGGATTCGCTGTATGA	CTGTAGCCTTGAGTATGGGATG
IFNGR1	3459	IFNγR1	TCTTTGGGTCAGAGTTAAAGCCA	TTCCATCTCGGCATACAGCAA
IFNGR2	3460	IFNγR2	CTCCTCAGCACCCGAAGATTC	GCCGTGAACCATTTACTGTCG
GAPDH	2597	GAPDH	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG

DC-T cell co culture

Allogeneic $CD4^+$ T cells were isolated from buffy coats by negative selection using the MACS $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec). DCs from all conditions were harvested after 24hrs, washed, and plated in 96-well round bottom plates at a starting ratio of 1:40 with 100,000 T cells/well. Cells were cultured for 5 days and supernatant was harvested to measure IFNγ production by means of ELISA. Proliferation was assessed by the addition of ^{3}[H]-thymidine (0, 5 μCi/well)[24].

Western Blot DCs were plated at 1×10^6 cells/ml, and stimulated at indicated time points with either IFNγ or IL-27. Cells were harvested, washed in ice-cold phosphate-buffered saline and lysed in medium stringency lysis buffer for 30 minutes. The lysates were centrifuged at 13,000 rpm and the supernatants were harvested. Protein concentration was determined by a Pierce assay and 30ug was loaded per lane. The samples were boiled for 5 min in 1x loading buffer under reducing conditions, and SDS-PAGE analysis was performed. Blots were probed for p-STAT1, total STAT 1 (both Cell signaling technologies), Properdin (Quidel) and factor H (Santa Cruz biotech). Anti β-actin (Abcam, Cambridge, UK) was used as a loading control. Blots were stripped using western blot stripping buffer (Thermo Scientific, Illinois, USA).

Confocal microscopy

Cells were cultured on 8 well chamber slides (Nunc) and stimulated overnight with IFNγ followed by Brefeldin-A for the last 5 hours of culture. The slides were subsequently washed and fixed in PBS containing 4% formaldehyde, 1% heat inactivated FCS followed by washing with PBS containing 1% BSA and permeabilised with perm buffer (PBS, 0.5 % saponin, 0.1% BSA) for 10 mins. The DCs were then stained using purified anti-fP or anti-fH followed by goat anti-mouse-Alexa 488. Hoechst was used for nuclear staining. Stains were visualised using Leica TCS SP5 or Zeiss LSM710 NLO.

Complement binding assays

For determination of properdin function, necrotic cells were exposed to supernatant of DC or tolDC for 1 hour at 4°C, followed by detection of properdin binding by flow cytometry and microscopy. Purified human C3b was coated overnight on a 96 well Nunc plate followed by exposure to DC or tolDC supernatants and detection of properdin binding with rabbit anti human fP. To determine specificity of the assay tolDC sups were fP depleted by incubating with anti-properdin pre-coupled A/G beads overnight at 4°C. Both depleted and control non-depleted tolDC supernatant was assessed for ability to bind C3b as above. For determination of Factor H

binding, DCs were either unstimulated or stimulated with IFNγ prior to supernatant exposure to coated human C3b, followed by detection by mouse anti factor H (Abcam).

Cytokine and Complement production

DCs or neutrophils were plated at $1x10^6$/ml in RPMI and treated as per mentioned earlier. Cell culture supernatants were harvested after indicated time points and frozen at −20°C until analysis. Subsequently they were tested for the presence of IL-12p40 and IL-10 by ELISA (Biolegend, Sanquin respectively) according to manufacturer's instructions, or properdin and fH using in-house specific sandwich ELISAs[26,27] Cell culture medium with 10% FCS unexposed to cells was negative in all complement assays used.

RNA interference

DCs were transfected with 50 nM siRNA through the use of the transfection reagent Lipofectamine 2000 (Life Technologies) and were used for experiments 24hr after transfection. The following SMARTpool siRNAs were used (Dharmacon): CFP, CFH and nontargeting siRNA as a control. Silencing of expression was verified by fP and fH ELISA.

Statistical analysis

Statistical analysis was performed with Graph Pad Prism (Graph Pad Software, San Diego, CA) using a one-tailed t-test. P-values ≤ 0.05 were considered statistically significant.

Results

TolDCs display elevated levels of properdin and fH.

