Multifaceted role of the complement system in health and disease: a focus on properdin
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Human macrophages produce and secrete complement regulators factor H and factor H-like protein 1 and complement stabilizer properdin

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Abstract

Macrophages are innate immune cells, involved in the maintenance of tissue homeostasis and the promotion of tissue repair. These cells are potent phagocytes expressing complement receptors to respond to complement factors and opsonized targets. Most complement factors are produced by the liver, however, also extrahepatic sources of complement factors have been described. Here we show that human monocyte-derived macrophages secrete the positive regulator of complement, properdin, and that levels were upregulated by LPS and CD40L stimulation and downregulated by IFN-γ stimulation. Expression of factor H and factor H-like protein 1 (FHL-1), negative regulators of the complement system, was comparable in the liver, however, in normal human serum, FHL-1 protein levels were lower compared to factor H. FHL-1 was detected in human renal tissue, which suggests renal clearance of this 42 kDa protein. Human monocyte-derived macrophages were able to express and produce factor H and FHL-1, and IFN-γ stimulation upregulated factor H and FHL-1 levels. In all donors, secreted FHL-1 levels were higher when compared to factor H. These data identify macrophages as potent producers of properdin, factor H and FHL-1. Preferential expression and secretion of FHL-1 by cells of the myeloid compartment suggests a local role in the regulation of complement activation.

Introduction

Macrophages are potent phagocytic cells playing an important role in homeostasis, tissue repair and immune responses (1–3). In the inflamed tissue, the local production and release of cytokines, chemokines and complement factors (anaphylatoxins C3a/ C5a) is responsible for the recruitment of immune cells, including monocytes (1,3). Proinflammatory cytokines like interferon gamma (IFN-γ), pathogen-associated molecular patterns (PAMPs) like lipopolysaccharide (LPS) and danger-associated molecular patterns (DAMPs) can polarize monocytes into proinflammatory macrophages. Recruited monocytes can also adopt an anti-inflammatory phenotype, involved in suppressing inflammation and inducing tissue repair (1,3). In addition, these pro- and anti-inflammatory macrophages function as antigen-presenting cells, directing T cell differentiation into T helper 1 (Th_1) and Th_2 cells, respectively (1,4).

The complement system plays an important role in the defense against microorganisms and the opsonization of dead cells (5,6). Most complement factors are predominantly produced by the liver, however extrahepatic sources like production by myeloid cells have also been described (7,8). Some complement factors are mainly produced extrahepatically, like C1q (9) and properdin (10). Properdin, the only known positive regulator of the complement system, is able to stabilize the C3 convertase enhancing its half-life 5-10 times (11), and is produced by myeloid cells like neutrophils and dendritic cells (10,12–14). In contrast, negative complement regulator factor H, a ~155 kDa soluble factor, is involved in complement regulation by causing the decay of the C3 convertase and in addition contains cofactor activity (15,16). Alternative splicing of the factor H gene results in the generation of ~42 kDa factor H like protein 1 (FHL-1), containing complement control protein (CCP) domains 1-7 similar to factor H, but lacks the C-terminal domains 8-20 (17–21). Factor H and FHL-1 are produced by the liver, however, information on extrahepatically production is rather limited. Factor H production was detected in some non-hepatic cells, amongst them dendritic cells, endothelial cells, keratinocytes and skin fibroblasts (10,22–24). FHL-1 was found synthesized by mononuclear phagocytes, fibroblast, endothelial cells ((24–26) reviewed by (27)) and retinal pigment epithelium (RPE cells), contributing to local complement regulation in the eye (28). However, the physiological role for FHL-1 remains largely unknown (21). Whether cells produce equal amounts of factor H and FHL-1 is currently unknown and also the impact of differential expression is unresolved. However, it is conceivable that the bulky factor H will have different functional properties as compared to the slim FHL-1 especially in the context of tissues outside the blood compartment or in cell-cell contact. Recently, it was shown that immune cells are able to produce various complement factors and express complement receptors like C3aR and C5aR to respond

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to anaphylatoxins C3a and C5a (7,29). In addition, C3a and C5a generated locally were shown to enhance local T cell proliferation and activation in antigen-presenting cell (APC) - T cell interaction (30). Downregulation of the decay accelerating factor (DAF) also increased T cell proliferation in APC-T cell interaction (31).

We investigated systemic and local production and secretion of soluble complement factors factor H, FHL-1 and properdin. Comparable factor H and FHL-1 expression were observed in the liver, however, FHL-1 levels were lower in normal human serum. Analysis in renal biopsies showed deposition of FHL-1 but not factor H, suggesting renal clearance of FHL-1. Next to the liver, also pro- and anti-inflammatory macrophages were able to produce properdin, factor H and FHL-1 and levels were altered upon stimulation. Preferential expression and protein secretion of FHL-1 by cells from the myeloid cell compartment was observed, suggesting a role for FHL-1 in local complement regulation.

