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Immature mouse granulocytic myeloid cells are characterized by production of ficolin-B

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ABSTRACT

Ficolins activate the lectin pathway of the complement system upon binding to carbohydrate patterns on pathogens. To characterize the producer cells of ficolin-B the expression of mouse ficolin-B, the orthologue of human M-ficolin, was studied in macrophages and dendritic cells during differentiation from bone marrow cells, in primary granulocytes, and during differentiation of granulocytes derived from ER-Hoxb8 cells. Expression of ficolin-B mRNA declined in all myeloid cell types to low levels during terminal differentiation. However, in contrast to macrophages and dendritic cells, ficolin-B expression was enhanced upon activation in granulocytes. High expression of ficolin-B was observed in primary immature neutrophilic CD11b^{+} Ly-6C^{int} Ly-6G^{high} granulocytes when isolated from the bone marrow, in particular during sepsis. Ficolin-B was demonstrated in lysates of primary granulocytes, ER-Hoxb8-derived granulocytes, bone marrow-derived macrophages, and dendritic cells. Native ficolin-B from cell lysates and supernatants of granulocytes activated the lectin pathway as measured by binding to MASP-2 and inducing C4 deposition. Specific staining demonstrated intra-cellular or cell associated ficolin-B protein in activated immature granulocytes deposited in a granular fashion. This study shows that ficolin-B is stored in and set free from immature granulocytic myeloid cells indicating a role in the early infection-induced cellular response of these inflammatory cells.

1. Introduction

The complement system provides a first line of defense in innate immune responses and can be activated by three different routes: the classical, the lectin, and the alternative pathway. The classical and the lectin pathways are initiated by pattern-recognition molecules such as C1q, mannan-binding lectin (MBL), or ficolins. While the classical pathway depends on C1q interacting with immune complexes to initiate the enzymatic cascade, recognition of target carbohydrate structures on microbial surfaces by MBL and ficolins mediates activation of MBL-associated serine proteases, MASP-1 and MASP-2, to initiate the lectin pathway (Matsushita and Fujita, 2002; Thiel, 2007; Endo et al., 2011; Degn et al., 2012).

The oligomeric protein family of ficolins is named after their primary structure which is composed of a C-terminal fibrinogen-like and a N-terminal collagen-like domain (Ichijo et al., 1993). Ficolins recognize acetylated pathogen-associated patterns with their globular fibrinogen-like domains (Endo et al., 2011; Runza et al., 2008; Krarup et al., 2004). They have been identified in many species and differ in expression site and ligand-binding specificity, suggesting a specific role for each ficolin. While in humans three ficolins are known, L-, H-, and M-ficolin, in mice and rats only two types of ficolins, termed ficolin-A and ficolin-B (FcnB), have been identified. Mouse ficolin-A is a serum protein like human L- and H-ficolin, whereas mouse FcnB, which is the orthologue of the human M-ficolin (Ohashi and Erickson, 1998), was mainly described to be cell-associated (Runza et al., 2006; Endo et al., 2011). The human M-ficolin is located in secretory granules of neutrophils and monocytes (Liu et al., 2005b; Rorvig et al., 2009; Kjaer et al., 2013) and has also been detected in serum (Honore et al., 2008). M-ficolin serum concentrations of about 0.5 μg/ml in healthy humans are significantly lower than the concentrations of L- and H-ficolin, but are

Abbreviations: BMDC, bone marrow-derived dendritic cells; BMDM, bone marrow-derived macrophages; CLP, cecal ligation and puncture; FcnB, ficolin-B; GlcNAc, N-acetyl glucosamine; GlcNAc-BSA, N-acetyl glucosamine-coupled BSA; MBL, mannan-binding lectin; MASP, MBL-associated serine protease; MDSC, myeloid cell-derived suppressor cells; NET, neutrophil extracellular trap; PEC, peritoneal exudate cells; PMN, polymorphonuclear neutrophils.

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comparable to those of MBL (Wittenborn et al., 2010; Sallenbach et al., 2011).

Mouse FcnB mRNA was strongly expressed in bone marrow and spleen by Gr-1-positive myeloid cells (Ohashi and Erickson, 1998; Liu et al., 2005a). The protein was located in lysosomes of activated macrophages where it co-localized with the lysosomal marker Lamp-1 (Runza et al., 2006), in cell lysates of bone marrow cells (Endo et al., 2012a; Hunold et al., 2012), and recently, similar to M-ficolin, was also found in low concentrations in serum (Endo et al., 2012b). In contrast to all other known ficolins, mouse FcnB was considered to fail in forming complexes with MASP-2 due to a single amino acid change in the putative MASP-binding site and, therefore, seemed incapable of activating complement (Girija et al., 2011; Endo et al., 2005). However, recently we and others reported that FcnB can interact with MASP-2 and induce C4 deposition, thus behaving like the other known starter molecules of the lectin pathway (Hunold et al., 2012; Endo et al., 2012a, 2012b).

In this study, by screening myeloid cell types during differentiation, we characterized the main producer cell of FcnB. Additionally, the complement-activating capacity of recombinant *versus* natural FcnB was compared and the cellular localization re-investigated.

2. Material and methods

2.1. Mice

BALB/C or C57BL/6 mice (20–30 g; Janvier, Le Genest, France) and FcnB-deficient mice, kindly provided by T. Fujita and Y. Endo, Fukushima, Japan (Endo et al., 2012b), were kept under standard conditions in the local animal facility of the University of Regensburg. Experiments were performed according to institutional and governmental regulations.

2.2. Reagents

Recombinant FcnB (rFcnB) was expressed and purified as reported earlier (Runza et al., 2006). The cDNA encoding the complete FcnB open reading frame was cloned into the pMT/Bip/V5/His-A vector (Invitrogen, Darmstadt, Germany) for inducible eukaryotic expression in *Drosophila* S2 cells. The soluble, secreted protein was purified under native conditions over a Chelating Sepharose Fast Flow resin (GE Healthcare Biosciences, Freiburg, Germany). The mutated form of FcnB (rFcnB E72A) was generated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) and expressed as rFcnB. Lipopolysaccharide (LPS) from *E. coli* 0127:B8 (Sigma–Aldrich, Munich, Germany) was used for stimulation of ER-Hoxb8 cells and LPS from *S. abortus equi* (Sigma–Aldrich) for stimulation of bone marrow-derived dendritic cells (BMDC) and bone marrow-derived macrophages (BMDM) (100 ng/ml). Monoclonal antibodies against recombinant FcnB (1A4 and 11A1, IgG2b) were generated and characterized by western blotting analysis as described recently (Schmid et al., 2012; Hunold et al., 2012). Medium (RPMI 1640), fetal calf serum (FCS), penicillin and streptomycin, phosphate buffered saline (PBS), β-mercaptoethanol (all from PAN BioTech GmbH, Aidenbach, Germany), GlcNAc-BSA (Dextra Laboratories, Reading, UK), acetylated LDL (AcLDL, Biomedical Technologies, Stoughton, USA), EDTA, poly-L-lysine, bovine serum albumin (BSA), acetylated BSA (AcBSA), fibrinogen, fetuin, phorbol myristate acetat (PMA), saponine, and cold water fish gelatin (all from Sigma–Aldrich) were commercially obtained. Genomic DNA was extracted from *E. coli* (PureGene Gentra DNA Purification Kit, Quiagen, Hilden, Germany).

