HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2020 May 15.

Published in final edited form as:

J Immunol. 2019 May 15; 202(10): 2982–2990. doi:10.4049/jimmunol.1701443.

Deletion of Biliverdin Reductase A in myeloid cells promotes chemokine expression and chemotaxis in part via a complement C5a-C5aR1 pathway

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Abstract

Biliverdin reductase A (BVR-A) is a pleotropic enzyme converting biliverdin (BV) to bilirubin (BR), and a signaling molecule that has cytoprotective and immunomodulatory effects. We recently showed that BV inhibits the expression of complement activation fragment 5a receptor (C5aR1) in 264.7 RAW macrophages. In this study, we investigated the role of BVR-A in determining macrophage inflammatory phenotype and function via regulation of C5aR1. We assessed expression of C5aR1, M1-like macrophage markers including chemokines (RANTES, IP-10) as well as chemotaxis in response to LPS and complement 5a (C5a) in bone marrow derived macrophages from $BVR^{fl/fl}$ and $LysM\text{-}Cre{:}BVR^{\mathbf{fl/fl}}$ mice (conditional deletion of BVR-A in myeloid cells). In response to LPS, macrophages isolated from $LysM\text{-}Cre{:}BVR^{fl/fl}$ showed significantly elevated levels of C5aR1 as well as chemokines (RANTES, IP10) but not pro-inflammatory markers such as iNOS and TNF. An increase in C5aR1 expression was also observed in peritoneal macrophages and several tissues from $LysM\text{-}Cre{:}BVR^{fl/fl}$ mice in a model of endotoxemia. In addition, knockdown of BVR-A resulted in enhanced macrophage chemotaxis towards C5a. Part of the effects of BVR-A deletion on chemotaxis and RANTES expression were blocked in the presence of a C5aR1 neutralizing antibody, confirming the role of C5a-C5aR1 signaling in mediating the effects of BVR.

In summary, BVR-A plays an important role in regulating macrophage chemotaxis in response to C5a, via modulation of C5aR1 expression. In addition, macrophages lacking BVR-A are

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characterized by the expression of M1 polarization-associated chemokines whose levels depend in part on C5aR1 signaling.

Keywords

macrophage; inflammatory mediators; complement; chemokines

Introduction

BVR-A (referred in a literature as BVR) is a multifunctional protein, which mediates the reduction of biliverdin (BV) to bilirubin (BR) and regulates intracellular signalling by acting as a kinase and transcriptional regulator (1–3). The conversion of BV to BR occurs in many cellular compartments; however the majority of BVR-A reactivity is detected in the endoplasmic reticulum (ER) and cell membrane (4). We have previously showed that BVR-A present on the surface of macrophages is crucial for mediating anti-inflammatory effects of BV through Akt-IL-10 signaling (5). Our previous work showed that knockdown of BVR-A also leads to the development of a pro-inflammatory phenotype in macrophages, which is characterized by elevated production of TNF due to increased basal expression of TLR-4 (6). Deletion of BVR-A by RNA interference promotes cell death and oxidative stress in response to H_2O_2 in a similar manner observed with depletion of glutathione (7, 8). Similarly, *BVR-A*$^{-/-}$ mice demonstrate greater oxidative damage to blood components due to lower levels of circulating BR (9). Recent studies using conditional deletion of BVR-A in the mouse liver show the importance of BVR-A in protecting against hepatic steatosis by inhibiting glycogen synthase kinase 3β (GSK3β) and activating the peroxisome proliferator-activated receptor α (PPARα) (10, 11). Further, BVR-A deficient animals are more prone to proximal tubular injury in a model of saturated fatty acid-induced lipotoxicity (12).

The complement system plays a key role in immunity by facilitating pathogen elimination by opsonization, augmenting antibody production and inflammatory responses (13). It also facilitates clearance of apoptotic and necrotic cells in tissues (14, 15). Activation of complement occurs by one of four different pathways: classical, lectin, alternative or extrinsic, and all pathways lead to the cleavage of the central fragments C3 and C5 to generate the anaphylatoxins, C3a and C5a (16, 17). These small protein fragments act primarily through their cellular receptors C3aR, C5aR1 and C5aR2, respectively (17–19). The C5a-C5aR1 axis has been identified as a crucial player in inflammation-associated pathologies such as ischemia reperfusion injury (IRI), neurodegenerative disorders, atherosclerosis, rheumatoid arthritis and sepsis (13, 20–26). We have previously shown that BV inhibits the expression of C5aR1 in RAW 264.7 macrophages in part *via* mTOR signaling (27).

In the present study, we sought to determine a crosstalk between immunomodulatory BVR protein and the C5a-C5aR1 axis in macrophages, which are key cells involved in mediating responses in septic shock.

Material and methods

Animals

$BVR^{fl/fl}$ mice were described before (10–12). We replicated most of the experiments in these mice based on our original observation in mice generated in-house on the 129 background. In house generated $BVR^{fl/fl}$ mice (conditional knockout mice) were based on a targeting construct that was designed based on PGK Neo FRT/loxp vector. A targeted sequence of exons IV and V of the mouse BVR-A gene was inserted into the SacII site, located upstream of neomycin resistance gene and flanked by two lox sites. The same exons were targeted in $BVR^{fl/fl}$ mice from Dr. Stec's colony. The fragment of 3' (part of intron IV) arm and 5' (exon VI) arm of homology were inserted outside the loxp sites between Hpa-I and Sal-I sites respectively. Blunt-end cloning was applied for all inserts. The construct was linearized with Not-I and electroporated into embryonic stem (ES) cells (Children's Hospital Core Facility, Harvard Medical School, Boston, MA). Homozygotes $BVR^{fl/fl}$ mice (129S background) were crossed with *LysM-Cre* mice (C57Bl6) to generate myeloid linage specific knockout of BVR (mixed background). These mice are not available in our colony due to difficulty with breeding.

Isolation and differentiation of bone marrow-derived macrophages (BMDM)

Male and female C57BL/6 (Jackson Laboratories, Bar Harbour, Maine, USA), $BVR^{fl/fl}$ controls and *LysM-Cre:* $BVR^{fl/fl}$ mice were used at 7–10 weeks of age. Animals were held under specific pathogen free conditions and all experiments were approved by the BIDMC Animal Care and Use Committee. BMDMs were isolated as previously described (5). BMDMs were isolated from the femur by crushing and washing the femurs with RPMI medium (Thermo Fisher, Logan, UT, USA) supplemented with Antibiotic-Antimycotic (Thermo Fisher, Logan, UT, USA). Isolated cells were differentiated with murine recombinant macrophage colony stimulating factor, M-CSF (ProSpec, East Brunswick, NJ) at final concentration of 20 ng/mL in RPMI medium supplemented with 15% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA) and Antibiotic-Antimycotic for five days (M-CSF medium). The medium was changed to fresh M-CSF medium on the third day of culture. Macrophages were harvested after five days and then cultured in RPMI medium supplemented with 15% FBS and Antibiotic-Antimycotic prior to experimentation. For macrophage polarization experiments, cells were treated with lipopolysaccharide, LPS (100 ng/mL; *E. Coli* Serotype 0127:B8, Sigma Aldrich, St. Louis, MO) and IFN-γ (20 ng/mL; Peprotech Inc. Rocky Hill, NJ) to induce M1 polarization for 24 hours.

Blocking experiments with anti-mouse C5aR1 (also known as CD88) were performed by pre-incubating cells with LEAF™ anti-mouse C5aR1 (1 μg/mL; clone 20/70, Biolegend, San Diego, CA) or anti-mouse IgG (Cell Signaling, Beverly, MA) for 30 minutes prior to experimentation.

Stable Transfection of RAW 264.7 cells with mir-BVR shRNA

RAW264.7 cells were stably transfected as described previously (6). Briefly, microRNA adapted short hairpin RNA (shRNAmir) against BVR was generated from a pSM2 vector (Open Biosystems, USA). shRNAmir BVR was subcloned to MSCV-LTRmir30-PIG (LMP)

vector (Open Biosystems, USA) with XhoI and EcoRI restriction enzymes (Life Technologies). Cloning was verified by restriction site analysis and sequencing. For retroviral production, HEK293T cells were transiently transfected with shRNAmir BVR-1α-LMP, VSVG, and Gag-Pol plasmids using Lipofectamine 2000 (Life Technologies, USA). Medium with viruses were collected at 12 hours post-transfection and the supernatants were used for transduction of RAW 264.7 cells. After 14 hours incubation with viruses, RAW cells were selected with 5 μg/ml of puromycin (Sigma-Aldrich, USA) for one week. Knockdown of BVR was confirmed by western blot and quantitative PCR.