Materials and methods

Generation of macrophages

Macrophages were derived from monocytes as described previously (32,33). In brief, peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors (Sanquin, Amsterdam, The Netherlands) using Ficoll density gradient centrifugation (Pharmacy, LUMC, Leiden, The Netherlands). Monocytes were isolated by positive magnetic cell sorting using anti-CD14 MACS microbeads (130-050-201, Miltenyi Biotec, Leiden, The Netherlands). Cultures were supplemented with medium (RPMI 1640 (524000-025, Gibco/Life technologies, Breda, The Netherlands) +10% heat-inactivated fetal calf serum (FCS; Bodinco, Alkmaar, The Netherlands), 90 U/mL penicillin and 90 µg/mL streptomycin (Gibco/Life technologies)) containing 10 ng/mL GM-CSF (Premium grade, 130-093-868, Miltenyi Biotec) or 100 ng/mL M-CSF (Premium grade, 130-096-493, Miltenyi Biotec) for the generation of pro- and anti-inflammatory macrophages, respectively. Cultures were refreshed at day three and five, and cells were harvested at day seven using trypsin (T4174, Sigma-Aldrich, Saint Louis, MO, USA). Differentiation was determined by flow cytometry (LSR-II 4L Full, BD Biosciences, San Jose, CA, USA) using mouse anti-human CD163 (PE, 1:20, Clone GHI/61, 333606, Biolegend, San Diego, CA, USA) and mouse anti-human CD14 (APC, 1:200, Clone MφP9, cat no. 345787, BD). Results were analyzed using FlowJo Software version 10.6.1 (Tree Star, Ashland, OR, USA). In addition, differentiation was analyzed by microscopy (DMI6000 B, Leica, Wetzlar, Germany) using LASX software.

Stimulation of macrophages

Harvested pro-and anti-inflammatory macrophages were stimulated with lipopolysaccharide (LPS, final concentration of 200 ng/mL, *E.coli* Serotype EH100, Enzo Life Sciences, Zandhoven, Belgium), irradiated (7500 Rad, Gammacell 1000 Elite, Best Theratronics, Kanata, ON, Canada) mouse fibroblast L-cells stably transfected with CD40L (1:10 ratio, (34)) or control cells (L-Orient) or with interferon-gamma (IFN-γ, 100 ng/mL, PeproTech, London, UK). After 48 h, the supernatants were harvested and kept frozen at -20°C until further usage.

Cytokine, properdin and FH measurements

Secretion of IL-6 and IL-10 by macrophages was analyzed by ELISA following manufacturer's instructions (IL-6: M9316, Sanquin, IL-10: M9310, Sanquin, or 555157, BD Biosciences). Absorbance was measured at 450nm (iMark Microplate Reader, Bio-Rad, Hercules, CA, USA).

Properdin was measured by coating ELISA plates with mouse monoclonal anti-human properdin (0.3 µg/mL, A233, Quidel, San Diego, CA, USA) overnight at room temperature. Plates were blocked (PBS-1% bovine serum albumin (BSA; Sigma-Aldrich, Saint Louis, MO, USA) and diluted supernatants (dilution buffer: PBS-0.05% Tween-20 (Sigma-Aldrich)-1%BSA) were incubated for 1 h at 37°C. Next, plates were washed with wash buffer (PBS containing 0.05% Tween-20) and incubated with rabbit anti-human properdin-digoxigenin (DIG; in-house derived, 1:2500) for 1 h at 37°C. After washing, plates with peroxidase-conjugated IgG fraction monoclonal mouse anti-DIG (0.1 µg/mL, 200-032-156, Jackson ImmunoResearch, Cambridgeshire, UK) for 1 h at 37°C and developed using 3,3',5,5'-tetramethylbenzidine (TMB, Sigma-Aldrich). The reaction was stopped using 1 M H_2SO_4 and absorbance was measured as described above.

Factor H/FHL-1 levels were measured by ELISA. In brief, mouse monoclonal anti-human factor H (19.5 µg/mL, OX23) was coated overnight. Plates were blocked (PBS-1% BSA) and supernatants were diluted in dilution buffer and incubated for 1 h at 37°C. Next, plates were washed and incubated with rabbit anti human-factor H-DIG (1:10.000, in-house derived) for 1 h at 37°C. After washing, plates were incubated with peroxidase-conjugated IgG fraction monoclonal mouse anti-DIG (0.1 µg/mL, 200-032-156, Jackson ImmunoResearch) for 1 h at 37°C and developed using TMB. The reaction was stopped with 1 M H_2SO_4 and the absorbance was measured at 450 nm.

Gene transcription levels in pro- and anti-inflammatory macrophages

mRNA was isolated from ~0.5 x 10^6 pro- and anti- inflammatory macrophages using the RNeasy Micro kit (Qiagen) following the manufacturer's instructions. mRNA was isolated

from ~3 x 10^6 monocytes, HuH-7 (gift from L.J.A.C. Hawinkels/M.A.C. Mieremet-Ooms) and HepG2 cells (gift from J. Kroon/H.C.M. Sips) using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. mRNA from liver tissue was isolated using Trizol followed by the RNeasy Mini Kit (Qiagen). On column genomic DNA digestion and cDNA generation was performed as described previously (35).

For qPCR analysis, 2 ng cDNA was pipetted into a 384 hard-shell PCR plate (thin wall, Bio-Rad) in combination with primers diluted in IQ SYBR green mix (Bio-Rad). Specific primers were used to determine human *GAPDH* (10), *CFP* (10), *CFH* and *FHL1* expression (Table 1, final primer concentration of 1.25 µM, Biolegio, Nijmegen, The Netherlands). Samples were run (C1000 Thermal Cycler, (Bio-Rad)) and gene expression levels were determined by calculating the mean values of triplicates followed by the ΔCT (gene of interest – *GAPDH*). Copies per *GAPDH* were calculated using 2$^{-\Delta CT}$.