2.3. Cells

For generation of bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC) bone marrow cells from femura and tibiae were washed and resuspended in RPMI medium containing macrophage colony stimulating factor (M-CSF) or granulocyte-macrophage colony stimulating factor (GM-CSF) for generation of BMDM or BMDC, respectively, as described (Lutz et al., 1999). Granulocytes from mouse bone marrow cell suspensions were separated on a discontinuous Ficoll gradient resulting in a cell fraction consisting of >80% CD11b$^+$ Ly-6C^{int} Ly-6G^{high} granulocytic neutrophils (PMN) as determined by differential staining and flow cytometrical analysis (Ermert et al., 2009). The remaining fraction consisted mainly of precursors expressing the Ly-6C marker (non-PMN). Myeloid CD11b$^+$ splenocytes were separated by magnetic cell separation (MACS) following the manifacturer's protocol (Milteniyi Biotech GmbH, Bergisch Gladbach, Germany). Up to 1×10^8 cells were stained in 1 ml master mix containing double-concentrated antibodies or streptavidine-dye conjugates, washed and resuspended for cell sorting on a cell sorter (FACS Aria I, BD Biosciences, Heidelberg, Germany). Alternatively, myeloid cells from bone marrow or spleens were first depleted of non-CD11b$^+$ cells using PE-conjugated antibodies to CD11c and CD4 (eBioSciences, Frankfurt, Germany), CD8, B220, NK1.1, and cKit (BD Biosciences), and CD3 (BioLegend, San Diego, USA) by MACS. The resulting CD11b-enriched cells were further separated by positive selection using anti-Ly-6G$^+$ microbeads (Milteniyi Biotech GmbH) by MACS, resulting in a granulocytic myeloid cell fraction (CD11b$^+$ Ly-6G$^+$ Ly-6C^{int}) of 87–94% purity (Suppl. Fig. 1) and a monocytic myeloid cells fraction (CD11b$^+$ Ly-6G$^-$ Ly-6C$^+$) of 57–60% purity (Delano et al., 2011; Youn et al., 2008; Movahedi et al., 2008). Peritoneal exudate cells (PEC) were obtained by lavage of the peritoneal cavity of mice 16 h after PBS (1 ml, i.p). About 50% of the PEC were macrophages as determind by microscopy. ER-Hoxb8 cells were kindly provided by H. Häcker, Memphis, Tennessee and granulocytes were generated in SCF-containing culture medium without estradiol according to the protocol of Wang et al. (2006).

2.4. Flow cytometry

Cells were incubated with antibodies against CD16/32 (anti-mouse Fcγ-receptor II/III, clone 2.4G2) to block non-specific antibody binding and stained with fluorochrome-labeled antibodies detecting MHCII, CD80, CD11c, CD11b, Gr-1 (clone RB6-8C5), Ly-6C, Ly-6G, streptavidin (all from BD Biosciences), CD11b (Milteniyi Biotech GmbH), F4/80 (AbD Serotec MorphoSys, Düsseldorf, Germany), and B220 (eBioScience). Flow cytometric analysis of stained cells was performed using the BD LSR II or a FACSCalibur instrument (BD Biosciences) and analyzed using the FacsDiva or CellQuest softwares (BD Biosciences). Purity of the sorted cell populations was >98%.

2.5. Cecal ligation and puncture (CLP)

Mice were anesthetized by injecting Ketamin (75 mg/kg, Parke, Davis & Company, Munich, Germany) and Xylazin (16 mg/kg, Bayer AG, Leverkusen, Germany) i.p. The cecum was exteriorized and 30% of the distal end was ligated and punctured once with a 27 gauge (0.4 mm in diameter) needle to achieve a sublethal CLP (Pollak et al., 2005).

2.6. Real time PCR

Total mRNA was isolated from cells using the RNeasy Kit (QIAGEN) or Nucleospin RNA II isolation Kit (Macherey Nagel, Düren, Germany) and subjected to a standard real-time

quantitative RT-PCR (Promega Kit, Madison, USA) with the FcnB-specific primers: 5′-CCCGAATTCCCAGCCATGGCC-CTGGGATCTGCTGCAC-3′ (forward) and 5′-CCCCTCGAGCTAGA-TGAGCCGCACCTTCATC-3′ (reverse) on a Bio-Rad iQ5 iCycler using the iQ SYBR Green Supermix (Bio-Rad, Munich, Germany). Relative mRNA levels were calculated using the delta–delta–Ct method. Results are given as x-fold relative to the expression of house-keeping gene 18S or as percentage of stimulated cells compared to non-stimulated cells.

2.7. Western blotting

For characterization of FcnB protein by Western blot analysis cell lysates were prepared using either RIPA buffer (Sigma–Aldrich, 10^6 cells in 50 μl of 50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1% Nonidet P40; 0.5% sodiumdesoxycholate; 10% SDS) or M-PER Protein Extraction Reagent (Pierce, Rockford,IL USA) containing protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Diagnostics, Penzberg, Germany), centrifuged (14,000 × g, 15 min, 4 °C) and either used in the experiment immediately or stored at −80 °C. Protein concentrations in the lysates were determined by a BCA Protein Assay Kit (Pierce). After SDS-PAGE (10% polyacrylamid gels under reducing conditions using the method of Laemmli) proteins were transferred to PVDF membranes (Immobilon, Millipore, Schwalbach, Germany), the membranes were blocked with 10% skim milk powder in TBS/Tween, probed with either the purified monoclonal anti-ficolin-B antibody (1A4, 10 μg/ml) and as secondary antibody HRP-conjugated goat-anti-rat IgG (1:8000 diluted, Sigma–Aldrich) or with HRP-conjugated anti-β-actin (20 ng/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBS/Tween with 1% skim milk powder for 1 h at room temperature for each step, washed with TBS/Tween, and developed with ECL-Plus Substrate (Pierce). Filters were analyzed using Image Quant LAS 4000 mini (GE Healthcare, Munich, Germany).

2.8. FcnB binding and complement activation

FcnB was measured by time-resolved immunofluorometric assay (TRIFMA) based on the ELISA protocol as described in detail (Hunold et al., 2012) adapted to time-resolved fluorometry (Kjaer et al., 2013; Wittenborn et al., 2010). Wells (NUNC, Roskilde, Denmark) were coated overnight with AcBSA or other reagents as indicated (10 μg/ml), blocked with 5% skim milk powder, incubated with FcnB-containing sample dilutions in TBS/Tween (0.14 M NaCl, 10 mM Tris, pH 7.4, 0.05% Tween 20) with 10 mM calcium chloride (TBS/Tween/Ca) for 2 h at room temperature, washed, and monoclonal rat anti-FcnB 1A4 (3 μg/ml) or mouse anti-V5 IgG (1:10,000, Invitrogen) in TBS/T/Ca was added for 2 h. After washing, biotinylated goat anti-rat IgG (1 μg/ml, BD Biosciences) or biotinylated anti-mouse IgG (1:20,000, Sigma–Aldrich), respectively, were added for 2 h, washed, and europium-labeled streptavidin (Streptavidin-Eu^{3+}, 1:1000, Perkin Elmer, Rodgau, Germany) was added in TBS/T/25 μM EDTA for 1 h. After washing the europium was eluted and encapsulated with Enhancement Buffer (Perkin Elmer) and the amount of bound europium was measured at 340 nm exitation and 615 nm emmission wave lengths (Mithras LB 940, Berthold Technologies, Bad Wildbad, Germany) and is given as counts/sec. MASP-2-binding and C4b, deposition were determined on AcBSA-coated, FcnB adsorbed plates as described (Hunold et al., 2012). Human MASP-2 (500 ng/ml) (Vorup-Jensen et al., 2000) and after washing biotinylated anti-MASP-2 antibody (Moller-Kristensen et al., 2003) were added and MASP-2 binding was determined with europium-labeled streptavidin as indicated above. For determination of C4 deposition, after overnight incubation at 4 °C and washing, human C4 was added to the FcnB-MASP-2 complex for 90 min at 37 °C, washed, and developed with anti-human C4 clone 162-2 (0.5 μg/ml) followed by biotinylated rabbit anti-mouse IgG and developed as indicated above. Human MASP-2, anti-MASP-2 antibodies, and human C4 were generous gifts from J. Jensenius, Aarhus, Denmark.