Source of antibodies

The following antibodies were used for western blot: rabbit anti-BVR (Enzo Life Sciences), rabbit anti-IκB and anti-GAPDH (Cell Signaling), mouse anti-β-actin (Sigma-Aldrich), rabbit anti-iNOS (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse IgG (Cell Signaling, Beverly, MA) or anti-rabbit IgG (Cell signaling). For flow cytometry, PE anti-mouse CD88 and PE rat IgG2a (Biolegend), FITC anti-mouse F4.80 and FITC rat IgG2a (Biolegend), APC anti-mouse CD11b and APC rat IgG2a (Biolegend) were used. For immunohistochemistry, rat anti-mouse CD88/C5aR1 antibody (clone 10/92, LifeSpan Biosciences, Seattle, WA) was applied.

Animal treatment

BVR$^{fl/fl}$ controls and *LysM-Cre:BVR*$^{fl/fl}$ mice were administrated LPS (5 mg/kg, intraperitoneal) and tissues were harvested 24 hours after injection. Peritoneal cells were isolated by flushing the mouse peritoneum with 10 mL of ice-cold PBS and spun down at 1200 × g for 5 minutes. Cells were stained with selected antibodies for 30 minutes on ice or at RT and thereafter were immediately analyzed by flow cytometry.

RNA extraction and RT-PCR

Total RNA was isolated from cultured cells using RNeasy® Plus Mini Kits (Qiagen, Valencia, CA, USA) and qPCR was performed as previously described (6). Primers were purchased from Life Technologies. The following oligonucleotides were used: β-actin: F: 5'CCACGGATTCCATACCCAAGA, R 5' TAGACTTCGAGCAGGAGATGG; BVR: F 5' ATTCTGCCACCATGGAAA, R 5' CTCCAAGGACCCAGATTTGA; C5aR1: F 5' CATTGCTCCTCACCATTCCA, R 5'CACCACTTTCGTTGG; iNOS: F 5' CAGCTGGGCTGTACAAACCTT, R 5' CATTGGAAGTGAAGCGGTTCG; COX-2: F 5' CAAAAGAAGTGCTGGAAAAGGTT, R 5' TCTACCTGAGTGTCTTTGACTGTG; IP10: F 5'CTTGAAATCATCCCTGCGAGC, R 5' TAGGACTAGCCATCCACTGGG; RANTES: F 5' CATATGGCTCGGACACCA, R 5' ACACACTTGGCGGTTCCT; IL10: F 5' CCAAGCCTTATCGGAAATGA, R 5' TTTTCACAGGGGAGAAATCG; IL1β: F 5' TGGGCCTCAAAGGAAAGA, R 5' GGTGCTGATGTACCAGTT; CD206: F 5' TCTTTGCCTTTCCCAGTCTCC, R 5' TGACACCCAGCGGAATTTC; Arginase: F CTCCAAGCCAAAGTCCTTAGAG, R AGGAGCTGTCATTAGGGACATC; TNF: F: 5' TCCCAGGTTCTCTTCAAGGGA 3', R: 5' GGTGAGGAGCACGTAGTCGG 3'.

Briefly, RNA was reverse transcribed using iScript™ cDNA synthesis kit (BioRad, Hercules, CA) and qPCR was performed with Mx3000P QPCR system (Agilent

Technologies, Santa Clara CA). Expression levels of BVR were quantified by using SYBR® Select Master Mix (Life Technologies, NY). The relative quantification of gene expression was analysed using ΔC_T method, normalized to the housekeeping gene and expressed as $2^{-\Delta\Delta CT}$.

Isolation of cells from the tissues

Fragments of tissues were digested in 500 μl of Liberase solution (200 μg/ml) at 37°C for 1 hour, vortexing the samples every 10 minutes. After digestion, tissues were further disrupted by pipetting and by using a 25G needle to obtain a single cell suspension. Cells were then washed twice in PBS, filtered through a 70 μm strainer and then incubated with the corresponding antibodies for further analysis.

Flow cytometry analysis of C5aR1

After harvesting and washing BMDM cells with PBS, cells were stained with PE labelled anti-mouse C5aR1 (CD88) antibody or PE-labelled IgG (1μg/10^6 cells) and myeloid markers for 30 min at RT or on ice. Cells were analyzed immediately using a FACS Calibur flow cytometer (Becton and Dickinson, San Jose, CA). The number of positive cells (%) was derived and analyzed using CellQuest ProTM software (Becton and Dickinson, San Jose, CA).

Immunohistochemistry

Liver, lung and spleen tissue samples were formalin-fixed followed by paraffin embedding and immunostaining of 5 μm sections as previously described (5). Mouse antibody against C5aR1 was used at a concentration of 5 μg/mL. Secondary antibody was used as negative control. Briefly, sections were processed for antigen retrieval with high pressure-cooking in citrate buffer for 1 hour. Sections were then blocked for 30 minutes in 7% horse serum (Vector Laboratories. Burlingame, CA). Primary antibody against C5aR1 was then applied to the sections for overnight at 4°C. The following day, sections were incubated with biotin-labelled secondary antibody (1.5 μg/mL in PBS; Vector Laboratories) for 1 hour at room temperature, followed by Vectastain Elite ABC kit and detection with ImmPact DAB (Vector Laboratories). The images were captured using Nikon Eclipse E600 microscope (Nikon Instruments, Melville, NY, USA).

Cell migration assay

Chemotaxis was evaluated in 24-well Transwell plates (Corning Inc. Corning, NY) using polycarbonate membranes (8 μm pore size). BMDM from $BVR^{fl/fl}$ and $LysM\text{-}Cre{:}BVR^{fl/fl}$ were resuspended in serum free RPMI medium at 1×10^5 cells/ml. 100 μl of cell suspension was added to the upper chamber and 500 μl of serum free RPMI medium containing recombinant mouse C5a (100 nM, R & D Systems, Minneapolis, MN), SDF-1 (50 ng/ml) or MCP-1 (15 ng/ml) was added to the lower chamber. Cells were incubated for 24 hours. Thereafter, the cells from the upper chamber were removed and cells on the lower side of the chamber were stained with Crystal Violet (Sigma Aldrich) for 10 min, followed by extensive washing with water. Cells were dried and visualized at 40 × magnification. Cells were then dissolved in 10% acetic acid, followed by measurement of absorbance at 562 nm on the

ELISA plate reader. For blocking experiments with anti-mouse C5aR1, cells were pre-incubated with LEAF™ anti-mouse C5aR1 (1 μg/ml) or IgG (1 μg/ml) for 30 minutes.

Immunoblotting

Cell lysates were prepared in ice-cold RIPA buffer (50 mM Tris-HCl, [pH 7.4], 50 mM sodium fluoride, 150 mM NaCl, 1% Nonident P40, 0.5 M EDTA [pH 8.0]) supplemented with the protease inhibitor cocktail Complete Mini (Roche, Indianapolis, IN). Samples were centrifuged at 14,000 × g at 4 °C for 20 min and supernatants were collected. Protein concentrations of supernatants were measured using a bicinchoninic acid protein assay kit (BCA; Thermo Fisher Scientific, Tewksbury, MA). Forty μg of each protein sample was then electrophoresed on NuPAGE 4–12% Bis-Tris Gel (Life Technologies) in NuPAGE MES SDS running buffer (Life Technologies) for 90 min at 100 V. The membranes were blocked with 5% non-fat dry milk in 1 × Tris buffered saline buffer (TBS; Boston Bio Products, Ashland, MA) for 1 hour and then probed with appropriate primary antibodies (diluted at 1:1000 in 1 × TBS with 5 % non-fat milk) overnight at 4°C. Membranes were then washed in 1 × TBS buffer and membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at a dilution of 1:5000 in 1 × TBS with 5% non-fat milk for 1 hour at RT. Membranes were visualised using Super Signal West Pico chemiluminescent substrate (Thermo Fisher Scientific) or Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), followed by exposure to the autobioradiography film (BioExpress, Kaysville, UT).

ELISA analysis

TNF cytokine concentrations were measured in supernatants using Quantikine Immunoassays (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Nitric oxide measurement

Supernatants from BMDM cultures were harvested at 24 hours after treatment. Griess Reagent Kit for Nitrite Determination (Life Technologies) was used to measure nitrate in the supernatants. 150 μL of the supernatant was incubated with 20 μL of Griess Reagent and 130 μL of deionized water for 30 minutes at RT following manufacturer's protocol. The reference samples were prepared at the same time. Absorbance was measured at 548 nm.