To investigate the ability of DCs to produce properdin (fP) and factor H (fH), we first assessed by RT-PCR if fP and fH were expressed in both DC and tolDC populations. We demonstrate that DCs and tolDCs expressed both factors, and that tolDCs showed more than 10 fold higher transcription of both fP and fH compared to DCs (Fig.1A,B).Western blotting was performed to analyse protein expression. Under reducing conditions for fP an expected band of approx. 55kDa was found, with no discernible changes noted with IFNγ stimulation over time (Fig.1C). The same cell lysates were assessed for the presence of fH, showing a band of approx. 155kDa in lysates of tolDCs (Fig.1D). In this case, expression of fH clearly increased over time with IFNγ stimulation (Fig.1D). We further assessed fP and fH production within DCs and tolDCs using intracellular microscopy. Using this method we could demonstrate expression of fP and fH protein in both DCs and tolDCs (Fig.1E). Incubation with IFNγ reduced the presence of fP, whereas at the same time it appeared to increase the expression of fH. To further quantify these observations, we performed intracellular flow cytometry, and confirmed these opposing effects of IFNγ on fP and fH expression (Fig.1F).

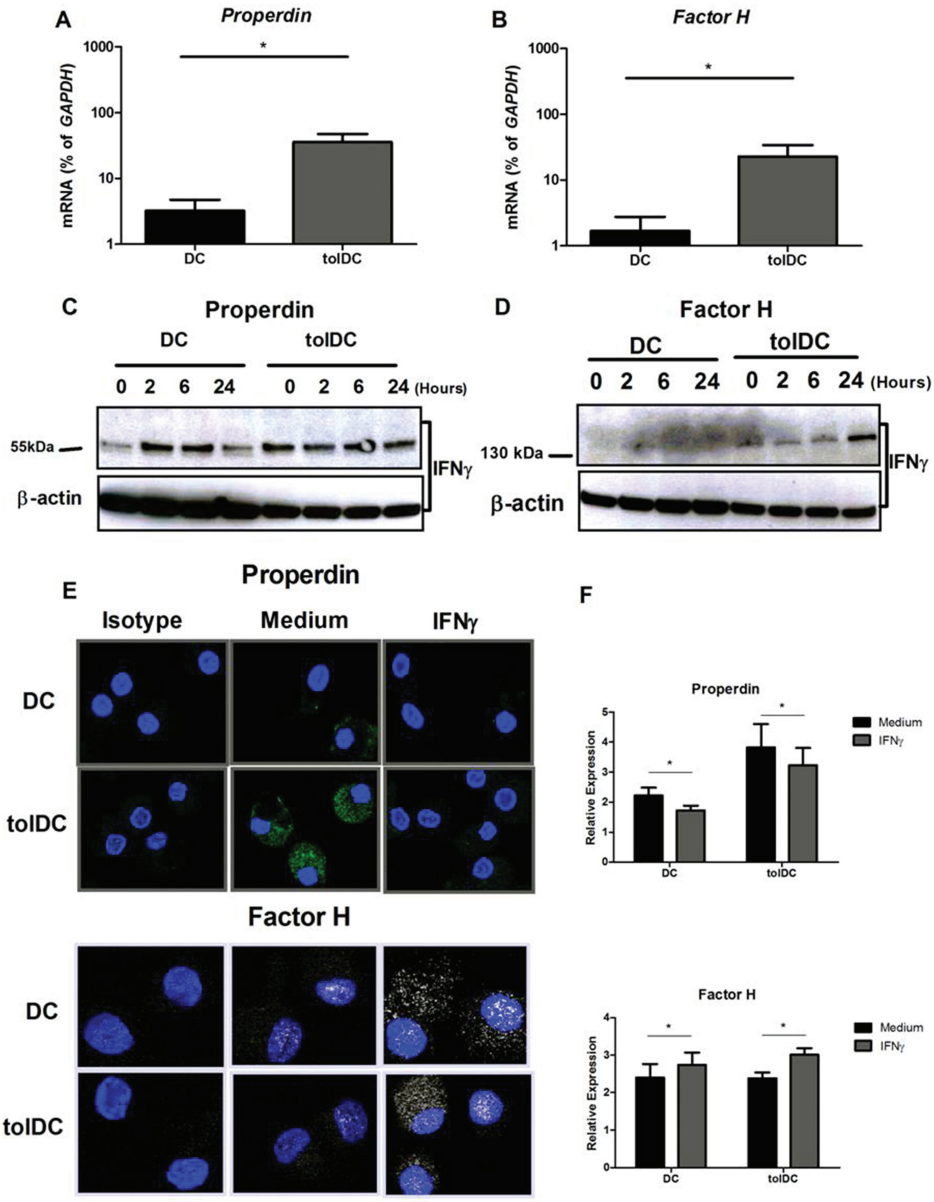

Figure 1: Expression and production of properdin and fH by human DC and tolDC. Dendritic cells were harvested after 6 days of culture after which mRNA was isolated followed by cDNA synthesis. The transcript levels of (A) Properdin (B) Factor H were determined by RT-PCR. *GAPDH* mRNA expression from the same samples was used as an endogenous reference gene (relative mRNA expression). Data shown is mean ± SD of 8 independent experiments. DCs and tolDCs were either untreated or stimulated for indicated time points with IFNγ. Cells were harvested, lysed and samples were loaded on 10% SDS gel followed by transfer to nitrocellulose membranes. Blots were probed with antibodies directed against either (C) properdin or (D) Factor H. Blots shown are