2.9. Microscopy

For confocal microscopy, PMN isolated from bone marrow were stimulated with PMA (120 ng/ml RPMI) for 16 h, washed, fixed with paraformaldehyde (1% in PBS, 30 min), washed thrice, permeabilized with Triton X-100 (0.5%, 1 min at room temperature), washed thrice and blocked with blocking buffer (3% cold water fish gelatin, 5% heat inactivated goat serum, 1% BSA, 0.25% Tween 20, in PBS, 30 min, 37 °C). Cells were stained with anti-FcnB (biotinylated 11A1, 10 μg/ml) in blocking buffer (1 h, at room temperature), washed thrice, mouse anti-Lamp-1 (FITC conjugated, 1:40, 12.5 μg/ml, BD Bioscience) and Streptavidin-Alexa 546 (1:400, Invitrogen) in blocking buffer were added (1 h, at room temperature), washed thrice, cytospun onto microscope slides and mounted using the anti-fade reagent Fluoromont (Sigma–Aldrich). Images were acquired by confocal laser scanning microscopy using a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) equipped with a Plapon 60X/1.42 objective. For neutrophil extracellular trap (NET) detection, PMN isolated from bone marrow (BALB/C) were seeded (10^6 cells per slide) on poly-L-lysine coated Lab-Tek chambered coverglass slides containing a borosilicate glass base of 170 mm thickness (NUNC) and stimulated with PMA (0.2 μM, 120 ng/ml in complete medium, 16 h, 37 °C, 5% CO_2), thoroughly washed with PBS, stained with anti-FcnB (biotinylated 11A1, 10 μg/ml) and mouse anti-nucleosome (1 μg/ml, BD Pharmingen) diluted in blocking buffer (1 h, 37 °C). After washing, Streptavidin-Alexa 488 (1:400; 2.5 μg/ml, Invitrogen) for detection of anti-mouse FcnB and Dylight 549-conjugated goat anti-mouse IgG for detection of anti-nucleosome IgG (1 μg/ml, Jackson ImmunoResearch, Newmarket, UK) were added (1 h, 37 °C). Cells were washed 3-times and extracellular DNA was stained with ToPro3 (1:1000 final, 15 min, Invitrogen).

2.10. Statistics

Results are given as representative data of 2 or more independent experiments as indicated in the legends. Statistical analyses were calculated using PRISM software (GraphPad) and one-way ANOVA with Dunnett's multiple comparison test, two-way ANOVA with Bonferroni post-test, or paired or un-paired Student's *t*-test as indicated.

3. Results

3.1. Expression of FcnB mRNA by bone marrow-derived macrophages and dendritic cells

We analyzed the expression of mouse FcnB mRNA in cells of the monocyte/macrophage lineage since expression of the human (M-ficolin) and the porcine (ficolin-β) orthologues of FcnB was reported in monocytes, macrophages, and neutrophils (PMN) and mouse FcnB was located in peritoneal exudate macrophages, bone marrow tissue, and PMN (Runza et al., 2006; Hunold et al., 2012; Endo et al., 2012a). Expression of FcnB mRNA was monitored during culture of bone marrow cells in the presence of either M-CSF or GM-CSF. In bone marrow-derived macrophages (BMDM), cultured in medium containing M-CSF, the levels of FcnB mRNA were stable during the first three days and then decreased during differentiation (Fig. 1A). A further 40% reduction was induced by stimulation with LPS (Fig. 1B). Also in bone marrow cells, cultured in the presence of GM-CSF, expression of FcnB mRNA remained almost stable,

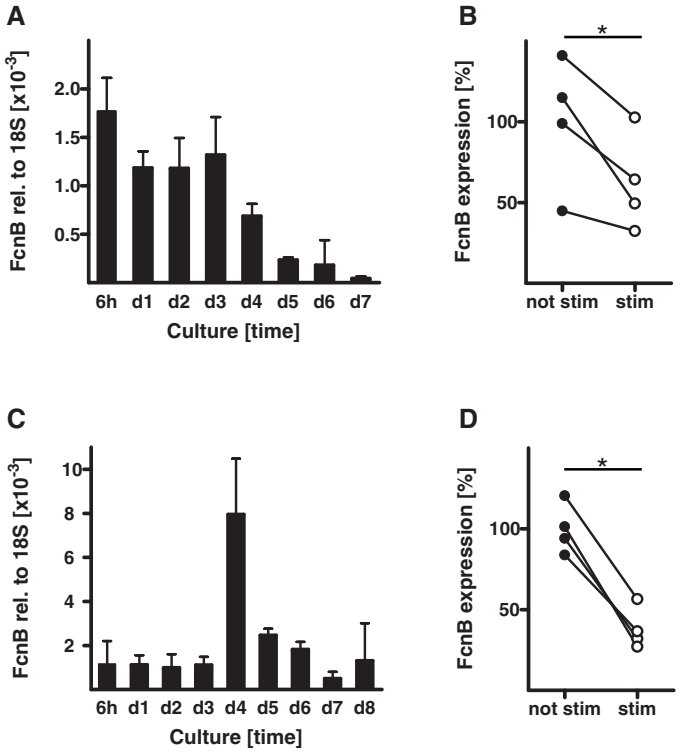

Fig. 1. FcnB mRNA is expressed in bone marrow-derived macrophages and dendritic cells. (A) Bone marrow cells from C57BL/6 mice were cultured for 7 days in the presence of M-CSF for differentiation in BMDM. After a 6 h adherence period FcnB mRNA levels were determined in 3 independent cell cultures at the indicated time points and shown as mean + SD. Significant differences of FcnB expression compared to cells in culture at day 0 (+6 h) were calculated on day 1, day 2, and from day 4 on ($p < 0.05$, one-way ANOVA with Dunnett's multiple comparison test). The experiment was performed >3-times. (B) BMDM from day 6 in (A) was activated for additional 24 h in the absence or presence of LPS (*E. coli*, 100 ng/ml) before FcnB mRNA levels were determined. BMDM were independently generated from 4 mice. Results are given as percentage setting the FcnB mRNA expression relative to the mean of the non-stimulated cultures to 100%. The difference in FcnB mRNA expression was significant ($p = 0.0405$, paired *t*-test). (C) Bone marrow cells from C57BL/6 mice were cultured (after a 6 h adherence period) for 8 days in the presence of GM-CSF for differentiation in BMDC. FcnB mRNA levels were determined in 3 independent cell cultures at the indicated time points. Difference of FcnB expression compared to cells in culture at day 0 (+6 h) was calculated for day 4 ($p < 0.05$, one-way ANOVA with Dunnett's multiple comparison test). The experiment was performed >3-times. (D) BMDC from day 7 in (C) was activated for additional 24 h in the absence or presence of LPS (*E. coli*, 100 ng/ml) before FcnB mRNA levels were determined. BMDC were independently generated from 4 mice. Results are given as percentage setting the FcnB mRNA expression relative to the mean of the non-stimulated cultures to 100%. The difference in FcnB expression was significant ($p = 0.0016$, paired *t*-test).