Statistical analysis

All data are reported as average ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA; Post-hoc Tukey test) or Mann-Whitney test using Graph pad Prism, unless otherwise stated.

Results

Knockdown of BVR-A in RAW 264.7 or deletion of BVR-A in bone marrow-derived macrophages increases C5aR1 expression in vitro

We first established whether C5aR1 expression is associated with macrophage differentiation. BM-derived myeloid cells stimulated with M-CSF were used. We show

significantly increased C5aR1 surface expression starting as early as 1 day after addition of M-CSF and reaching plateau at day 4–5 (Fig. 1A–B).

To assess the role of BVR-A in regulating C5aR1 expression, we employed RAW 264.7 macrophages with stable knockdown of BVR (shRNA-BVR-A) and control cells (28). We confirmed lower expression of BVR-A in shRNA-BVR-A cells compared to control cells by RT-PCR and western blotting (Fig. 1C–D). Knockdown of BVR-A in these cells resulted in significantly higher expression of C5aR1 as compared to control cells (shRNA control) (Fig. 1E–F).

To study the role of BVR-A on C5aR1 expression in primary macrophage culture, we deleted BVR-A by crossing $BVR^{fl/fl}$ mice *to LysM-Cre* mice. We show that BVR protein levels were significantly decreased in BMDM isolated from *LysM-Cre:*$BVR^{fl/fl}$ mice as compared to BMDM isolated from $BVR^{fl/fl}$ control mice (Fig. 2A). This corresponded with a significant increase in C5aR1 protein expression in BMDM isolated from *LysM-Cre:*$BVR^{fl/fl}$ mice compared to the control BMDM isolated from $BVR^{fl/fl}$ mice (Fig. 2B). These results were confirmed by incubation of cells with antibody against C5aR1 at RT or on ice to prevent internalization of antibody (Fig. 2B). Additionally, there was a significant increase in the number of C5aR1+/F4.80+ cells in the BMDM population at 5 days compared to 3 days after M-CSF and a further increase in the number of C5aR1+/F4.80+ cells within the BMDM isolated from *LysM-Cre:*$BVR^{fl/fl}$ mice compared to the control BMDM isolated from $BVR^{fl/fl}$ mice at 5 days (Fig. 2C). However, in BMDM, unlike in RAW247.4 cells, we observed no change in gene expression of C5aR1 (Supplementary Fig. 1A).

Deletion of BVR in BMDM increases chemotaxis towards C5a

C5a is a potent chemoattractant and the ligand for C5aR1 that mediates macrophage chemotaxis (29). Therefore, we next assessed the effect of BVR deletion on BMDM migration towards C5a. BMDM isolated from mice lacking BVR showed significantly increased migration towards C5a at 24 hours compared to control cells (Fig. 2D). Similarly, BMDM M1-polarized with LPS/IFNγ and lacking BVR-A showed greater chemotaxis towards C5a (Fig. 2E). Interestingly, the effect of BVR-A on chemotaxis was specific towards C5a, as SDF-1- or MCP-1-induced chemotaxis was not associated with BVR-A deletion (Supplementary Fig. 1B)

BMDM from LysM-Cre:$BVR^{fl/fl}$ mice express greater levels of chemokines: RANTES and IP10, markers of an M1-like phenotype

Having shown that BMDM from *LysM-Cre:*$BVR^{fl/fl}$ mice have increased expression of C5aR1 and enhanced C5a-mediaed chemotaxis, we next assessed the phenotype of BMDM isolated from $BVR^{fl/fl}$ and *LysM-Cre:*$BVR^{fl/fl}$ mice in response to LPS, which polarizes macrophages towards an M1 phenotype (30). Stimulation of BMDM with LPS and IFN-γ leads to increased expression of M1 markers including: iNOS and TNF, IP-10 (CXCL10), IL-1β, IL-6, COX-2, RANTES (CCL5) or MCP-1 among others (31, 32).

Treatment with LPS/IFNγ for 24 hours led to increased markers of M1 polarization (Fig. 3A–H). We assessed expression of iNOS (by RT-PCR or activity of iNOS by NO

production) and TNF (by RT-PCR and ELISA), however, no change was observed in BMDM from *LysM-Cre:BVR*$^{fl/fl}$ compared to *BVR*$^{fl/fl}$ mice treated with LPS/IFNγ (Fig. 3A–D). Interestingly, M1-polarized BMDM (LPS/IFNγ-treated) from *LysM-Cre:BVR*$^{fl/fl}$ mice showed significantly enhanced expression level of RANTES and IP-10 compared to M1-polarized BMDM from *BVR*$^{fl/fl}$ mice (Fig. 3E, 3G). A pro-inflammatory cytokine, IL1β significantly induced in M1-polarized BMDM from *LysM-Cre:BVR*$^{fl/fl}$ mice compared to untreated BMDM but not in BMDM from *BVR*$^{fl/fl}$ mice (Fig. 3F). No difference in COX2 or anti-inflammatory M2 marker, IL-10 was observed (Supplementary Fig. 2A–B). Similarly, we also measured arginase and CD206 by RT-PCR and demonstrated no difference in the expression of these M2 markers between the BMDM isolated from these strains (Supplementary Fig. 2C–D).

Many of these pro-inflammatory markers are regulated by NFκB signaling in response to LPS, and therefore we assessed the levels of IκB in BMDM from *LysM-Cre:BVR*$^{fl/fl}$ mice treated with LPS (Fig. 3H). The levels of IκB degradation did not differ between the strains.

Increased expression of C5aR1 in vivo after LPS administration in LysM-Cre:BVR$^{fl/fl}$ mice

We next evaluated C5aR1 protein expression *in vivo* in various organs basally or after treatment with LPS (5 mg/kg, *i.p.*, 24 hours) of *LysM-Cre:BVR*$^{fl/f}$ and *BVR*$^{fl/fl}$ mice. Total staining of the C5aR1 in the spleen, liver and lungs in untreated (Fig. 4A) and LPS-treated (Fig. 4B) mice was assessed by IHC. The number of C5aR1+ cells in analyzed organs was increased upon instillation of LPS and further elevated in LPS-treated *LysM-Cre:BVR*$^{fl/f}$ compared to LPS-treated *BVR*$^{fl/fl}$ mice (Fig. 4C–E). There was no difference in expression of C5aR1 in any of the organs tested at basal conditions.

We next evaluated the effects of BVR on C5aR1 expression in peritoneal macrophages isolated from *BVR*$^{fl/fl}$ and *LysM-Cre:BVR*$^{fl/fl}$ mice with and without LPS administration (Fig. 5A). We found significant upregulation of C5aR1 in *LysM-Cre:BVR*$^{fl/fl}$ mice upon administration of LPS compared to the control groups analyzed (Fig. 5A). However, in contrast to the increased number of C5aR1+ cells in the total population of peritoneal isolates, we detected a lower number of C5aR1+/F.480+ residential macrophages in the peritoneal cavity upon administration of LPS (Fig. 5B). There was slight but not significant increase in a number of C5aR1+/F4.80+ in *LysM-Cre:BVR*$^{fl/f}$ compared to *BVR*$^{fl/fl}$ mice (Fig. 5B).

We also performed an extensive analysis of a surface expression of C5aR1 in organs by flow cytometry (Fig. 5C–H). LPS administration increased the number of C5aR1+ and C5aR1+/F4.80+ populations in both strains in the spleen (Fig. 5C–D). The size of the spleen was increased after LPS administration but did not differ between the strains (Supplementary Fig. 3). There was no difference in the basal number of C5aR1+, C5aR1+/F4.80+ or C5aR1+/CD11b+ in the organs between *LysM-Cre:BVR*$^{fl/f}$ compared to *BVR*$^{fl/fl}$ mice (Fig. 5, Supplementary Fig. 4A). We did not see any difference in the numbers of C5aR1+ cells in the strains or after LPS treatment in the liver (Fig. 5E–F). There was, however, a significant elevation in the number of C5aR1+ cells in the lungs of LPS-treated animals, but there was no difference between the strains (Fig. 5G–H).

The effects of BVR on chemotaxis and chemokine expression are in part mediated by C5aR1

To evaluate the role of C5aR1 in the effects seen upon deletion of BVR, BMDM were pre-incubated with neutralizing antibody against C5aR1 or control IgG. Confirmation of neutralization of C5aR1 was performed by flow cytometry (Fig. 6A). C5a-induced chemotaxis in *LysM-Cre:BVR*$^{fl/fl}$ BMDM was significantly inhibited by this neutralizing antibody against C5aR1 (Fig. 6B–C), suggesting a functional role of this receptor in BVR-mediated effects in BMDM.