Table 1: Oligonucleotide sequences used for real-time PCR

Target gene	GenBank accession no.	Product size (bp)	Forward primer (5'- 3')	Reverse primer (5'-3')
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	**NM_001357943.2**	175	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG
CFP	**NM_001145252.3**	181	TTGCGGCTTCGTGTGTCTCC	GTAATCACCCTGCTCCCAAG
CFH	**NM_000186.4**	128	GGACACAGTGAAGTGGTGGAAT	CCACAGGTACTCTCCTCCACA
FHL1	**NM_001014975.3**	99	TCCTGGCTACGCTCTTCCAA	CAGAGGGTAAAGCTGACACGG

Factor H and FHL-1 staining on renal tissue

Biopsies of healthy renal tissue were stained for factor H and FHL-1. In brief, biopsies were sectioned (3µm), acetone fixed and frozen. Sections were thawed and endogenous peroxidase (PBS-0.1% NaN$_3$-0.3% H$_2$O$_2$) was blocked for 30 min at room temperature. Next, sections were washed and blocked in PBS-1%BSA-1% heat inactivated normal goat serum (NGS). Slides were incubated with OX24 (0.1 µg/mL, gift from L.A. Trouw), staining both factor H and FHL-1, and with FH.16 (0.4 µg/mL, clone anti-FH.16, binding factor H domains 16/17, gift from I. Jongerius (36)) diluted in PBS-1%BSA (PB) overnight at room temperature. Next day, slides were washed, incubated for 1 h with goat-anti-mouse HRP (5 µg/mL, P0447, DAKO) diluted in PB. All slides were washed and developed using Nova RED (SK-4800, Vector Labs, Peterborough, UK). Slides were washed (PBS) and counterstained with Mayers hematoxylin (1.09249.0500, Merck, Darmstadt, Germany). Slides were dried overnight at room temperature before being covered using enthellan

(1.07961, Merck, Darmstadt, Germany). Slides were imaged (Pannoramic Midi scanner, 3DHISTECH, Budapest, Hungary) and images were taken (CaseViewer from 3DHISTECH).

Western blot analysis

Purified factor H (75 ng, A410, Quidel), recombinant FHL-1 (75 ng, kindly provided by C.Q. Schmidt, (37)), and normal human serum (3 µl) were prepared for separation using SDS PAGE (24 µl sample + 12 µl blue protein loading dye, (B7703S, New England BioLabs Inc., Ipswich, MA, USA) and boiled for 5 min). All samples were loaded onto a mini-Protean 10% gel (4568033, Bio-Rad) with a marker (10µl, PageRuler Plus Prestained Protein Ladder, 10-250 kDa, 26619, ThermoFisher Scientific, Waltham, MA, USA). Next, samples were blotted using the trans-blot turbo transfer system (PVDF, Bio-Rad, 1704156). Blots were blocked (PBS-5% skim milk (70166, Sigma-Aldrich) overnight at 4°C and incubated for 1 h at room temperature with either rabbit anti-human factor H-DIG (1:5000, in-house DIG labelled) or OX24 (5 µg/mL) diluted in PBS-5% skim milk. Blots were washed (PBS-0.05% Tween-20) and incubated with either peroxidase-conjugated IgG fraction monoclonal mouse anti-DIG (1:1000, Jackson) or goat-anti-mouse-HRP (50 ng/mL, P0447, DAKO) for 1 h at room temperature. Blots were washed and developed (SuperSignal West Pico PLUS Chemiluminescent Substrate, 34580, ThermoFisher Scientific) and imaged with the Chemidoc Touch (Bio-Rad). Figures were prepared using Image Lab software (Bio-Rad).

Immunoprecipitation

Factor H and FHL-1 were immunoprecipitated from the supernatant from pro- and anti-inflammatory macrophages (stimulated with IFN-γ for 48 h) following the protocol. In brief, per sample 50 µl magnetic Dynabeads Protein G (30mg/mL, 10004D, Invitrogen, Waltham, MA, USA) were mixed with 5 µg OX24 or an isotype control (5 µg, mouse IgG1, DAKO, X0931) diluted in PBS-0.02% Tween-20 and incubated for 10 min at room temperature rotating. Magnetic bead-antibody complexes were washed (placed tube on magnet, removed supernatant, added PBS-0.02% Tween-20) and cross-linked using the cross-linking immunoprecipitation (CLIP) protocol. In brief, the antibody-bead complexes were washed twice with 200 µl conjugation buffer (20 mM sodium phosphate, 0.15 M NaCl, pH 7.4). The complexes were resuspended in 250 µl 5 mM BS^3 (dissolved in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4) and incubated for 30 min at room temperature rotating. The reaction was quenched using 12.5 µl/sample (1 M Tris HCl, pH 7.5) and incubated for 15 min at room temperature rotating. The cross-linked antibody-beads complexes were washed three times with 200 µl PBS-0.02% Tween-20. Next, the antigen was immunoprecipitated. Supernatant (1 mL containing ~ 15 ng factor H/FHL-1) was incubated for 15 min at room temperature rotating. Supernatant was removed (placing the tube containing the magnetic beads-antibody-antigen complexes