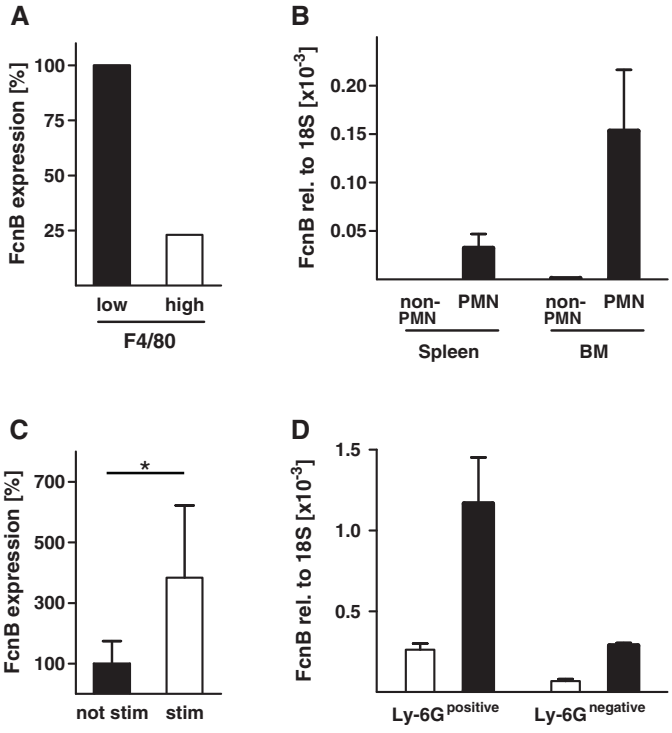

Fig. 2. FcnB mRNA is expressed in PMN isolated from bone marrow and spleen. (A) Splenic CD11b$^+$ cells from C57BL/6 mice were sorted according to the expression intensity of the marker F4/80. FcnB mRNA expression was determined in the F4/80^{low} and F4/80^{high} cell fractions. Results are given as percentage setting the mean of FcnB mRNA expression measured in the F4/80^{low} fraction to 100%. This experiment was done only once. (B) FcnB mRNA expression of granulocytes (PMN) and remaining non-granulocytes (non-PMN) from bone marrow and spleen separated on a Ficoll gradient was determined. Results are given as mean + SD from duplicate cultures. The experiment was performed >3-times. (C) The granulocytes from bone marrow in (B) were cultured in the absence or presence of LPS (*E. coli*, 100 ng/ml) for 30 min and FcnB mRNA expression was determined. Results of FcnB mRNA expression in 6 independent cultures are given as mean + SD of FcnB mRNA expression relative to the mean + SD of the un-stimulated cultures set to 100%. The cells expressed significantly higher levels of FcnB mRNA after stimulation ($p = 0.0194$, unpaired *t*-test). (D) CD11b$^+$ cells from bone marrow of either naïve (white bars) or septic mice (black bars, d5 after CLP) were separated according to their expression of Ly-6G. FcnB mRNA expression was determined in the granulocytic Ly-6G-positive cell fraction (purity of 87–94% CD11b$^+$ Ly-6G$^+$ Ly-6C$^+$) and the monocytic Ly-6G-negative fraction (purity of 57–60% CD11b$^+$ Ly-6G$^-$ Ly-6C$^+$). Results are given as mean + SD of triplicates. FcnB mRNA expression was significantly higher in PMN isolated from CLP-treated mice compared to naïve mice ($p = 0.0159$, two-way ANOVA with Bonferroni post-test). The experiment was performed twice.

suddenly increased on day 4 of culture and was low in terminally differentiated bone marrow-derived dendritic cells (BMDC) (Fig. 1C). Similar to BMDM, additional stimulation with LPS also reduced FcnB mRNA expression of BMDC by about 60% (Fig. 1D). The expression of FcnB mRNA in BMDC was further analyzed in BMDC sorted according to activation markers (CD80, CD86, and MHC-II). Although FcnB mRNA was present in all cell fractions, the more immature BMDC fractions contained about twice the amount of FcnB mRNA (data not shown). Thus, expression of FcnB mRNA was detected in not yet fully differentiated myeloid cells during differentiation of bone-marrow precursors to mature macrophages (BMDM) and to dendritic cells (BMDC). Bone marrow-derived mast cells did not show FcnB mRNA expression at any time of culture (data not shown).

3.2. Expression of FcnB mRNA by PMN

Expression of FcnB mRNA has been well documented in Gr-1-positive cells isolated from the spleen (Ohashi and Erickson, 1998; Liu et al., 2005a). To distinguish granulocytic from monocytic cells, CD11b$^+$ myeloid splenocytes were sorted for the macrophage maturation marker F4/80. F4/80^{low} myeloid splenocytes expressed 4-fold higher FcnB mRNA levels compared to F4/80^{high} mature macrophages (Fig. 2A). CD11b$^+$ myeloid cells from bone marrow and spleens of naïve mice were separated on a discontinuous Ficoll gradient resulting in Ly-6G-negative and more than 80% Ly-6G-positive granulocytic cell fractions characterized as CD11b$^+$ Ly-6C^{int} Ly-6G$^+$ cells. Only the granulocytic cells (PMN) from both bone marrow and spleen expressed FcnB mRNA identifying granulocytic cells, in particular from the bone marrow, as FcnB producer cells (Fig. 2B). FcnB mRNA expression was further enhanced in bone marrow-derived PMN after stimulation with LPS (Fig. 2C).

Since sepsis induces recruitment of PMN into the periphery (Delano et al., 2007), we measured FcnB mRNA in PMN in sepsis.