representative of 2 independent experiments. (E) DCs were cultured on 8 well chamber slides and either untreated or stimulated with IFNγ for 16 hours followed by incubation with Brefeldin A for the last 5 hours of culture. The cells were then fixed and permeabilised, followed by incubation with anti-fP or anti-fH followed by detection with GaM-Alexa^{488}. (F) DCs were either untreated or stimulated with IFNγ for 16 hours followed by incubation with Brefeldin A for the last 5 hours of culture. The cells were then fixed and permeabilised, followed by incubation with purified anti-fP or anti-fH followed by detection by flow cytometry with GaM-APC. Data shown is mean ± SD of 3 independent experiments.

IFNγ, but not LPS, exerts opposing effects on the production of fP and fH by DC and tolDC.

To further assess the differential regulation of fP and fH protein, we performed ELISAs on cell culture supernatants. In line with Q-PCR data (Fig.1A,B), we found that tolDCs produced significantly more fP and fH compared to DCs (Fig.2A,B). For comparison, supernatants from neutrophils, a widely accepted source of fP, were shown to produce levels of fP similar to DCs, but with only minimal production of fH.

To investigate whether the differential regulation of fP and fH was a general feature of mature DCs or was activation dependent, we compared stimulation with either IFNγ or LPS. Both stimuli induced the typical cytokine profile, with DCs producing high IL-12 and low IL-10, and tolDCs producing no IL-12 but high levels of IL-10 (Fig.2C,D). In view of the large differences in fP and fH production (Fig.2A,B), we calculated the relative change upon stimulation for each donor measured, with the immature/medium state set at 100%. In both DC and tolDC we observed that fP production was significantly reduced in the presence of IFNγ (Fig.2E,F). This effect was not observed with LPS stimulation where no clear regulation was noted. Regulation of fH demonstrated the opposite effect, where both cell types showed a significant increase in fH production upon treatment with IFNγ (Fig.2G,H). Also here, despite the strong induction of cytokine production (Fig.2C,D), LPS did not show any effect on the production of fH.

TolDCs express elevated levels of IFNγ R, IL-27R and STAT-1.

We observed that IFNγ displayed its most striking differences regarding fP and fH when incubated with tolDCs (Fig.1E). IL-27 is a member of the IL-12 family of cytokines, but possesses some effector properties in line with IFNγ stimulation [28, 29]. We investigated the level of expression of both IFNγR and IL-27R on DC and tolDCs, and found that tolDCs express higher levels of both IFNγR1 and R2, together making the IFNγ receptor (Fig.3A). In addition, both DC and tolDCs expressed the IL-27R, with tolDCs expressing significantly higher levels of both gp130 and WSX-1, together generating the IL-27R (Fig.3B).