on the magnet with consecutive removal of the fluid) and the beads were incubated again with 1 mL supernatant. The magnetic beads-antibody-antigen complexes were washed 3 times using PBS by putting the tube on the magnet. The mixture was transferred to a new tube and the antigen was eluted by incubating the complexes with 20 μl elution buffer (50 mM glycine pH 2.8) for 4 min at room temperature. The eluted protein was transferred to a clean tube and 4 μl 1M Tris (pH 7.5) was added to adjust the pH of the sample. The eluted samples were prepared for separation using SDS PAGE (to 24 μl elution buffer 12 μl blue protein loading dye (B7703S) was added). In addition, purified factor H (15 ng, A410, Quidel) and recombinant FHL-1 (15 ng) were prepared and all samples were loaded and blotted as described above. Factor H and FHL-1 were detected using rabbit anti-human factor H-DIG (1:5000, in-house DIG labelled) and peroxidase-conjugated IgG fraction monoclonal mouse anti-DIG (1:1000, Jackson). This protocol was also used to immunoprecipitate factor H and FHL-1 from supernatant from unstimulated pro- and anti-inflammatory macrophages generated from two donors (after concentrating the supernatant). Figures were prepared using Image Lab software (Bio-Rad) and factor H/FHL-1 levels were quantified using volume tools after global background subtraction.

Statistics

Statistical analysis between two groups was assessed by a paired, Wilcoxon matched-pairs signed rank test. Significance was defined as $P \leq .05$. For statistical analysis and graphical representations, GraphPad Prism v.9.01 was used (GraphPad Software, San Diego, CA, USA).

Results

Generation of monocyte derived macrophages

Polarization of monocytes into either pro- or anti-inflammatory macrophages was analyzed using microscopy. Proinflammatory macrophages, generated with GM-CSF, showed a "fried-egg" phenotype, whereas anti-inflammatory macrophages, generated with M-CSF, showed a spindle-shaped phenotype (Figure 1a) (38–40). Polarization was also followed using flow cytometry, showing the intermediate expression of CD14 and CD163 on proinflammatory macrophages, whereas anti-inflammatory macrophages express homogeneous and higher levels of CD14 and CD163 (Figure 1B-C). Functional polarization of macrophages was confirmed by significantly higher levels of IL-6 secretion by proinflammatory macrophages following LPS stimulation (Figure 1D). Anti-inflammatory macrophages were able to secrete IL-6, however, to a 100-fold lower extent when compared to proinflammatory macrophages (Figure 1D). Both pro- and anti-inflammatory macrophages produced similar amounts of IL-10 upon stimulation with LPS.
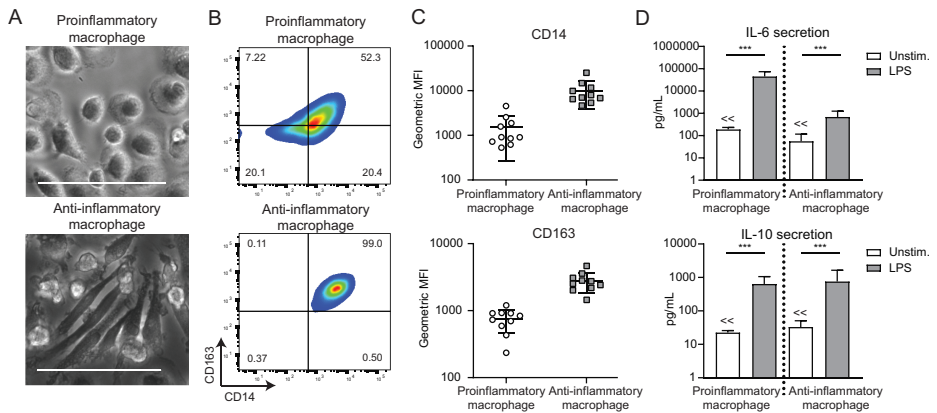
Figure 1: Phenotypic analysis of monocyte-derived pro- and anti-inflammatory macrophages. Monocytes were isolated and stimulated for 7 days with GM-CSF or M-CSF for the generation of proinflammatory or anti-inflammatory macrophages, respectively. **(A)** Microscopic analysis of pro- and anti-inflammatory macrophages at day 7. Scale bar: 100 μm. **(B)** Representative FACS plots for the phenotypic analysis of CD163 and CD14 expression. **(C)** Quantification of CD14 and CD163 expression on pro- and anti-inflammatory macrophages (n=10). **(D)** Analysis of IL-6 and IL-10 production by unstimulated and LPS stimulated pro-and anti-inflammatory macrophages (n=11). Data are presented as mean ± SD. Wilcoxon matched-pairs signed rank test, *** P ≤.001.
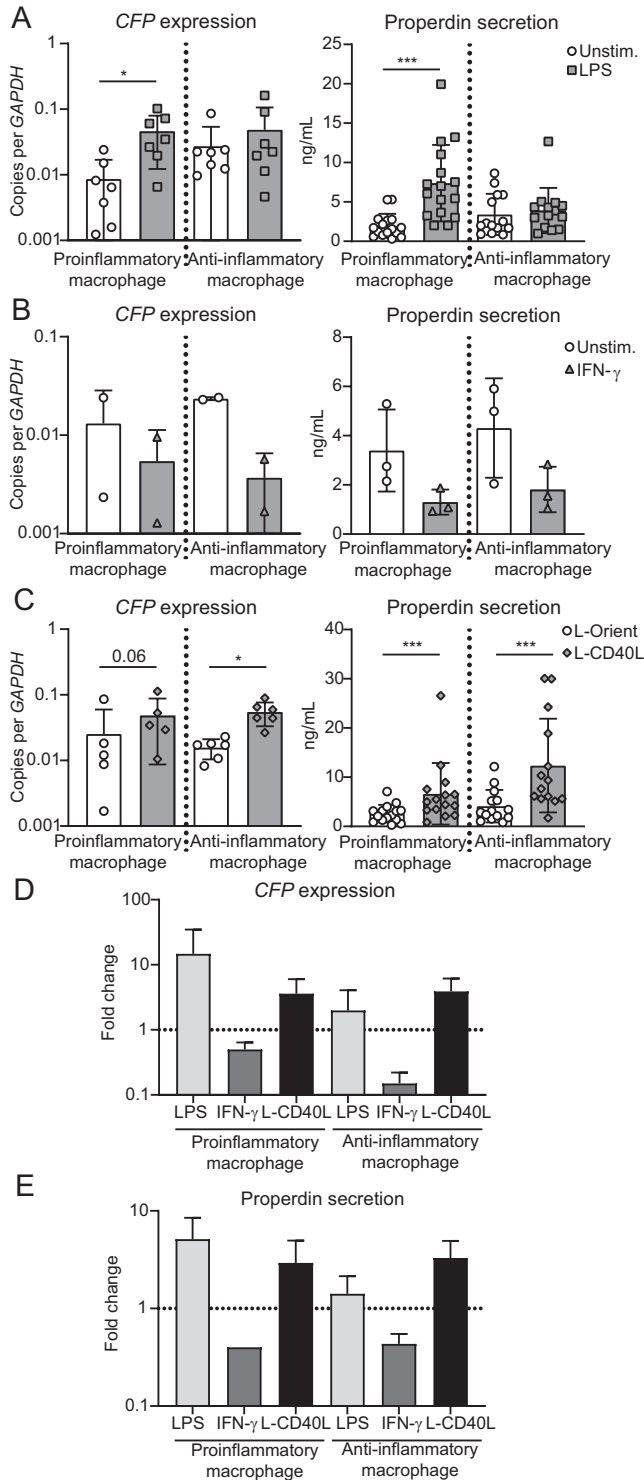
Properdin expression and secretion by macrophages