Polymicrobial septic peritonitis was induced in mice by sublethal cecal ligation and puncture (CLP) and PMN from bone marrow and spleen were isolated 5 days after CLP. The highly pure Ly-6G$^+$ cell fraction carrying the marker profile CD11b$^+$ Ly-6C^{int} Ly-6G$^+$ of immature myeloid cells (IMM) or PMN-myeloid cell-derived suppressor cells (PMN-MDSC) were compared to Ly-6G$^+$ IMM from naïve mice (Delano et al., 2011; Youn et al., 2008; Movahedi et al., 2008). Total spleen cell numbers were unchanged and increased only later in CLP-induced sepsis (data not shown). As expected, a larger proportion of splenocytes from septic mice had CD11b (17.7% in septic *versus* 10.7% in naive) and a larger fraction of these CD11b-positive splenocytes showed the surface marker Ly-6G (10.2% in septic *versus* 3.7% in naïve mice), documenting recruitment of PMN by polymicrobial sepsis (Delano et al., 2007). Also in bone marrow, the proportion of CD11b-positive cells increased after CLP (from 57.7% to 83.2%) and among these cells the proportion of Ly-6G-positive IMM increased as well (from 44.2% to 53.0%). A very pure CD11b$^+$ Ly-6G$^+$ IMM fraction from bone marrow (Suppl. Fig. 1) expressed highly increased FcnB mRNA levels after CLP (Fig. 2D). Low FcnB mRNA expression was detected in the less purified Ly-6G-negative monocytic cell fraction from bone marrow, again with higher FcnB mRNA expression in cells from septic compared to naïve mice. However, we were not able to reliably detect FcnB mRNA expression in the respective spleen cell preparations from septic mice (data not shown).

To analyze the expression of FcnB mRNA in PMN during differentiation granulocytes were generated *in vitro* from ER-Hoxb8 precursor cells (Wang et al., 2006). During differentiation cells were analyzed by flow cytometry for the expression of the markers CD11b, Gr-1, and Ly-6C, and Ly-6G (Fig. 3A). After 5 days of culture without estradiol more than 90% of the cells showed the typical morphology of granulocytes and stained strongly positive for CD11b, Gr-1, and Ly-6C. After 3 days of culture cells became increasingly positive for Ly-6G and also for the macrophage marker F4/80 (data not shown). A strong increase of FcnB mRNA expression was detected on days 2–4 demonstrating transiently up-regulated expression of FcnB during differentiation (Fig. 3B). Stimulation with LPS of mature (d5) and immature (d3) ER-Hoxb8-derived granulocytes enhanced FcnB mRNA expression (Fig. 3C and data not shown). This was also seen after stimulation with PMA but not with fMLP or aggregated IgG (data not shown).

3.3. Presence of FcnB in myeloid cells

Since FcnB has been demonstrated in activated peritoneal macrophages, in bone marrow tissue, and in PMN (Runza et al., 2006; Endo et al., 2012a; Hunold et al., 2012), lysates of different myeloid cell types were tested for the presence of FcnB by western blot. Positive staining with the FcnB-specific monoclonal antibody 1A4 was obtained with lysates from BMDM, BMDC, PMN, and peritoneal exudate cells (PEC) (Fig. 4A). Lysates from PMN seemed to contain more FcnB than lysates from BMDM, BMDC, or PEC as judged by the staining intensity. A prominent band with an apparent molecular size of about 37 kDa was reproducibly stained by 1A4 after gel electrophoresis under reducing conditions corresponding to the expected molecular weight of monomeric FcnB (Endo et al., 2012a). More FcnB was detected in immature (d3) compared to mature (d5) ER-Hoxb8-derived cells when the staining intensity from cell lysates was compared (data not shown). After electrophoresis under reducing conditions the most prominent band detected with 1A4 was at about 37 kDa while using non-reducing conditions 1A4 stained a band of about 70 kDa corresponding to a dimer in the rFcnB preparations as well as in ER-Hoxb8 (d3) and bone marrow-derived PMN cell lysates (Fig. 4B). Thus, rFcnB and

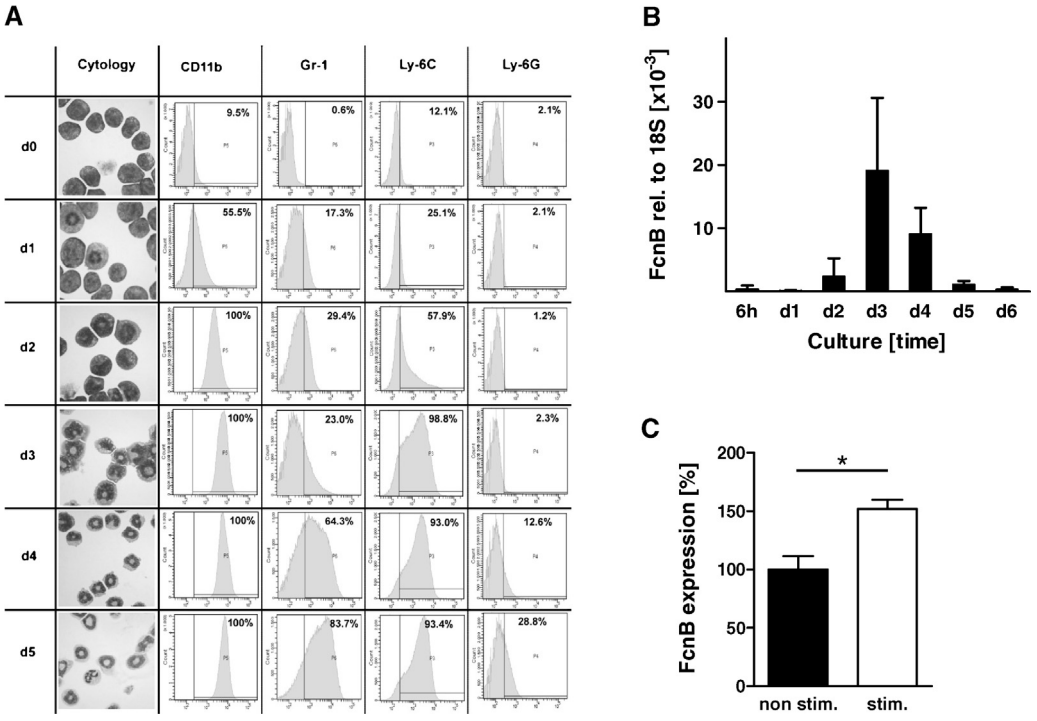

Fig. 3. FcnB mRNA is expressed in ER-Hoxb8-derived PMN. (A) Morphological changes (left panel) and surface markers of ER-Hoxb8 cells during 5 days of differentiation visualized by Giemsa–May–Grünwald staining and FACS analysis. Marker profiles of CD11b, Gr-1, Ly-6C, and Ly-6G were determined by flow cytometrical analysis and the percentages of positive cells for the respective markers are given from one representative experiment. The experiment was performed >3-times. (B) FcnB mRNA levels were determined in cells from 5 independent cultures at each indicated time point after withdrawal of estradiol and shown as mean + SD. Significant differences of FcnB mRNA expression compared to cells in culture of day 0 (+6 h) were calculated for day 3 and day 4 ($p < 0.05$, one-way ANOVA with Dunnett's multiple comparison test). The experiment was performed >3-times. (C) ER-Hoxb8-derived PMN after 3 days of culture were stimulated with LPS (*E. coli*, 100 ng/ml) or not for 30 min and FcnB mRNA expression was determined. Results are given as mean + SD of FcnB mRNA expression in 3 independent cultures and expression of non-stimulated cells set to 100% ($p = 0.03$), unpaired *t*-test).