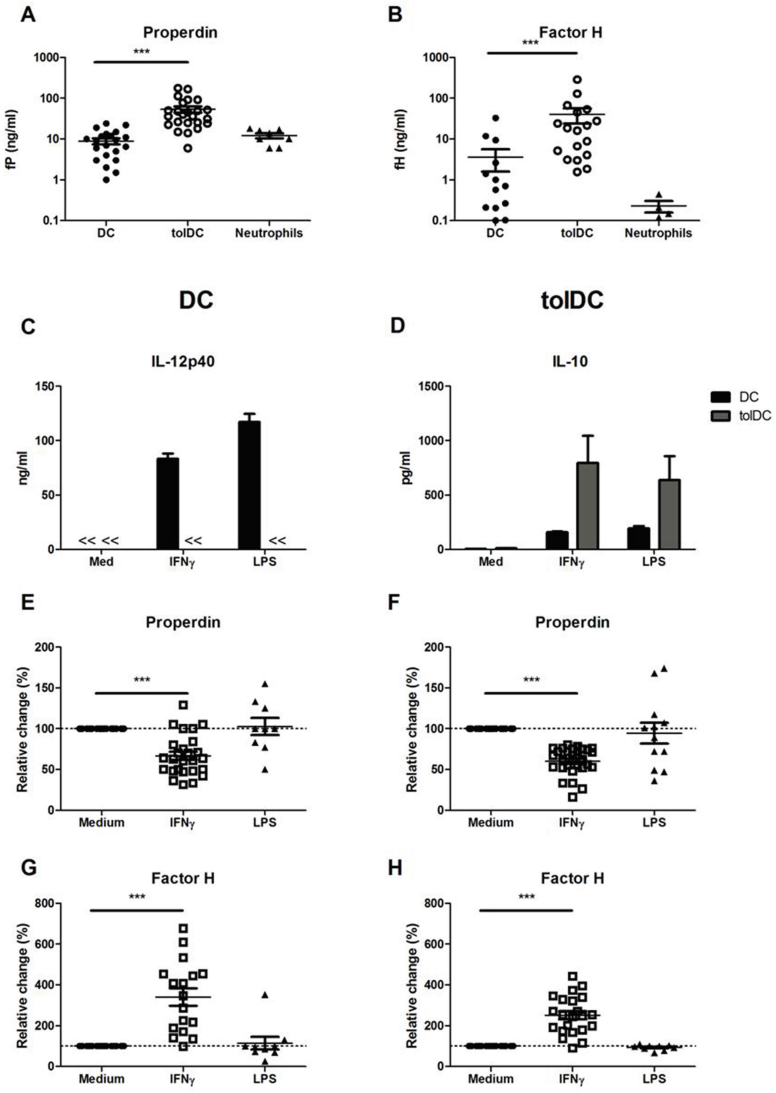

Figure 2: IFNγ exerts opposing effects on the production of fP and fH by DC and tolDC. DCs were harvested on day 6 and re-plated for another 24 hours. (A) fP and (B) fH production was measured using ELISA. Data shown is mean ± SD of 8-24 independent experiments. Dendritic cells were harvested after 6 days of culture and stimulated with IFNγ or LPS. After 24 hours the supernatants were harvested and (C) IL-12p40 and (D) IL-10 was measured. Data shown is the mean ±SD of 4 independent experiments. Cells were stimulated for 24 hours after which (E, F) fP and (G, H) fH was determined by ELISA. Levels upon stimulation were expressed as a percentage relative to medium values (relative change). Data shown is mean ± SD of 12-28 independent experiments.

Both IFNγ and IL-27 are known to mediate their downstream functions largely through phosphorylation of STAT-1. We observed a strong and prolonged phosphorylation of STAT-1 in tolDCs upon stimulation with both IFNγ and IL-

27, while in comparison, DCs had a much lower and transient level of p-STAT-1. Interestingly, total STAT-1 levels were elevated in tolDCs compared to DCs despite the loading control being similar amongst all conditions analysed (Fig.3C).

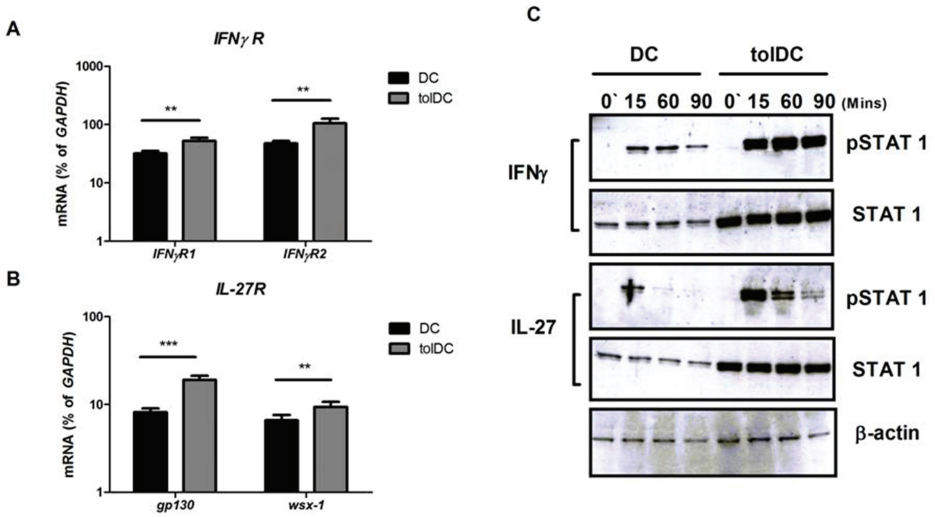

Figure 3: TolDCs express elevated levels of IFNγR, IL-27R and STAT-1. Dendritic cells were harvested after 6 days of culture after which mRNA was isolated followed by cDNA synthesis. The transcript levels of (A) IFNγR (B) IL-27R were determined by RT-PCR. GAPDH mRNA expression from the same samples was used as an endogenous reference gene (relative mRNA expression). Data shown is mean ± SD of 9 independent experiments. DCs and tolDCs were either untreated or stimulated for indicated time points with IFNγ or IL-27. Samples were loaded on 10% SDS gel followed by transfer to nitrocellulose membranes. Blots were probed with antibodies directed against either (C) total or phosphorylated STAT-1.

Type I IFNs do not possess the same opposing properties as IFNγ, while IL-27 specifically increases fH.