We investigated the expression and secretion of properdin by pro- and anti-inflammatory macrophages and mimicked the proinflammatory milieu during inflammation by stimulating the cells with LPS or IFN-γ. Properdin (*CFP*) was expressed spontaneously by pro- and anti-inflammatory macrophages and in proinflammatory macrophages, *CFP* levels were upregulated by LPS. Similar trends were observed when examining properdin secretion, which was significantly upregulated by proinflammatory macrophages after LPS stimulation (Figure 2A). After stimulation with IFN-γ, *CFP* expression was downregulated in both pro- and anti-inflammatory macrophages. Properdin protein levels were also reduced after IFN-γ stimulation by both cell types (Figure 2B). Stimulation with CD40L, a molecule expressed on activated T cells thereby mimicking APC-T cell interaction, increased *CFP* expression in both types of macrophages, which also resulted in increased properdin secretion (Figure 2C).

We calculated the effect of the stimulation in the individual donors and showed an increased *CFP* expression after LPS (14.8-fold) and L-CD40L stimulation (3.6-fold) in proinflammatory macrophages. In anti-inflammatory macrophages, *CFP* expression was also increased after stimulation with LPS (2.0-fold) and L-CD40L (3.9-fold). In both types of macrophages, stimulation with IFN-γ lowered *CFP* expression (proinflammatory macrophages: 2-fold decrease; anti-inflammatory macrophages: 5-fold decrease)

(Figure 2D). Similar effects were observed for secreted properdin levels, where fold change comparison showed increase properdin secretion after stimulation with LPS (proinflammatory macrophages: 5.1-fold; anti-inflammatory macrophages: 1.4-fold) and L-CD40L (proinflammatory macrophages: 2.9-fold; anti-inflammatory macrophages: 3.3-fold) and reduced properdin secretion after IFN-γ in both types of macrophages (2.5-fold decrease) (Figure 2E).

Figure 2: Properdin expression and secretion by pro- and anti-inflammatory macrophages. Pro- and anti-inflammatory macrophages were generated and stimulated with 200 ng/mL LPS, 100 ng/mL IFN-γ or with CD40L expressing L-cells (1:10 ratio). Transcript levels of *GAPDH* and *CFP* were determined by qPCR. Properdin protein levels were determined by ELISA. Both properdin mRNA and protein levels were determined 48 h after stimulation. **(A)** *CFP* expression in unstimulated and LPS stimulated pro- and anti-inflammatory macrophages (n=7). Secretion of properdin in unstimulated and LPS stimulated pro-(n=16) and anti-inflammatory macrophages (n=14). **(B)** *CFP* expression in unstimulated and IFN-γ stimulated pro- and anti-inflammatory macrophages (n=2). Secretion of properdin in unstimulated and IFN-γ stimulated pro- and anti-inflammatory macrophages (n=3). **(C)** *CFP* expression in control (L-Orient) and L-CD40L stimulated pro- (n=5) and anti-inflammatory macrophages (n=6). Secretion of properdin in L-Orient and L-CD40L stimulated pro- (n=15) and anti-inflammatory macrophages (n=14). **(D)** Fold change in *CFP* expression of LPS (proinflammatory macrophage, n=7; anti-inflammatory macrophage n=7) and IFN-γ (proinflammatory macrophage, n=2; anti-inflammatory macrophage, n=2) stimulated cells compared to unstimulated pro-and anti-inflammatory macrophages. *CFP* fold change expression for L-CD40L (proinflammatory macrophage, n=5, anti-inflammatory macrophage, n=6) stimulated cells compared to control cells (L-Orient). Data are presented as mean ± SD. **(E)** Fold change in properdin secretion of LPS (proinflammatory macrophage, n=16; anti-inflammatory macrophage, n=14) and IFN-γ (proinflammatory macrophage, n=3; anti-inflammatory macrophages, n=3) stimulated cells compared to unstimulated pro-and anti-inflammatory macrophages. Fold change in properdin secretion for L-CD40L (proinflammatory macrophage, n=15; anti-inflammatory macrophages, n=14) stimulated cells compared to control cells (L-Orient). Wilcoxon matched-pairs signed rank test, * P < .05, *** P < .001.   ------→