Next we assessed whether the BVR-modulated RANTES and IP-10 levels were dependent on C5aR1 signaling (Fig. 6D–E). LPS/IFN-γ-induced RANTES expression in BMDM from *LysM-Cre:BVR*$^{fl/fl}$ was blunted after treatment with the C5aR neutralizing antibody. However, a similar effect was not noted for IP-10 expression (Fig. 6D–E).

Discussion

In the present study, we show that BVR is a regulator of macrophage chemotaxis towards C5a and its effects are partially dependent on C5aR1. Conditional deletion of BVR not only augmented C5aR1 expression in primary macrophages but also enhanced C5a-mediated chemotaxis. We showed similar effects of BVR deletion on C5aR1 in an endotoxemia model *in vivo*. Further, the LPS-induced chemokine RANTES was elevated in the absence of BVR-A and was subsequently blunted by C5aR1 blockade. We suggest that the remarkable effects of BVR on C5a-C5aR1 signaling and macrophage polarization are crucial in the negative regulation of innate immune responses.

We have previously shown that BV inhibits TLR-4 expression in part via BVR binding to AP-1 and GATA-4 sites (6). BV is also known to block C5aR1 expression in part via the mTOR pathway (27), however whether the effects are dependent on the activity of BVR has not been studied before. Silencing of surface BVR with RNA interference abrogated BV-induced Akt (protein kinase B) phosphorylation (5) and IL-10 expression, and thus was implicated in the induction of an anti-inflammatory phenotype of macrophages (5, 33). The anti-inflammatory effects of BV in models of sepsis (5, 34), transplantation and ischemic injury (35) have also been demonstrated, however, the effects of BVR deletion on complement receptor and macrophage polarization status have not been studied before.

Increased expression of C5a and its receptor C5aR1 are strongly associated with acute and chronic inflammation and inflammatory disorders (20, 21, 23). C5aR has also been implicated in controlling of M2 polarization of macrophages in the tumor microenvironment (36). Our studies indicate the importance of C5aR1 in regulation of M1-like chemokine release upon deletion of BVR. C5aR1 is a G-protein coupled receptor and expressed in both myeloid and non-myeloid cells and increased expression of C5aR1 has been observed in inflamed tissues (21, 37). Indeed, we observed an increased number of C5aR1+ cells in the spleens, lungs and livers upon administration of LPS. However, the number of residential macrophages expressing C5aR1 was not different between strains, although they were increased by LPS treatment. C5aR1 is expressed on multiple cells types beside myeloid cells (38). It is possible that other myeloid cells, such as granulocytes in which Cre is expressed

may exhibit changes in C5aR1 expression upon deletion of BVR. The complexity in expression of C5aR1 in different cell types within the tissue has been observed. In the asthma model, C5aR was increased in lung tissue eosinophils but decreased in airway and pulmonary macrophages as well as in pulmonary CD11b+ conventional dendritic cells and monocyte-derived DC cells (39). Further, deletion of BVR in myeloid cells may impact tissue microenvironment and expression of C5aR1 in other cells (38). The number of cells expressing C5aR1 is increased in BVR^{flfl}:*LysM-Cre* mice compared to BVR^{flfl} mice at average of ~10%. It is important to note that this is a baseline expression of C5aR on the surface that changes upon deletion of only one metabolic gene, BVR-A. Even small changes in number of C5aR1 expressing cells or the levels of C5aR1 are highly relevant to the physiology of myeloid cells as they have large consequences (40–43). Further, we might not be detecting all cells mediating an increase in levels of C5aR1 at specific time points as this is not a synchronized population of cells.

Hu et al. (33) reported that BVR overexpression or ablation had no effect on TNF expression and release in non-polarized macrophages, however, over expression of BVR with adenovirus in non-polarized BMDM increased both iNOS and arginine-1 expression, suggesting that BVR had no involvement in macrophage polarization (33). C5aR1 regulates the LPS-induced production of pro-inflammatory cytokines such as IL-6 and IL-12p40 (45). We show here that incubation of macrophages lacking BVR with LPS and IFN-γ stimulated expression of RANTES, IP-10 and IL1β in macrophages. Indeed, C5a stimulated RANTES and IL1β expression in endothelial cells (44) and the effects of BVR on RANTES were indeed partially dependent on C5aR in this study. C5aR1 also plays a key role in disrupting blood brain barrier integrity via regulating mRNA expression of iNOS in brain endothelial cells (46). We did not see an effect of BVR on TNF or iNOS expression. However, our previous work indicates that long-term depletion of BVR by shRNA in RAW macrophages increases TNF levels basally and upon LPS stimulation (28). Interestingly, as much as RNA levels of C5aR1 were elevated in RAW264.7 shRNA BVR cells compared to control cells, we demonstrated changes in C5aR1 protein expression only in BMDM. The differences between BMDM and RAW cells may be due to typical variation between the primary versus cultured cell lines, however this may also emphasize the importance of BVR deletion for extended periods of time or to secondary effects of BVR deletion in RAW264.7 macrophages.

Lack of BVR in BMDM from *LysM-Cre:*$BVR^{fl/fl}$ mice resulted in higher protein expression of C5aR1. Our *in vitro* findings are supported by *in vivo* studies, in which we report higher total C5aR1 expression in the spleen, liver and lung from *LysM-Cre:*$BVR^{fl/fl}$ mice and surface C5aR1 in peritoneal macrophages from *LysM-Cre:*$BVR^{fl/fl}$ mice treated with LPS. Transcriptional activation of C5aR1 promoter basally and in the presence of LPS, requires nuclear factor-Y (NF-Y) (47), which was induced upon treatment with heme, a precursor of biliverdin (48). We cannot exclude the possibility that C5aR1 levels are regulated on the cell surface in addition to mRNA levels. Further, BMDM isolated from *LysM-Cre:*$BVR^{fl/fl}$ mice and treated with LPS did not show any difference in NFκB signaling, which indicates that other signaling pathways are implicated in BVR-regulated chemokine and cytokine expression in response to LPS.

Activation of C5aR1 promotes recruitment of neutrophils and macrophages at the site of infection, trauma and inflammation (49, 50). Indeed, higher levels of C5aR1 in BMDM depleted of BVR corresponded to higher chemotaxis activity. Soruri et al. showed that blockade of C5aR1 with neutralizing antibody against C5aR1 (clone 20/70) inhibited the migration of rat basophilic leukemia RBL-2H3 cells towards C5a (29). A more recent study by Staab et al. (50) also demonstrated that inhibition of C5aR1 with PMX205 (peptidomimetic antagonist) reduced influx of eosinophils and neutrophils, as well as production of pro-inflammatory cytokines in response to OVA allergen sensitization and challenge, indicating the immunomodulatory potential of C5a/C5aR1 signaling (50).

Collectively, our data demonstrate that BVR is an important molecule required for regulating macrophage chemotaxis towards C5a and deletion of BVR in macrophages promotes chemotaxis towards C5a. We report that the increased cell migration of BMDM towards C5a in *LysM-Cre:BVR*$^{fl/fl}$ mice was suppressed after treatment with a C5aR1 neutralizing antibody, suggesting a partial role for C5aR1 in BVR-mediated modulation of chemotaxis. Moreover, we translated our findings to an *in vivo* model, where we observed that peritoneal cells from LPS treated *LysM-Cre:BVR*$^{fl/fl}$ mice expressed more C5aR1 compared to *BVR*$^{fl/fl}$ mice.

In summary, we show that BVR modulates C5a-C5aR1 signaling and macrophage phenotype in part via regulation of C5aR1 expression. We identified here BVR as a target for regulating responses to LPS, and complement activation products. Alteration in BVR expression may therefore modulate macrophage polarization and contribute to the development of inflammatory pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding information

This research was supported by: NIDDK R01 DK104714, start-up funds from Department of Surgery at BIDMC and Eleanor Shore HMS Foundation to BW.