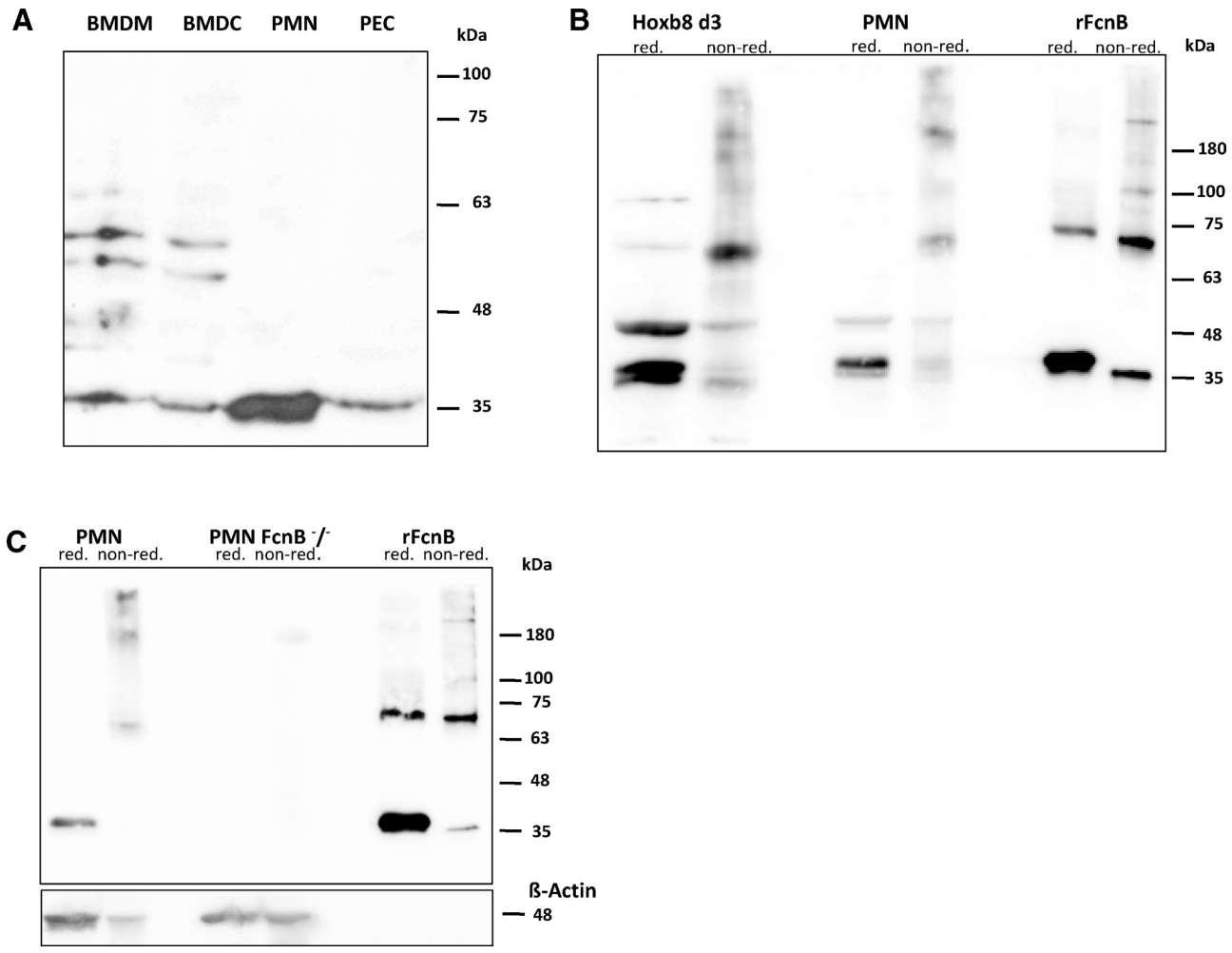

Fig. 4. FcnB protein is detectable in myeloid cells. (A) Proteins in lysates (prepared with RIPA buffer) from BMDM, BMDC, bone marrow-derived PMN, and peritoneal exudate cells (PEC) (50 μg of protein, corresponding to 1.5×10^6 cells, each) were separated by gel electrophoresis under reducing conditions, blotted, and stained with the specific anti-FcnB antibody 1A4. (B) Proteins in lysates (prepared with M-PER buffer) from ER-Hoxb8-derived PMN from day 3 of culture (50 μg of protein, corresponding to 1.3×10^6 cells) or bone marrow-derived PMN (50 μg of protein, corresponding to 5×10^6 cells) were separated by gel electrophoresis under reducing and non-reducing conditions and the western blot was stained as in (A). Recombinant FcnB (50 ng) was used as positive control. (C) The same as in (B) with proteins in lysates (prepared with M-PER buffer) from bone marrow-derived PMN either from WT or from FcnB-deficient mice (50 μg of protein each).

natural FcnB from cell lysates showed a similar banding pattern under reducing and non-reducing electrophoretic conditions. No staining was obtained in PMN lysates from FcnB-deficient mice documenting the specificity of the monoclonal antibody 1A4 for FcnB (Fig. 4C). No increase of FcnB staining was observed in lysates of BMDM, BMDC, or ER-Hoxb8-derived PMN after stimulation with LPS at different times (data not shown).

3.4. Functional activity of natural FcnB

We have recently shown that rFcnB expressed by insect cells has a much less complement activating capacity *in vitro* compared to M-ficolin. Therefore, we reasoned that complement activation by rFcnB might be improved by substitution of the glutamate to alanine which is the natural amino acid residue in sequence of the MASP-2-binding site of rat FcnB (Girija et al., 2011; Endo et al., 2005). We generated a mutated form of rFcnB, named rFcnB E72A, by site-directed mutagenesis. Comparison of the rFcnB with the rFcnB E72A preparation by measuring adsorption on AcBSA-coated plates and detection with antibodies directed against the molecular tag revealed that the FcnB concentration in both preparations was comparable (Fig. 5A). The Western banding pattern of the two preparations, rFcnB and rFcnB E72A, was almost identical under reducing and non-reducing conditions of SDS-PAGE when stained with anti-tag antibodies (data not shown). The binding epitope of the monoclonal antibody 1A4 seems to be specific for the proper mouse MASP2-binding site since 1A4 hardly recognized rFcnB E72A (Fig. 5B). This also held true when instead of AcBSA other substances for coating were used such as AcLDL, fetuin, GlcNAc-BSA, bacterial DNA or fibrinogen (Suppl. Fig. 2). Even though not well recognized by 1A4 (Fig. 5C), rFcnB E72A served in our experiments as a biologically active control for MASP-2 binding (Fig. 5D) and C4 deposition (Fig. 5E) demonstrating complement activation. Compared to rFcnB E72A, reduced MASP-2 binding and negligible C4 deposition was measured with rFcnB (Fig. 5D and E). Interestingly, while secreted rFcnB was biologically inactive, cell lysates of FcnB-transfected Drosophila S2 insect cells contained complement-activating rFcnB (Suppl. Fig. 3). Natural FcnB in lysates of bone marrow-derived PMN, ER-Hoxb8 (d3) cells, or a concentrated supernatant of Hoxb8 cell cultures reproducibly bound to AcBSA (Fig. 5F) and induced C4 deposition (Fig. 5G).