A

CFP expression

Properdin secretion

B

CFP expression

Properdin secretion

C

CFP expression

Properdin secretion

D

CFP expression

E

Properdin secretion

**Clearance of FHL-1 results in lower systemic levels**

Most circulating complement factors are produced by the liver and we examined *CFH* and *FHL1* expression in unstimulated liver cells and tissue. In tissue obtained from two liver biopsies, expression of *CFH* and *FHL1* was high and no difference in *CFH* and *FHL1* expression was observed. In the HuH-7 liver cell line, the expression of *CFH* and *FHL1* was lower when compared to the expression in liver biopsies. In line with the liver biopsy data, no difference in *CFH* and *FHL1* expression was observed. In the HepG2 cell line, *CFH* and *FHL1* expression were not detected as expected (41) (Figure 3A). Western blot analysis showed the detection of purified factor H and recombinant FHL-1 both by a monoclonal (OX24) and polyclonal antibody (Figure 3B, lane 2-3). Despite similar levels of mRNA for *CFH* and *FHL1* in the liver, at the protein level, as determined in serum, only minute amount of FHL-1 could be detected (Figure 3B, lane 5). A possible explanation for the observed equal production in liver and differential levels in serum may be related to renal clearance by the substantially smaller FHL-1. Therefore, we have stained tissue sections of human kidneys. Using OX24, a monoclonal antibody detecting both factor H and FHL-1, a very prominent staining at the tubular brush border was observed in biopsies (Figure 3C, arrowheads). In contrast, when using an antibody specific for factor H (FH.16), no deposition was observed in the tubular compartment (arrowheads), however, some vascular staining in the glomeruli was observed (arrow, biopsy 2). These results suggest that the FHL-1 produced by the liver is cleared from the circulation via the kidney (Figure 3C).