References

1. Miralem T, Lerner-Marmarosh N, Gibbs PE, Jenkins JL, Heimiller C, and Maines MD. 2016 Interaction of human biliverdin reductase with Akt/protein kinase B and phosphatidylinositol-dependent kinase 1 regulates glycogen synthase kinase 3 activity: a novel mechanism of Akt activation. FASEB J 30: 2926–2944. [PubMed: 27166089]
2. Wegiel B, and Otterbein LE. 2012 Go green: the anti-inflammatory effects of biliverdin reductase. Front Pharmacol 3: 47. [PubMed: 22438844]
3. Gibbs PE, Miralem T, Lerner-Marmarosh N, Tudor C, and Maines MD. 2012 Formation of ternary complex of human biliverdin reductase-protein kinase Cdelta-ERK2 protein is essential for ERK2-mediated activation of Elk1 protein, nuclear factor-kappaB, and inducible nitric-oxidase synthase (iNOS). The Journal of biological chemistry 287: 1066–1079. [PubMed: 22065579]
4. Salim M, Brown-Kipphut BA, and Maines MD. 2001 Human biliverdin reductase is autophosphorylated, and phosphorylation is required for bilirubin formation. The Journal of biological chemistry 276: 10929–10934. [PubMed: 11278740]

5. Wegiel B, Baty CJ, Gallo D, Csizmadia E, Scott JR, Akhavan A, Chin BY, Kaczmarek E, Alam J, Bach FH, Zuckerbraun BS, and Otterbein LE. 2009 Cell surface biliverdin reductase mediates biliverdin-induced anti-inflammatory effects via phosphatidylinositol 3-kinase and Akt. The Journal of biological chemistry 284: 21369–21378. [PubMed: 19509285]
6. Wegiel B, Gallo D, Csizmadia E, Roger T, Kaczmarek E, Harris C, Zuckerbraun BS, and Otterbein LE. 2011 Biliverdin inhibits Toll-like receptor-4 (TLR4) expression through nitric oxide-dependent nuclear translocation of biliverdin reductase. Proc Natl Acad Sci U S A 108: 18849–18854. [PubMed: 22042868]
7. Baranano DE, Rao M, Ferris CD, and Snyder SH. 2002 Biliverdin reductase: a major physiologic cytoprotectant. Proc Natl Acad Sci U S A 99: 16093–16098. [PubMed: 12456881]
8. Sedlak TW, Saleh M, Higginson DS, Paul BD, Juluri KR, and Snyder SH. 2009 Bilirubin and glutathione have complementary antioxidant and cytoprotective roles. Proc Natl Acad Sci U S A 106: 5171–5176. [PubMed: 19286972]
9. Chen W, Maghzal GJ, Ayer A, Suarna C, Dunn LL, and Stocker R. 2018 Absence of the biliverdin reductase-a gene is associated with increased endogenous oxidative stress. Free radical biology & medicine 115: 156–165. [PubMed: 29195835]
10. Hinds TD Jr., Burns KA, Hosick PA, McBeth L, Nestor-Kalinoski A, Drummond HA, AlAmodi AA, Hankins MW, Vanden Heuvel JP, and Stec DE. 2016 Biliverdin Reductase A Attenuates Hepatic Steatosis by Inhibition of Glycogen Synthase Kinase (GSK) 3beta Phosphorylation of Serine 73 of Peroxisome Proliferator-activated Receptor (PPAR) alpha. The Journal of biological chemistry 291: 25179–25191. [PubMed: 27738106]
11. Weaver L, Hamoud AR, Stec DE, and Hinds TD Jr. 2018 Biliverdin reductase and bilirubin in hepatic disease. American journal of physiology. Gastrointestinal and liver physiology 314: G668–G676. [PubMed: 29494209]
12. Adeosun SO, Gordon DM, Weeks MF, Moore KH, Hall JE, Hinds TD, and Stec DE. 2018 Loss of biliverdin reductase-A promotes lipid accumulation and lipotoxicity in mouse proximal tubule cells. American journal of physiology. Renal physiology 315: F323–F331. [PubMed: 29631357]
13. Trouw LA, Pickering MC, and Blom AM. 2017 The complement system as a potential therapeutic target in rheumatic disease. Nature Reviews Rheumatology 13: 538. [PubMed: 28794515]
14. Nayak A, Pednekar L, Reid KB, and Kishore U. 2012 Complement and non-complement activating functions of C1q: a prototypical innate immune molecule. Innate Immun 18: 350–363. [PubMed: 21450789]
15. Carroll MV, and Sim RB. 2011 Complement in health and disease. Adv Drug Deliv Rev 63: 965–975. [PubMed: 21704094]
16. Coulthard LG, and Woodruff TM. 2015 Is the complement activation product C3a a proinflammatory molecule? Re-evaluating the evidence and the myth. J Immunol 194: 3542–3548. [PubMed: 25848071]
17. Hawksworth OA, Li XX, Coulthard LG, Wolvetang EJ, and Woodruff TM. 2017 New concepts on the therapeutic control of complement anaphylatoxin receptors. Molecular immunology 89: 36–43. [PubMed: 28576324]
18. Ward PA 2009 Functions of C5a receptors. J Mol Med (Berl) 87: 375–378. [PubMed: 19189071]
19. Li R, Coulthard LG, Wu MC, Taylor SM, and Woodruff TM. 2013 C5L2: a controversial receptor of complement anaphylatoxin, C5a. FASEB J 27: 855–864. [PubMed: 23239822]
20. Grailer JJ, Fattahi F, Dick RS, Zetoune FS, and Ward PA. 2015 Cutting edge: critical role for C5aRs in the development of septic lymphopenia in mice. J Immunol 194: 868–872. [PubMed: 25539817]
21. Manthey HD, Thomas AC, Shiels IA, Zernecke A, Woodruff TM, Rolfe B, and Taylor SM. 2011 Complement C5a inhibition reduces atherosclerosis in ApoE−/− mice. FASEB J 25: 2447–2455. [PubMed: 21490292]
22. Oksjoki R, Kovanen PT, Meri S, and Pentikainen MO. 2007 Function and regulation of the complement system in cardiovascular diseases. Front Biosci 12: 4696–4708. [PubMed: 17485406]
23. Ward PA 2010 Role of C5 activation products in sepsis. ScientificWorldJournal 10: 2395–2402. [PubMed: 21170490]