We addressed whether IL-27 could, like IFN-γ, also possess dual roles in regulating alternative pathway components in DCs. In addition to IL-27 stimulation we used type I IFNs (IFN α and β) to discern whether fP and fH regulation was unique for type II IFN (IFNγ) or was more widely regulated across the IFN family. We observed that both DCs and tolDCs demonstrated reduced levels of fP upon IFNγ stimulation. IFN-α but not IFN-β did significantly reduce fP levels in tolDC supernatants, while no regulation of fP was observed in DC with type I IFN (Fig.4A,C). In contrast, IL-27 did not demonstrate any clear ability to regulate fP production. The levels of fH remained unchanged in both DC and tolDCs stimulated with either IFNα or IFNβ but were significantly upregulated upon IL-27 and IFNγ stimulation respectively (Fig.4 B,D).

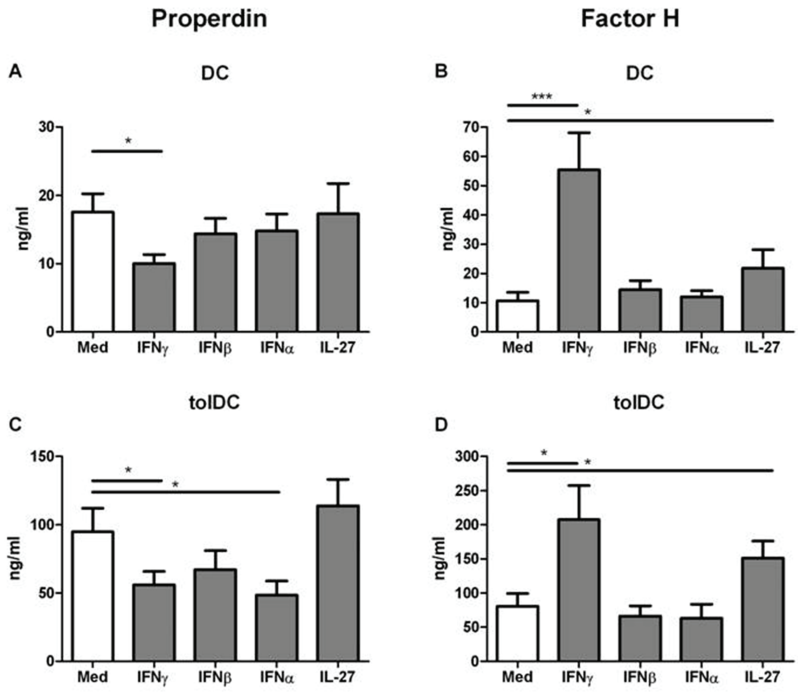

Figure 4: Type I IFNs decrease fP production but do not increase fH, while IL-27 specifically increases fH. Dendritic cells were harvested after 6 days of culture and stimulated with IFNγ, IFNβ, IFNα or IL-27. After 48 hours the supernatants were harvested and (A, C) fP and (B, D) fH was measured by sandwich ELISA. Data shown is the mean ±SD of 8-13 independent experiments.

DC derived fP and fH exhibit traditional functional characteristics.

A key characteristic of serum derived fH is its ability to bind C3b, so we assessed whether fH found in DC supernatants can also demonstrate functional activity by binding C3b. Although we found minimal binding in immature DC supernatants the binding was enhanced in the supernatants from IFNγ stimulated DCs. This binding was more pronounced in supernatants of IFNγ-stimulated tolDCs (Fig.5A). We assessed the ability of fP derived from DCs and tolDCs to bind its traditional ligand C3b and found that while the amount produced by DCs might be too little for detection, fP derived from tolDCs showed a strong and dose-dependent binding to C3b (Fig.5B). To confirm the specificity of this assay we show that binding is inhibited upon depletion of fP using pre-coupled anti-properdin beads (Fig.5C).Recent evidence suggests that fP possesses additional functions aside from C3b binding, and may act as an independent recognition molecule for necrotic and apoptotic cells. Necrotic cells were generated as previously described [30], and incubated with DC or tolDC conditioned supernatant followed by detection of fP binding. Using fluorescent microscopy, fP binding was detected on necrotic cells exposed to DC supernatant and this binding was

even more evident on necrotic cells exposed to tolDC supernatants (Fig.5D). This binding to necrotic cells was confirmed by flow cytometric analysis (Fig.5E), and quantification across a number of donors showed that tolDC supernatants demonstrated significantly higher levels of fP binding to necrotic cells compared to supernatant of DCs (Fig.5F).