Preferential expression of FHL1 in pro- and anti-inflammatory macrophages

To investigate whether cells from the immune system were able to produce and secrete FHL-1 locally, expression of *CFH* and *FHL1* by myeloid cells was examined. Monocytes did not seem to express *CFH* and *FHL1*, as was also indicated before (24). Compared to monocytes, both pro- and anti-inflammatory macrophages express high *CFH* and *FHL1* levels. *FHL1* expression was significantly higher compared to *CFH* expression in all donors tested, despite the variation between the different donors (Figure 4A, proinflammatory 4.5-fold increase and anti-inflammatory macrophages 5.2-fold increase). Using an ELISA to examine factor H and FHL-1 protein levels, it was observed that proinflammatory macrophages secreted higher levels of both factor H and FHL-1 compared to anti-inflammatory macrophages. Stimulation with LPS did not have an effect (Figure 4B, left graph). Stimulation with IFN-γ resulted in increased factor H/FHL-1 secretion in both cell-types, whereas L-CD40L only increased factor H/FHL-1 secretion by proinflammatory macrophages (Figure 4B). To increase sensitivity, factor H and FHL-1 were immunoprecipitated from supernatant of IFN-γ-stimulated pro- and anti-inflammatory macrophages and the eluate was analyzed by western blot (Figure 4C). Both factor H and FHL-1 were secreted. Quantification showed that secreted FHL-1 levels were higher when compared to factor H (Figure 4D, proinflammatory macrophages: 2.3-fold increase, n=3; anti-inflammatory macrophages: 2.9-fold increase, n=3).
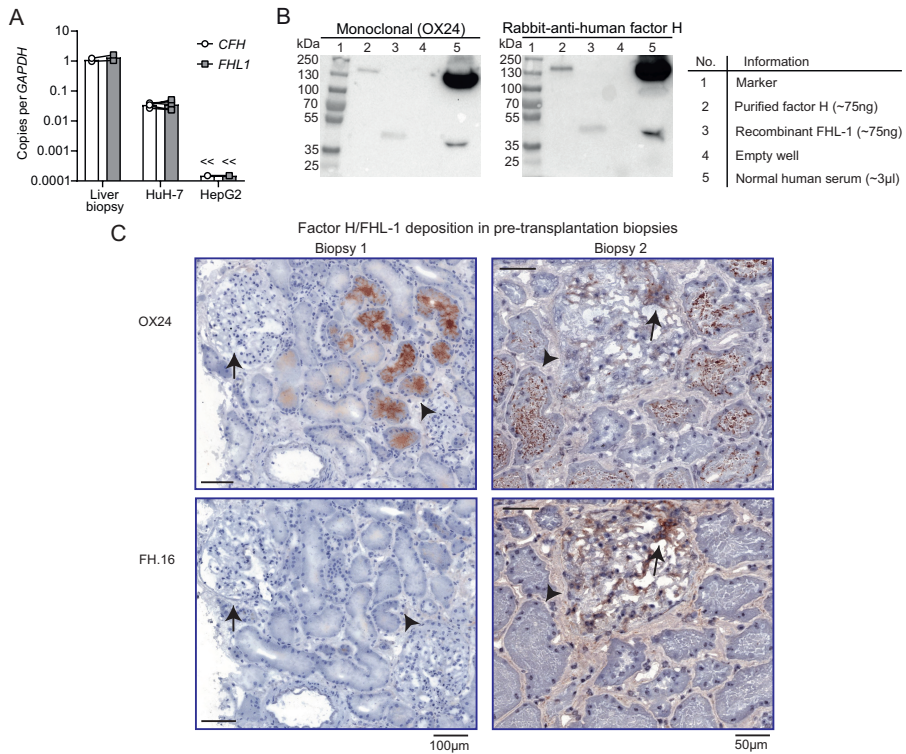
Figure 3: Detection of FHL-1, but not factor H, in healthy renal tissue. (A) Comparison of *CFH* and *FHL1* expression in the tissue (liver biopsy n=2) and liver cell lines (HuH-7 n=5, HepG2 n=4). (B) Western blot analysis of factor H and FHL-1 in normal human serum (NHS) compared to purified factor H and recombinant FHL-1. Factor H and FHL-1 were detected using both a monoclonal (OX24) and polyclonal (rabbit-anti-human factor H-DIG) antibody. (C) Staining for factor H/FHL-1 deposition (OX24) or factor H specifically (FH.16) in biopsies of healthy renal tissue. Representative images of factor H/FHL-1 deposition in two biopsies of healthy renal tissue. Arrowheads indicate tubular factor H/FHL-1 deposition, arrows indicate factor H/FHL-1 deposition in glomeruli.
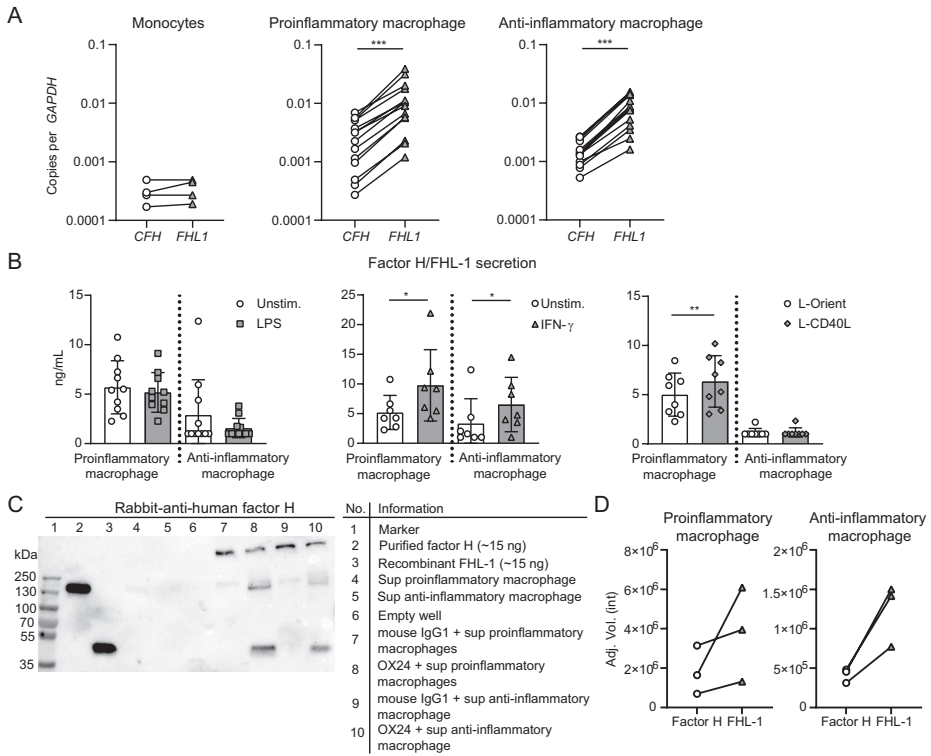
Figure 4: Expression and secretion of factor H and FHL-1 by myeloid cells. (A) *CFH* and *FHL1* expression by monocytes (*CFH* n=4/4 CT>32; *FHL1* n=2/4 CT>32, CT=32 used for calculations), proinflammatory macrophages (n=14; in 2/14 *CFH* CT>32, CT=32 used for calculations) and anti-inflammatory macrophages (n=14; in 2/14 *CFH* CT>32, CT=32 used for calculations). **(B)** Factor H/FHL-1 secretion by unstimulated and LPS stimulated pro- (n=10) and anti-inflammatory macrophages (n=11, no secretion detected in 7/11 donors), unstimulated and IFN-γ stimulated pro- (n=7) and anti-inflammatory macrophages (n=7, no secretion detected in 3/7 unstimulated and 1/7 IFN-γ stimulated anti-inflammatory macrophages). Factor H/FHL-1 secretion by L-Orient and L-CD40L stimulated pro- (n=8) and anti-inflammatory macrophages (n=8, no secretion detected in 7/8 stimulated anti-inflammatory macrophages). **(C)** Immunoprecipitation of factor H and FHL-1 from supernatant of IFN-γ stimulated pro- and anti-inflammatory macrophages, analyzed by western blot. **(D)** Factor H and FHL-1 secretion by pro- and anti-inflammatory macrophages, quantified after western blot analysis. Pro- and anti-inflammatory macrophages from one donor were stimulated with IFN-γ for 48 h before immunoprecipitation followed by western blot analysis. Pro- and anti-inflammatory macrophages from two other donors were incubated for 48 h without stimulation (total n=3). Supernatant from unstimulated macrophages was concentrated (proinflammatory macrophages 100 ng/mL and 106 ng/mL after concentration, 1 mL incubated with antibody-coupled beads; anti-inflammatory macrophages 14.2 ng/mL and 18.5 ng/mL after concentration, 1.25 mL incubated with the antibody-coupled beads). Quantification was performed with ImageLab using the volume tools after global background subtraction. Wilcoxon matched-pairs signed rank test, * P <.05, ** P <.01, *** P < .001.