24. Woodruff TM, Nandakumar KS, and Tedesco F. 2011 Inhibiting the C5–C5a receptor axis. Molecular immunology 48: 1631–1642. [PubMed: 21549429]
25. Mahajan SD, Parikh NU, Woodruff TM, Jarvis JN, Lopez M, Hennon T, Cunningham P, Quigg RJ, Schwartz SA, and Alexander JJ. 2015 C5a alters blood-brain barrier integrity in a human in vitro model of systemic lupus erythematosus. Immunology 146: 130–143. [PubMed: 26059553]
26. Mahajan SD, Tutino VM, Redae Y, Meng H, Siddiqui A, Woodruff TM, Jarvis JN, Hennon T, Schwartz S, Quigg RJ, and Alexander JJ. 2016 C5a induces caspase-dependent apoptosis in brain vascular endothelial cells in experimental lupus. Immunology 148: 407–419. [PubMed: 27213693]
27. Bisht K, Wegiel B, Tampe J, Neubauer O, Wagner KH, Otterbein LE, and Bulmer AC. 2014 Biliverdin modulates the expression of C5aR in response to endotoxin in part via mTOR signaling. Biochem Biophys Res Commun 449: 94–99. [PubMed: 24814708]
28. Otterbein LE, Hedblom A, Harris C, Csizmadia E, Gallo D, and Wegiel B. 2011 Heme oxygenase-1 and carbon monoxide modulate DNA repair through ataxia-telangiectasia mutated (ATM) protein. Proc Natl Acad Sci U S A 108: 14491–14496. [PubMed: 21849621]
29. Soruri A, Kim S, Kiafard Z, and Zwirner J. 2003 Characterization of C5aR expression on murine myeloid and lymphoid cells by the use of a novel monoclonal antibody. Immunol Lett 88: 47–52. [PubMed: 12853161]
30. Ter Horst EN, Hakimzadeh N, van der Laan AM, Krijnen PA, Niessen HW, and Piek JJ. 2015 Modulators of Macrophage Polarization Influence Healing of the Infarcted Myocardium. Int J Mol Sci 16: 29583–29591. [PubMed: 26690421]
31. Gerrick KY, Gerrick ER, Gupta A, Wheelan SJ, Yegnasubramanian S, and Jaffee EM. 2018 Transcriptional profiling identifies novel regulators of macrophage polarization. PLOS ONE 13: e0208602. [PubMed: 30532146]
32. Tarique AA, Logan J, Thomas E, Holt PG, Sly PD, and Fantino E. 2015 Phenotypic, Functional, and Plasticity Features of Classical and Alternatively Activated Human Macrophages. American Journal of Respiratory Cell and Molecular Biology 53: 676–688. [PubMed: 25870903]
33. Hu Z, Pei G, Wang P, Yang J, Zhu F, Guo Y, Wang M, Yao Y, Zeng R, Liao W, and Xu G. 2015 Biliverdin Reductase A (BVRA) Mediates Macrophage Expression of Interleukin-10 in Injured Kidney. Int J Mol Sci 16: 22621–22635. [PubMed: 26393580]
34. Sarady-Andrews JK, Liu F, Gallo D, Nakao A, Overhaus M, Ollinger R, Choi AM, and Otterbein LE. 2005 Biliverdin administration protects against endotoxin-induced acute lung injury in rats. Am J Physiol Lung Cell Mol Physiol 289: L1131–1137. [PubMed: 16155084]
35. Yamashita K, McDaid J, Ollinger R, Tsui TY, Berberat PO, Usheva A, Csizmadia E, Smith RN, Soares MP, and Bach FH. 2004 Biliverdin, a natural product of heme catabolism, induces tolerance to cardiac allografts. Faseb Journal 18: 765–767. [PubMed: 14977878]
36. Piao C, Zhang WM, Li TT, Zhang CC, Qiu S, Liu Y, Liu S, Jin M, Jia LX, Song WC, and Du J. 2018 Complement 5a stimulates macrophage polarization and contributes to tumor metastases of colon cancer. Experimental cell research 366: 127–138. [PubMed: 29551360]
37. Oksjoki R, Laine P, Helske S, Vehmaan-Kreula P, Mayranpaa MI, Gasque P, Kovanen PT, and Pentikainen MO. 2007 Receptors for the anaphylatoxins C3a and C5a are expressed in human atherosclerotic coronary plaques. Atherosclerosis 195: 90–99. [PubMed: 17234193]
38. Zwirner J, Fayyazi A, and Gotze O. 1999 Expression of the anaphylatoxin C5a receptor in non-myeloid cells. Molecular immunology 36: 877–884. [PubMed: 10698342]
39. Ender F, Wiese AV, Schmudde I, Sun J, Vollbrandt T, König P, Laumonnier Y, and Köhl J. 2017 Differential regulation of C5a receptor 1 in innate immune cells during the allergic asthma effector phase. In PloS one. e0172446. [PubMed: 28231307]
40. Floreani AA, Heires AJ, Welniak LA, Miller-Lindholm A, Clark-Pierce L, Rennard SI, Morgan EL, and Sanderson SD. 1998 Expression of receptors for C5a anaphylatoxin (CD88) on human bronchial epithelial cells: enhancement of C5a-mediated release of IL-8 upon exposure to cigarette smoke. J Immunol 160: 5073–5081. [PubMed: 9590258]
41. Herrmann JB, Muenstermann M, Strobel L, Schubert-Unkmeir A, Woodruff TM, Gray-Owen SD, Klos A, and Johswich KO. 2018 Complement C5a Receptor 1 Exacerbates the Pathophysiology of N. meningitidis Sepsis and Is a Potential Target for Disease Treatment. MBio 9.

42. Soruri A, Grigat J, Kiafard Z, and Zwirner J. 2008 Mast cell activation is characterized by upregulation of a functional anaphylatoxin C5a receptor. BMC Immunol 9: 29. [PubMed: 18559098]

43. Gu H, Fisher AJ, Mickler EA, Duerson F 3rd, Cummings OW, Peters-Golden M, Twigg HL 3rd, Woodruff TM, Wilkes DS, and Vittal R. 2016 Contribution of the anaphylatoxin receptors, C3aR and C5aR, to the pathogenesis of pulmonary fibrosis. FASEB J 30: 2336–2350. [PubMed: 26956419]

44. Monsinjon T, Gasque P, Chan P, Ischenko A, Brady JJ, and Fontaine MC. 2003 Regulation by complement C3a and C5a anaphylatoxins of cytokine production in human umbilical vein endothelial cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 17: 1003–1014. [PubMed: 12773483]

45. Zhang X, Kimura Y, Fang C, Zhou L, Sfyroera G, Lambris JD, Wetsel RA, Miwa T, and Song WC. 2007 Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo. Blood 110: 228–236. [PubMed: 17363730]

46. Jacob A, Hack B, Bai T, Brorson JR, Quigg RJ, and Alexander JJ. 2010 Inhibition of C5a receptor alleviates experimental CNS lupus. J Neuroimmunol 221: 46–52. [PubMed: 20207017]

47. Hunt JR, Martin CB, and Martin BK. 2005 Transcriptional regulation of the murine C5a receptor gene: NF-Y is required for basal and LPS induced expression in macrophages and endothelial cells. Molecular immunology 42: 1405–1415. [PubMed: 15950736]

48. Marziali G, Perrotti E, Ilari R, Testa U, Coccia EM, and Battistini A. 1997 Transcriptional regulation of the ferritin heavy-chain gene: the activity of the CCAAT binding factor NF-Y is modulated in heme-treated Friend leukemia cells and during monocyte-to-macrophage differentiation. Mol Cell Biol 17: 1387–1395. [PubMed: 9032265]

49. Godau J, Heller T, Hawlisch H, Trappe M, Howells E, Best J, Zwirner J, Verbeek JS, Hogarth PM, Gerard C, Van Rooijen N, Klos A, Gessner JE, and Kohl J. 2004 C5a initiates the inflammatory cascade in immune complex peritonitis. J Immunol 173: 3437–3445. [PubMed: 15322209]

50. Staab EB, Sanderson SD, Wells SM, and Poole JA. 2014 Treatment with the C5a receptor/CD88 antagonist PMX205 reduces inflammation in a murine model of allergic asthma. Int Immunopharmacol 21: 293–300. [PubMed: 24859057]

Key points:

1. Myeloid-specific conditional deletion of BVR-A results in increased response to LPS.
2. Macrophages isolated from *LysM-Cre:BVR*$^{fl/fl}$ showed elevated levels of C5aR1.
3. Deletion of BVR-A resulted in enhanced macrophage chemotaxis towards C5a.
4. BVR-A-modulated chemotaxis and RANTES levels are dependent in part on C5aR1.

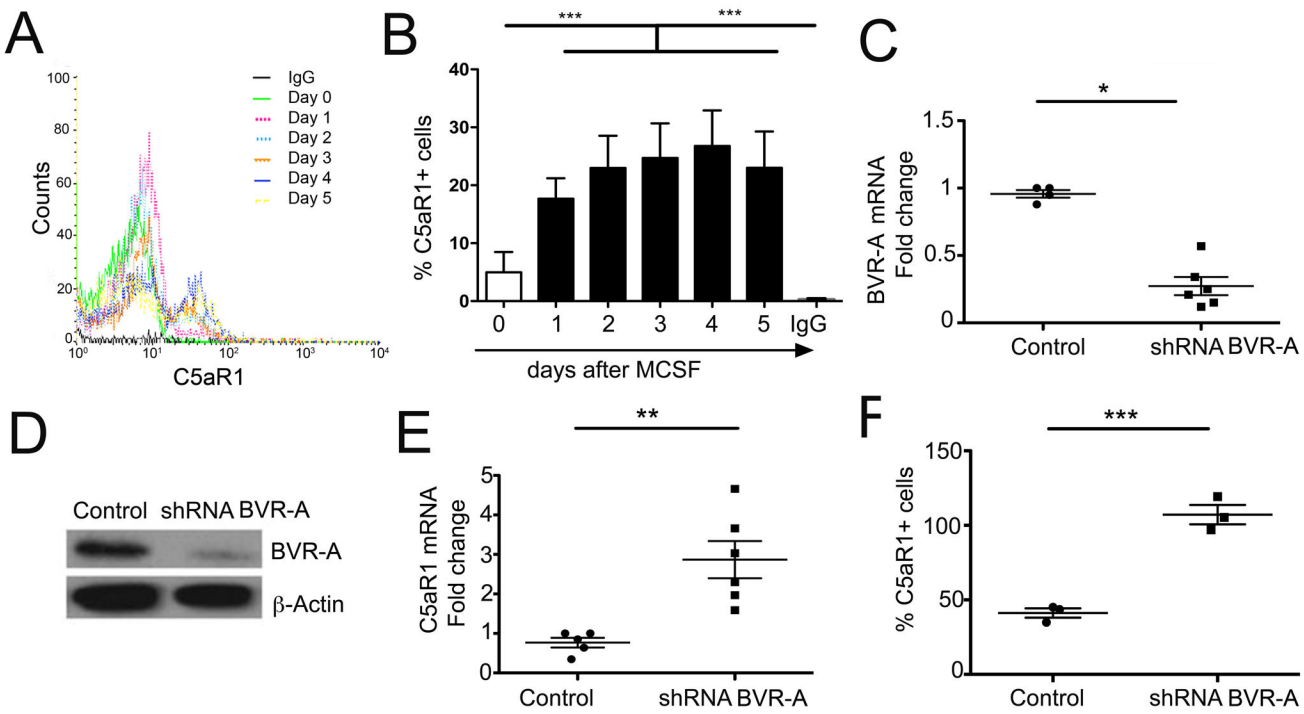

Figure 1. Increased expression of C5aR1 in RAW247.4 macrophages upon knockdown of BVR-A. A-B.