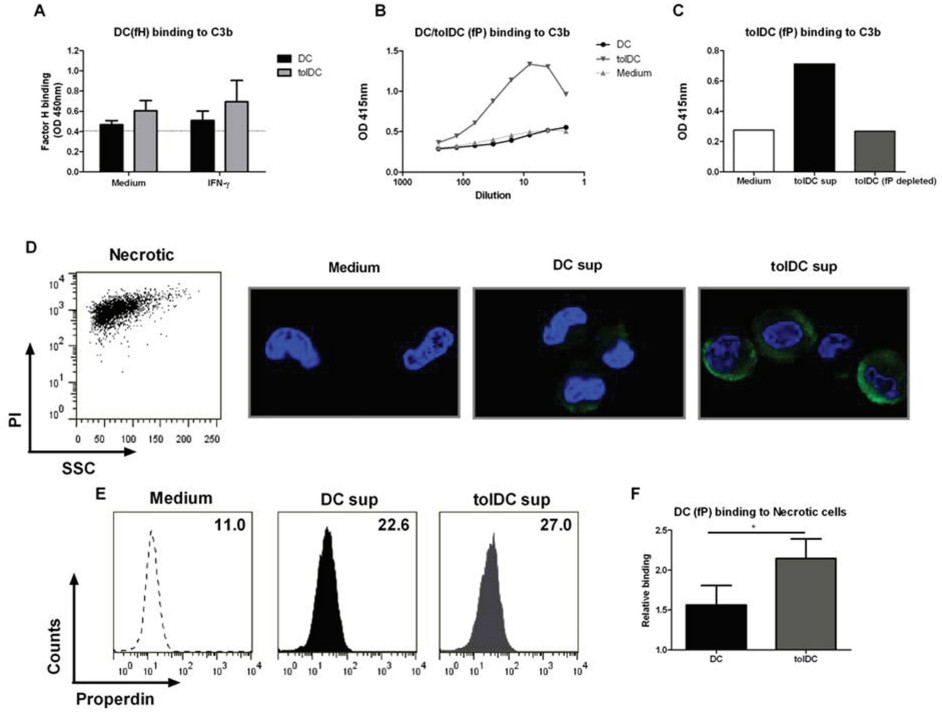

Figure 5: DC derived fP and fH exhibit traditional functional characteristics. Dendritic cells were harvested after 6 days of culture and either untreated or stimulated with IFNγ. After 48 hours the supernatants were harvested and incubated on a 96-well ELISA plate coated with C3b, followed by detection of (A) fH and (B) fP. (C) To determine the specificity of fP binding the same C3b binding assay was performed with tolDCs supernatants either untreated or depleted of fP using pre-coupled anti-properdin beads. Data shown is representative of 3 independent experiments. (D) Necrotic cells were generated and incubated with 48 hour supernatants from immature DC/ tolDC, followed by detection of fP binding by fluorescent microscopy. (F) The same experiment was performed followed by detection of fP binding by flow cytometry and calculated by MFI DC supernatant / MFI Medium alone.

Inhibition of fP in DCs decreases allogenic T cell proliferation, while inhibition of fH enhances the allostimulatory capacity of DCs.

In light of the recent evidence that AP components may play a role in the allostimulatory capacity of DCs, we investigated whether silencing of fP or fH in human DCs could alter T cell activation in an allogenic setting. We successfully and specifically silenced fP and fH in both immature and IFNγ-stimulated DCs

by up to 60% on average (Fig.6A,F). Cells treated with siRNA were harvested and co cultured with allogenic total CD4$^+$ T cells at indicated ratios for 5 days.