3.5. Cellular localization of FcnB

Specific staining of FcnB with 1A4 demonstrated localization of FcnB in granula inside ER-Hoxb8 d3 cells and also in bone

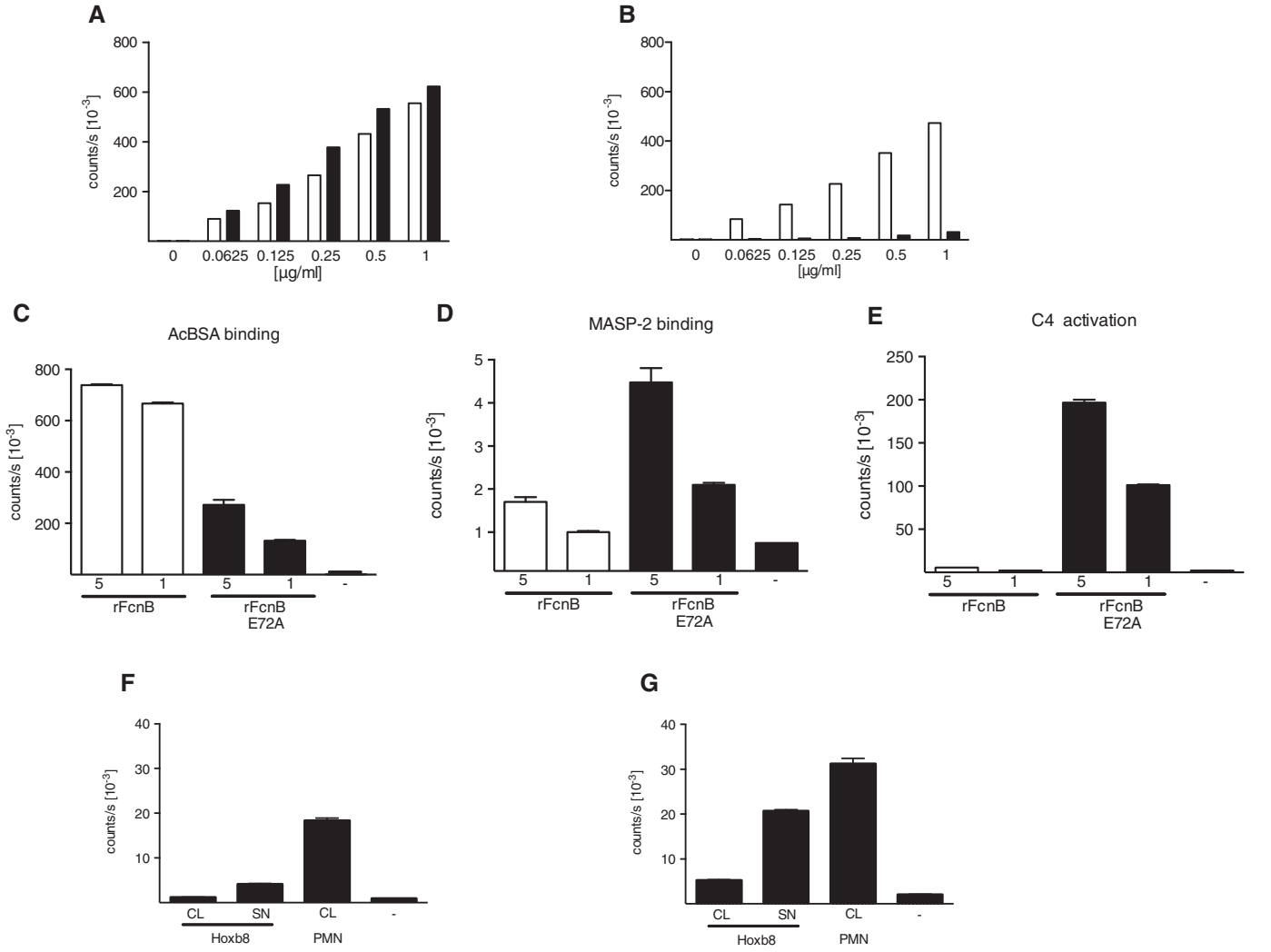

Fig. 5. FcnB E72A and native FcnB activate complement. Binding of rFcnB (white bars) and rFcnB E72A (black bars) to immobilized AcBSA was detected with monoclonal antibodies either to the tag (V5) (A) or to FcnB (1A4) (B). Binding of 5 or 1 µg/ml rFcnB (white bars) or rFcnB E72A (black bars) was detected with anti-FcnB 1A4 (C), binding of MASP-2 to the complex was detected with antibodies to MASP-2 (D), and C4-deposition onto the complex was measured with antibodies to C4 (E). Proteins from cell lysates of either ER-Hoxb8 d3 cells or PMN (1 mg/ml each) or supernatant of ER-Hoxb8 d3 cell cultures were compared for their capacity of binding to anti-FcnB 1A4 (F) or for C4-deposition (G). All experiments were performed by TRIFMA at least 3-times and results given as counts per second.

marrow-derived PMN extending our previous finding of intracellular FcnB in peritoneal macrophages (Runza et al., 2006) (Fig. 6A). Confocal microscopy using primary granulocytes activated with PMA also confirmed that FcnB was stored inside the PMN in a granular fashion (Fig. 6B) and co-localized with the lysosomal marker Lamp-1 (Fig. 6C). Some neutrophil extracellular trap (NET) structures and/or debris of cells that had died during the procedure were visualized. As preparations were neither fixed nor permeabilized, only extracellular FcnB and DNA exposed by cells undergoing NETosis or by ghosts was stained (Fig. 6D). Again, FcnB was detected as a granular staining in close proximity to the ghosts.

4. Discussion

Ficolins play an important role in recognition and elimination of pathogens in innate immunity by initiating the lectin pathway of complement activation. Ficolins not only recognize bacteria but also opsonize apoptotic cells, thus, playing a role in the clearance of both microorganisms and cellular debris. Among the ficolins of humans, rats, and mice, mouse FcnB was considered to be special first because, like M-ficolin, it has only very recently been detected in serum at low concentrations (Endo et al., 2012a). Second, due to an unusual consensus sequence in the putative MASP-2-binding site mouse FcnB did not seem to efficiently activate the complement cascade. Only very recently it was shown that natural FcnB as well as recombinant mouse FcnB expressed by mammalian cells activates the lectin pathway and protects from pneumococcal infection (Endo et al., 2012a, 2012b). We previously reported on a weak interaction of rFcnB with recombinant human MASP-2 and subsequent C4 deposition *in vitro* (Hunold et al., 2012) which is much lower compared to the activity of rFcnB E72A as shown here supporting previous findings by Endo et al. (Endo et al., 2012a). The expression system using insect cells obviously leads to a secreted form of ficolin-B which is only capable of weak binding to MASP-2 and hardly detectable C4 cleavage. However, natural FcnB from cell lysates of PMN or supernatant of ER-Hoxb8 cells as well as – unexpectedly – rFcnB from cell lysates of transfected S2 cells induced C4 deposition. It remains to be analyzed why rFcnB expressed by insect cells is biologically almost inactive after secretion. So far, acetylated BSA served as best binding substrate for rFcnB in the sensitive TRIFMA test system and also to a minor degree acetylated LDL and even less so fetuin. Whether this holds true for natural FcnB remains to be studied.