C5aR1 cell surface expression on BMDM harvested from C57BL/6 mice was measured at day 0–5 of their differentiation with M-CSF by flow cytometry. Data are representative of 3 independent experiments. ***p<0.001 (ANOVA, post-hoc Tukey test) at 1–5 days compared to untreated control cells. **C-F**. RAW264.7 cells were stably transfected with shRNA against BVR-A (shRNA BVR-A) or shRNA control (Control). Gene (**C**) and protein expression (**D**) of BVR were analyzed using RT-PCR and western blot, respectively. Results are representative of three independent experiments (n = 3/group); **p<0.01, *p<0.05 (Mann-Whitney test); control *vs* shRNA BVR-A. **E-F.** Gene expression (**E**) and cell surface expression (**F**) of C5aR1 were measured by RT-PCR and flow cytometry, respectively, in RAW264.7 shRNA-control and shRNA-BVR-A cells. The data are representative of three independent experiments (n = 3/group). ***p<0.001 (Student T test; p=0.1 using Mann-Whitney test) control *vs* shRNA BVR-A.

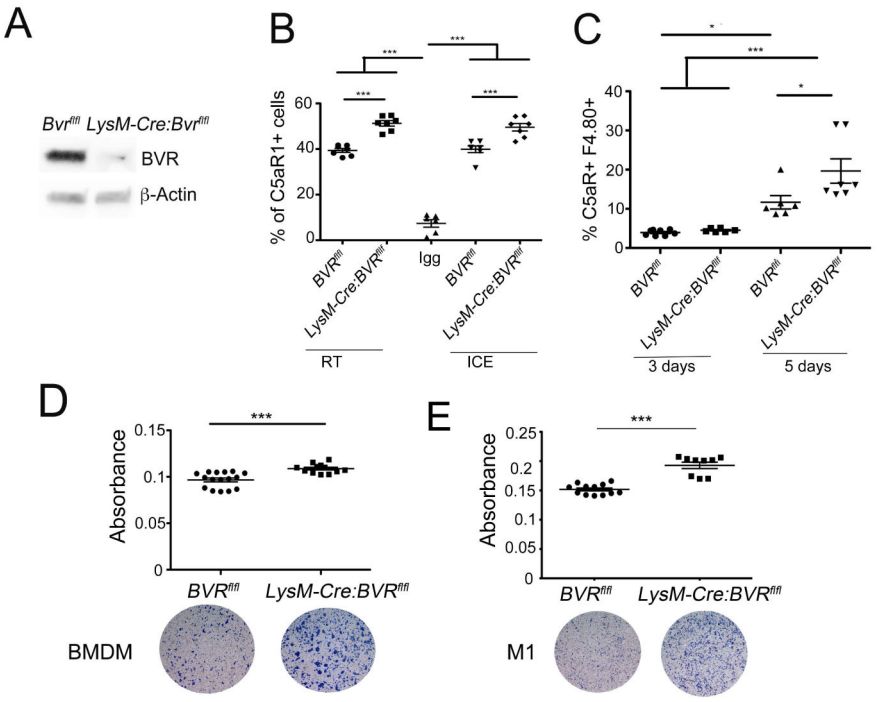

Figure 2. BMDM isolated from *LysM-Cre:BVR$^{fl/fl}$* mice are characterized by increased C5aR1 levels and chemotaxis towards C5a.

A. The deletion of BVR in BMDM isolated from *LysM-Cre:BVR$^{fl/fl}$* vs *BVR$^{fl/fl}$* mice was evaluated by western blot. **B-C**. Surface expression of C5aR1 was assessed by flow cytometry in BMDM from *BVR$^{fl/fl}$ and LysM-Cre:BVR$^{fl/fl}$*. % of C5aR+ cells is shown with results representative for n=4–6 mice per group. Staining was performed either at room temperature (RT) or on ice (ICE). The kinetics of C5aR1 expression at 3 and 5 days is shown in C. ***p<0.001, *p<0.05 (ANOVA, Tukey post-hoc); *LysM-Cre:BVR$^{fl/fl}$* vs *BVR$^{fl/fl}$*. **D-E**. Representative images and absorbance of unpolarized (**D**) or M1-polarized (**E**) BMDM from *BVR$^{fl/fl}$* and *LysM-Cre:BVR$^{fl/fl}$* mice that migrated through to the lower part of transwell chamber in response to C5a after 24 hours culture in serum free media. Results are representative of three independent experiments (n = 3/group). ***p<0.05 (Mann-Whitney test), *LysM-Cre:BVR$^{fl/fl}$* vs *BVR$^{fl/fl}$*.

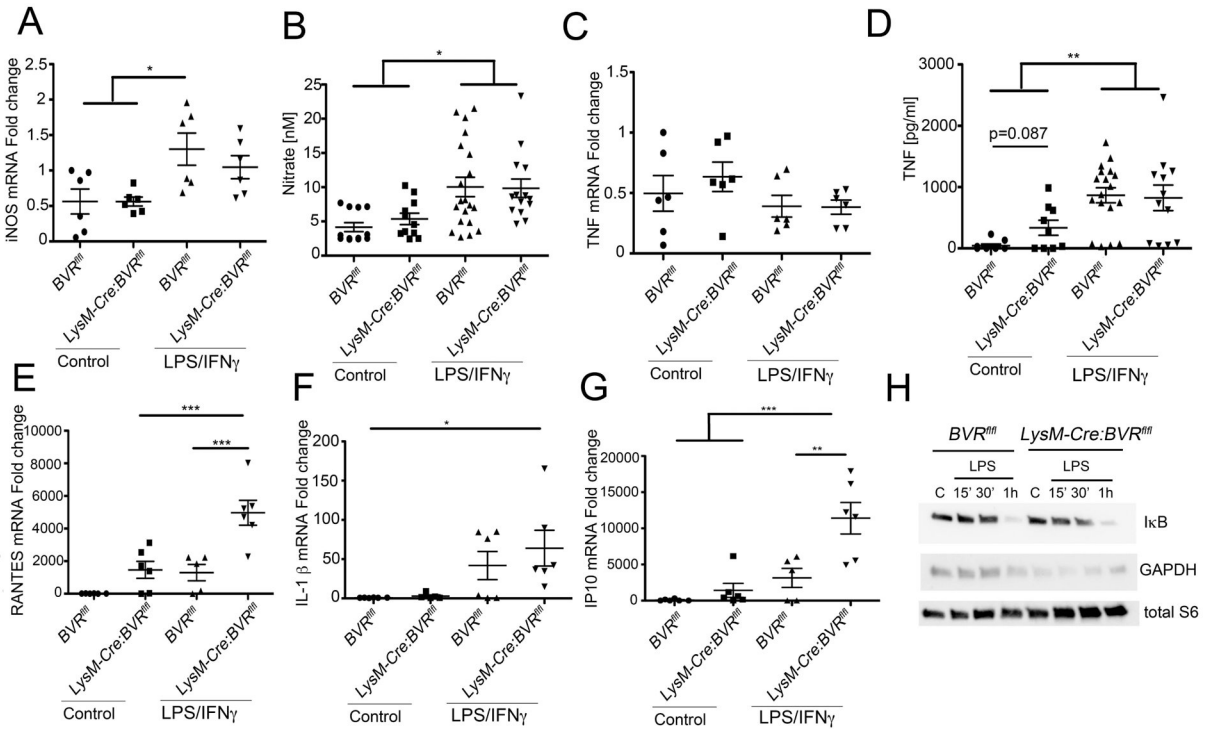

Figure 3. Induction of RANTES, IP-10 and IL1β expression in M1-polarized BMDM from *LysM-Cre:BVR$^{fl/fl}$* mice.