Discussion

In this study, we investigated the expression of *CFP*, *CFH* and *FHL1* in various primary cells and cell lines. Proinflammatory macrophages were shown to produce and secrete properdin and factor H/FHL-1, where stimulation with LPS increased properdin levels but did not affect FH/FHL-1 levels. Stimulation with IFN-γ reduced properdin levels, but increased expression and secretion of FH/FHL-1. L-CD40L stimulation increased both properdin and FH/FHL-1 levels. In anti-inflammatory macrophages, LPS did not alter properdin and FH/FHL-1 levels, whereas stimulation with IFN-γ reduced properdin levels but increased FH/FHL-1 levels. Stimulation with L-CD40L led to increased properdin secretion, however, FH/FHL-1 levels were not altered. Our results show that there are opposite effects of IFN-γ stimulation of macrophages on properdin and factor H/FHL-1 expression and secretion. This suggests a role for the local environment in balancing complement activation and regulation, thereby affecting local complement activation, e.g. during cell-cell interactions.

In liver tissue and HuH-7 cells, *CFH* and *FHL1* expression was comparable, whereas no expression was observed in HepG2 cells. Examination of serum showed high factor H protein levels, whereas low levels of FHL-1 were detected. Staining of human renal tissue showed that FHL-1 was found in the tubules – mainly at the tubular brush boarder – suggesting renal clearance of the FHL-1 protein. We showed that cells from the myeloid compartment, including pro- and anti-inflammatory macrophages and monocyte-derived dendritic cells (data not shown), preferentially express *FHL1* in comparison to *CFH* expression. Interestingly, the expression of *CFH* and *FHL1* was not detected in monocytes, the precursor cells we used for the *in vitro* generation of macrophages and dendritic cells. This suggests a different role for complement factors like factor H/FHL-1 systemically, e.g. monocytes in blood, and locally, e.g. monocyte-derived macrophages in tissues.

Using OX24 for immunoprecipitation, we were able to enrich for factor H and FHL-1 levels secreted by pro- and anti-inflammatory macrophages, either in unstimulated cells or after stimulation with IFN-γ. As a control, magnetic beads coupled to control mouse-IgG1 were also incubated with the supernatant, which did not result in immunoprecipitation of factor H and FHL-1 (Figure 4). The antibody-coupled beads we used remained at the sample well (Figure 4C, lane 7-10, top band). An additional small band was observed (~160kDa), which was present in samples from both OX24 (lane 8 and 10) and mouse-IgG1 (lane 7 and 9) coupled magnetic beads incubated with supernatant from macrophages and therefore would probably be a consequence of the immunoprecipitation process. Quantification showed that more FHL-1 was secreted by

pro- and anti-inflammatory macrophages generated from three different monocyte-donors, when compared to factor H levels, corresponding with the preferential expression of *FHL1*.

The factor H protein is involved in complement regulation, causing the decay of the C3 convertase and contains cofactor activity (15,16,42–45). Factor H is able to discriminate between host and foreign cell surfaces, regulating complement activation locally (46). A local role for produced factor H has been implicated, since addition of factor H during the generation of monocyte-derived dendritic cells resulted in the generation of tolerogenic dendritic cells (47). Secretion of factor H by macrophages might play a role in local regulation of complement activation, potentially initiating an anti-inflammatory phenotype.

Alternative splicing of factor H results in the generation of FHL-1 (17–19). A local role for FHL-1 has been described. In contrast to factor H, the smaller FHL-1 protein was able to diffuse through Bruch's membrane, contributing to local complement regulation in the eye (28). The differences in *CFH* and *FHL1* expression have already been indicated by others, for example in the context of rheumatoid arthritis (RA) and the local expression in the eye by RPE cells (26, 28,48). *CFH* and *FHL1* are located on the same gene and share their promotor and transcription start site. Despite this, systemic FHL-1 levels are lower compared to factor H levels ((17,48,49) and reviewed by (21)). We also observed this when examining factor H and FHL-1 protein levels in serum (Figure 3B). This difference might be explained by clearance of the smaller FHL-1 protein, as we observed FHL-1 deposition in the tubules from healthy human kidney biopsies in which factor H was absent (Figure 3C). Pharmacokinetic analysis showed that when both human factor H and FHL-1 were injected in mice, FHL-1 clearance rate is ~6-fold higher compared to factor H clearance (49).

The small FHL-1 protein contains all domains needed for complement regulation and was shown to display overall comparable regulatory activity as factor H (37), although FHL-1 discriminates less between host and foreign surfaces than factor H does (49). This highlights the importance of FHL-1 in regulating AP activity at local compartments were FHL-1 molecules outnumbers factor H. Our findings show that the liver produces and secretes both the large factor H and small FHL-1 protein, contributing to the factor H and FHL-1 source in circulation. In contrast, in the local tissue, macrophages predominantly produce and secrete the small FHL-1. The intricate regulation of the production of the complement enhancer properdin and the complement inhibitor FHL-1 suggest a local role for AP activation in the context of the innate and adaptive immune responses.

Acknowledgments

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