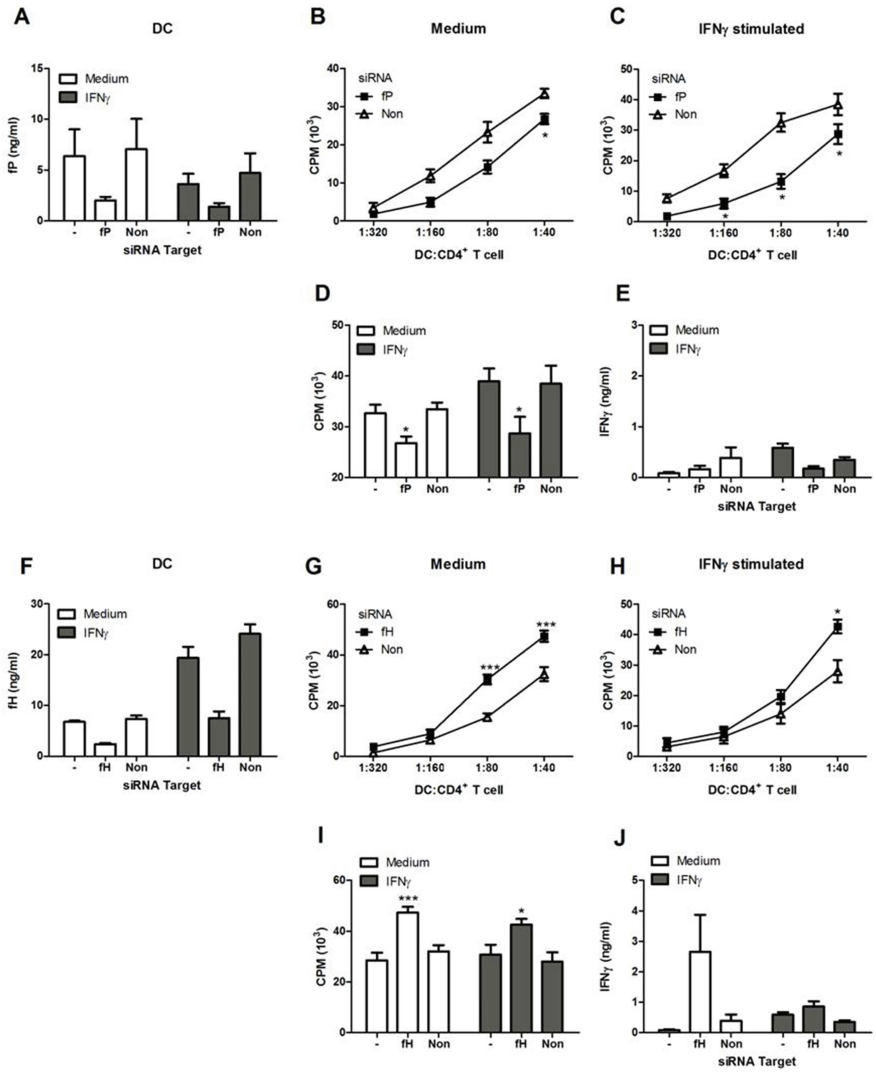

Figure 6: Inhibition of fP in DCs decreases the allostimulatory capacity of DCs while inhibition of fH enhances allogenic T cell proliferation. DCs were either non treated, or treated with siRNA targeting fP or non-specific (non) target. After 24 hours of culture, with or without IFNγ activation, supernatants were harvested and fP was measured by ELISA (A). DCs with either fP or control silencing, without (B) or with IFNγ (C) stimulation were co-cultured with allogenic CD4$^+$ T cells at indicated ratios. T cell proliferation was determined at day 5 of culture by ^{3}[H] incorporation. Data shown are the mean ±SD of triplicate cultures. The mean ±SD of proliferation (D) and IFNγ production (E) at the 1:40 ratio of 3 independent experiments of cells silenced for fP is shown. DCs were either non treated, or treated with siRNA targeting fH or non-specific (non) target. After 24 hours of culture, with or without IFNγ activation, supernatants were harvested and fH was measured

by ELISA (F). DCs with either fH or control silencing, without (G) or with IFNγ (H) stimulation were co-cultured with allogenic $CD4^+$ T cells at indicated ratios. T cell proliferation was determined at day 5 of culture by ^{3}[H] incorporation. Data shown are the mean ±SD of triplicate cultures. The mean ±SD of proliferation (I) and IFNγ production (J) at the 1:40 ratio of 3 independent experiments of cells silenced for fH is shown.

Proliferation was determined by ^{3}[H]-thymidine incorporation. We observed that silencing of fP in DCs significantly hampered their ability to induce allogenic T cell proliferation compared with DCs with non-targeted siRNA (Fig.6B). This inhibition of proliferation was even more pronounced when fP silencing was combined with IFNγ stimulation (Fig.6C). The silencing procedure by itself did not affect the T cell stimulatory capacity. The reduced proliferation upon fP silencing was significant for different individual experiments (Fig.6D). In these co-cultures, IFNγ production was relatively low, but showed a trend for reduced production in IFNγ-activated, fP-silenced DC (Fig.6E).
Similar experiments were performed using DCs treated with siRNA targeting fH. These cells induced significantly more T cell proliferation compared to non-target (non) treated DCs (Fig.6G). This was also observed, but to a lesser degree, with DCs activated with IFNγ (Fig.6H). Both immature and IFNγ-stimulated DCs silenced for fH exhibited more T cell proliferation, in particular at a ratio of 1:40 (Fig.6I). We assessed the production of IFNγ in T cell supernatants and found that immature DCs silenced for fH induced more IFNγ production. In line with our proliferation data the stimulatory effect of silencing fH was less potent when the DCs were first activated with IFNγ (Fig.6J). Taken together loss of fH induced more T cell proliferation while loss of fP decreased T cell proliferation, suggesting that the local complement activation is important in regulating DC: T cell responses.