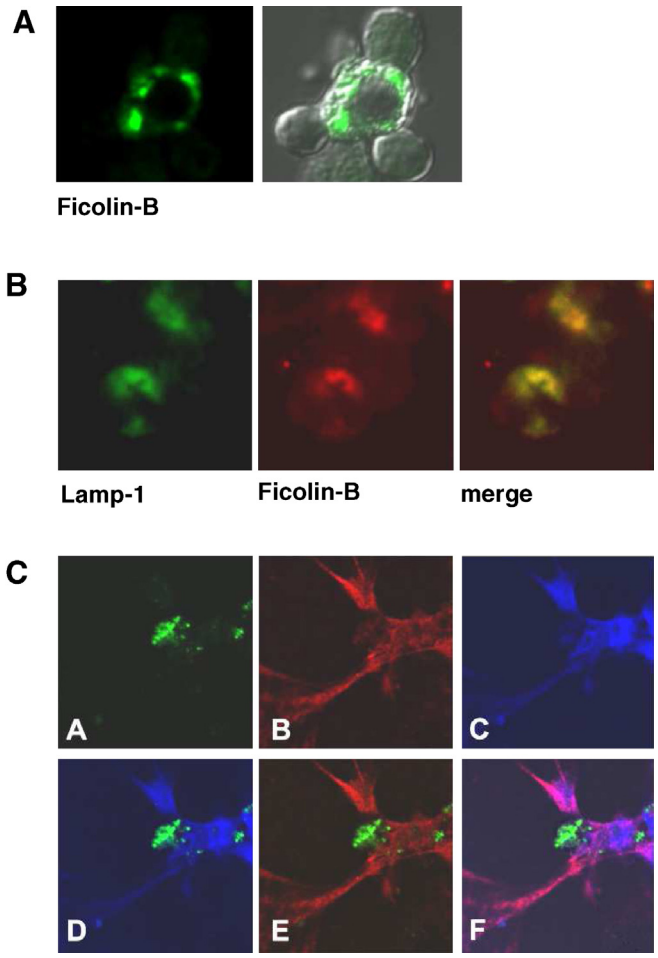

Fig. 6. FcnB protein is detectable in granulocytes. (A) Confocal microscopy was performed with PMA stimulated bone marrow-derived PMN from BALB/c mice, fixed, permeabilized and stained with anti-FcnB (biotinylated 11A1 and Streptavidin-Alexa 488, green). (B) Same as (A) stained with anti-Lamp-1-FITC (green, left) and with anti-FcnB (biotinylated 11A1 and Streptavidin-Alexa 546, red, middle). Merged pictures are shown in the right panel (magnification = 600×). (C) Stainings of neither fixed nor permeabilized PMN with anti-FcnB (biotinylated 11A1 and Streptavidin-Alexa 488, green), mouse anti-nucleosome and anti-mouse Dylight 549 (red), DNA-staining with ToPro3 (blue) are shown: (A) anti-FcnB; (B) anti-nucleosome; (C) DNA-staining; (D) overlay of FcnB and DNA stain; (E) overlay of FcnB and nucleosome stain; (F) overlay of FcnB, nucleosome, and DNA stain.

In respect to producer cell type and cellular localization our results also confirm extensive similarities between mouse FcnB and its human orthologue M-ficolin. The expression of human M-ficolin has been documented in monocytes, macrophages, and PMN with the protein located on the cellular surface (Teh et al., 2000), in secretory granules (Liu et al., 2005b; Rorvig et al., 2009), and also in serum in low amounts (Honore et al., 2008; Endo et al., 2012b). Expression of mouse FcnB mRNA by cells of the myeloid lineage from bone marrow and spleen has been reported earlier (Liu et al., 2005a) and, here, we confirm expression of FcnB mRNA in all phagocytic cell types. Highest mRNA expression levels were seen during differentiation from precursor cells to macrophages, dendritic cells, or PMN when the cells were not yet fully matured. Only PMN could be stimulated for enhanced FcnB expression while LPS stimulation reduced FcnB mRNA expression of bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC). This nicely parallels the observation of decreasing M-ficolin mRNA expression in human monocytes upon *in vitro* adherence of monocytes for longer than 24 h (Lu et al., 1996).

The detailed analysis of the cellular source among myeloid splenocytes indicated granulocytic rather than monocytic cells to be the main source of FcnB. Our finding of FcnB mRNA expression being highest in PMN carrying the marker profile of IMM or PMN-MDSC with CD11b^{+} CD11c^{-} Ly-6C^{int} Ly-6G^{high} and, in particular, when isolated from bone marrow of septic mice indicates that FcnB expression might be confined to early inflammatory phagocytes recruited by sepsis (Delano et al., 2011). Tumor-induced granulocytic MDSC have been described to be pathologically activated precursors of granulocytes generated in the bone marrow and recruited to the periphery with high activity of arginase and oxygen radical activity compared to mature granulocytes (Fridlender et al., 2009; Youn et al., 2012). Interestingly, the *Fcnb* gene was found among the most highly over-expressed genes in such tumor-induced granulocytic MDSC by gene array analysis (Youn et al., 2012). CLP did not recruit FcnB mRNA-expressing CD11b^{+} Ly-6C^{int} Ly-6G^{high} PMN to the spleen within 5 days in sufficient quantity for its isolation in septic mice. The relatively low FcnB expression signal in the monocytic MDSC (CD11b^{+} Ly-6C^{high} Ly-6G^{-}) population from bone marrow of septic mice in our study might be due to contaminating granulocytic MDSC because this fraction was not highly purified. Following the protocol of Wang et al. for differentiation of ER-Hoxb8 precursor cells to granulocytes (Wang et al., 2006), maximal FcnB mRNA expression as well as protein levels were detected before PMN were fully differentiated further supporting the immature character of FcnB-producing granulocytes. In addition, such ER-Hoxb8-derived PMN could also be activated for enhanced expression of FcnB mRNA by LPS or PMA but not by aggregated IgG or fMLP.

Specific staining of FcnB in primary granulocytes extend our previous demonstration of FcnB protein in lysosomes of activated peritoneal exudate macrophages (Runza et al., 2006) and in lysates from primary granulocytes (Hunold et al., 2012). The identification of the immature phenotype of PMN, such as immature ER-Hoxb8 (d3) or "pathologically activated granulocyte precursors", as the most important producer cells for FcnB suggests that FcnB could be set free upon stimulation and/or be attached to the so-called NET structures in a similar way as human M-ficolin (Rorvig et al., 2009). So far, however, we could not stain FcnB directly on the NET filaments while soluble natural FcnB was clearly detected in supernatants of ER-Hoxb8 cells. Since the monoclonal anti-rFcnB antibody 1A4 seems to recognize an epitope in the MASP-2-binding site of mouse FcnB we are currently investigating whether 1A4 neutralizes FcnB activity.

Taken together, our studies confirm the expression of FcnB mRNA in all myeloid cells tested and confined maximal expression to the immature state of such cells and in particular to immature granulocytes which phenotypically resemble the sepsis- or pathologically-induced inflammatory granulocytic cell population or granulocytic myeloid-derived suppressor cells (Delano et al., 2007, 2011; Movahedi et al., 2008; Youn et al., 2008). A comparable PMN subpopulation with suppressive potential has recently been described in humans during acute systemic inflammation (Pillay et al., 2012). How FcnB contributes to the local antimicrobial activity of phagocytes or whether FcnB is involved in any suppressive effect remains to be studied.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2013.06.015.

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