Discussion

Dendritic cells are a heterogeneous population of professional antigen presenting cells responsible for both the initiation of immunity and immunological tolerance. Recent murine studies have suggested that complement activation is a key determinant in the ability of APCs to direct T cell responses. In this study we show that production of fP and fH by DCs is subject to regulation by IFNγ and IL-27 and that modulation of these regulators of the AP determines the potency of the T cell response. This implicates the local cellular and cytokine microenvironment as crucial mediators of overall complement regulation and subsequent T cell activation.
Several exciting studies over the last decade have introduced complement as a system that actively plays a role in regulating T cell immunity. By generating bone marrow APCs from $C3^{-/-}$[31,32], factor $B^{-/-}$, factor $D^{-/-}$, $C3aR^{-/-}$[33] and $C5aR^{-/-}$[34]

mice, various groups demonstrated that DCs require local complement activation in order to become fully competent APCs. To date these studies have mainly focused on rodent models, however DCs from C3 deficient individuals[35] were also shown to have impaired DC differentiation. More recently, a role of C3a and C5a derived from human DC in T cell proliferation has been demonstrated in vitro and in a humanised mouse model[8]. Further work on human DCs and the contribution of other complement factors has remained finite with mostly transcriptional analysis and limited protein data.

We focused on monocyte-derived DCs and tolerogenic DCs and showed that both cells produced fP and fH in an immature state with tolDCs expressing much higher levels of both components. Some studies have demonstrated gene expression of fP and fH in plasmacytoid DCs[21]. Although we did not perform PCR analysis on pDCs, we did test cellular supernatants and did not find any detectable levels of both components analysed in this study (data not shown).

Several complement components have been shown to possess GAS and ISRE elements in their promoters[36,37], so we assessed the ability of IFNγ to regulate fP and fH. Interestingly, IFNγ possessed opposing roles by decreasing fP production, while increasing fH. Decrease of fP upon IFNγ stimulation has been observed in primary monocytes and THP-1 cell lines[38], but to our knowledge this is the first report of this dual effect on fH and fP in human DCs. IFNγ is the only member of the type II IFNs so we questioned whether the regulation of fP and fH was unique for this specific member, or was a broader feature of the IFN family, including type I IFNs. IFNα and IFNβ behaved comparably in terms of fP and fH regulation and did not possess the same regulatory properties of IFNγ.

The IL-12 family has been gaining attention as a key regulator of immunity and immunological tolerance[39,40], and one family member, IL-27 has been shown to exert some functions typically seen with IFNγ stimulation[28,29,41,42]. Interestingly IL-27 stimulation of DCs did increase production of fH but did not influence fP production again stressing the uniqueness of IFNγ in its differential regulation of these two opposing molecules of the complement system.

We observed in our study considerable variation using LPS stimulation, with no clear regulation of fP or fH evident across a number of donors, despite its ability to induce IL-12p40 and IL-10 production. This fluctuating effect of LPS on fP production was not observed for fH. There are some conflicting reports regarding the ability of LPS to regulate fP. While it has been shown that LPS can up-regulate fP in monocytic cells lines[38], decreased transcriptional expression has been observed in murine BMDCs[33]. Conversely again upregulation of fP mRNA has been seen in human DCs[21]. This may be explained by different strains of LPS used in set of experiments.

We performed western blot analysis to confirm that fP and fH possessed the

expected molecular structure. fP demonstrated an expected band at approx. 55kDa under reducing conditions. While we did observe a band of approx. 155kDa for fH in tolDC lysates no clear band was evident in DC lysates. Other studies demonstrating fH in DCs have focussed on DC supernatants[21] and it may be that our detection method was not sensitive enough to detect fH in DC cell lysates. Notably we did observe two smaller bands of approx. 30-35kDa (data not shown). Further work is needed to determine if these molecules are fH like or related proteins and what role they might play in DC biology, although a role in phagocytosis has been demonstrated previously[43]. Functionally both fP and fH were active in the traditional complement assessment by binding to C3b. In recent years fP has been shown to act as a recognition molecule[11,44,45] with the ability to bind to, amongst others, apoptotic[46] and necrotic cells[30]. We found that fP from DCs could bind necrotic cells though we did not test whether this was independent of complement activation.

We have shown that cytokines can profoundly and differentially regulate the opposing regulators of the AP, fP and fH. IFNγ stimulation of DCs also changes many phenotypical features of DCs including up regulating co-stimulatory molecules such as CD80 and CD86. In order to establish whether simply shifting the balance of production of fP and fH could alter the allostimulatory capabilities of DCs we silenced fP or fH in DCs and co-cultured these cells with allogenic $CD4^+$ T cells. We found that inhibition of fH led to a significant increase in T cell proliferation while inhibition of fP led to decreased proliferation. DCs silenced for fP and pre-matured with IFNγ were even further hampered in their allostimulatory capacity, likely because of the combined silencing by siRNA and decreased fP production by IFNγ. Further work is needed to fully establish whether the diverging T cell proliferation is a direct effect of fP and fH on the T cells or a consequence of altered local complement activation.

Taken together our results demonstrate a novel role for fP and fH production by DCs in the regulation of T cell activation. Both molecules can be influenced by IFNγ whereas only fH is increased by IL-27, indicating that the local cellular and cytokine environment can potentially influence the local complement activation and in turn local T cell responses.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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4