A, C, E-G. BMDM isolated from *LysM-Cre:BVR$^{fl/fl}$* or *BVR$^{fl/fl}$* mice were incubated in the presence or absence of LPS/IFNγ (M1) for 24 hours. Expression of iNOS (**A**), TNF (**C**), RANTES (**E**), IL-1β (**F**) and IP-10 (**G**) was measured by RT-PCR. Data are representative of three independent experiments (n = 4–6 per group). *p<0.05, **p<0.01, ***p<0.001 (ANOVA with Tukey post-hoc test). **B**. Nitrate levels were measured in the supernatants of BMDM from *BVR$^{fl/fl}$* and *LysM-Cre:BVR$^{fl/fl}$* mice incubated with LPS/IFNγ for 24 hours. *p<0.05 (ANOVA with Tukey post-hoc test) LPS/IFNγ-treated versus controls. n=5–6 per group. **D.** ELISA was applied to measure TNF levels in the supernatant of BMDM from *BVR$^{fl/fl}$* and *LysM-Cre:BVR$^{fl/fl}$* mice incubated with LPS/IFN-γ for 24 hours. Data are representative of three independent experiments (n = 3/group). ** *p*<0.01 Control *versus* LPS. **H**. Immunoblot with antibodies against IκB and GAPDH of the lysates of BMDM treated with LPS for 15'−1h. Data are representative for 2–3 independent experiments.

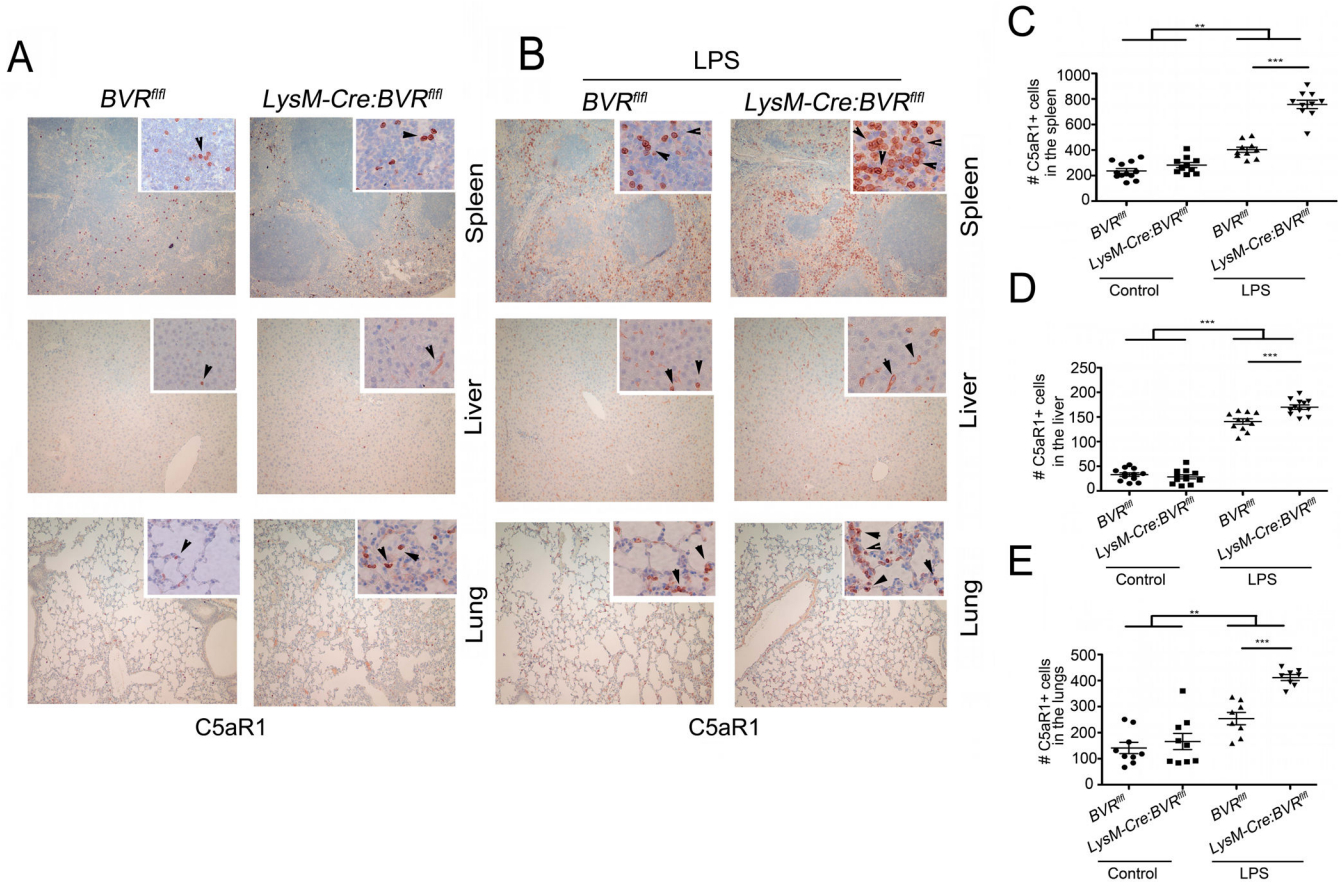

Figure 4. C5aR1-expressing cells are increased in *LysM-Cre:BVR$^{fl/fl}$* mice basally and in response to LPS.

A-B. *BVR$^{fl/fl}$* and *LysM-Cre:BVR$^{fl/fl}$* mice were treated with LPS (5 mg/kg, *i.p.*) and livers, lungs and spleens were harvested at 24 hours. C5aR1 expression was analyzed by immunohistochemistry. Representative pictures of migration assay are shown in **A-B**. Pictures were taken at 100 × magnification and insets at 400x. **C-E**. Semi-quantitative analysis for C5aR1 positive cells in multiple fields of view (n=4–6 mice per group, 2–3 sections per group) is shown. **p<0.05, ***p<0.001 (ANOVA, Tukey post-hoc test), *LysM-Cre:BVR$^{fl/fl}$* vs *BVR$^{fl/fl}$* mice.

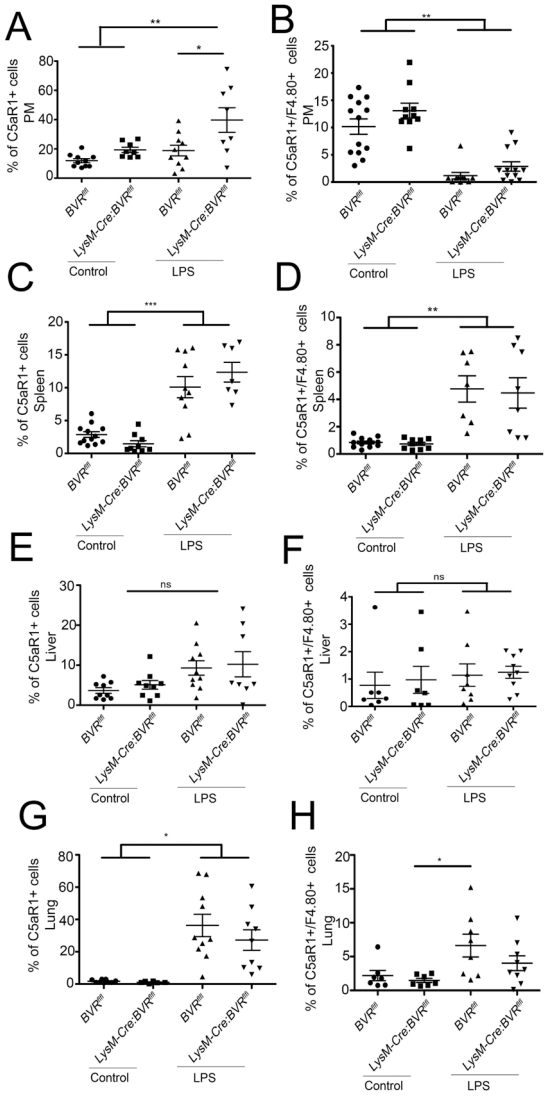

Figure 5: Lack of BVR promotes C5aR1 expression in peritoneal infiltrates in *LysM-Cre:BVR$^{fl/fl}$* in response to LPS. A-B.

Peritoneal cells were isolated from *BVR$^{fl/fl}$* and *LysM-Cre:BVR$^{fl/fl}$* mice 24 hours after LPS injection. The number of C5aR1+ cells was measured by flow cytometry in the total and F4.80+ cell populations. Results are expressed as average of n=6–10 mice per group. *p<0.05, **p<0.01, ***p<0.001 (ANOVA, Tukey post-hoc test). C-H. The flow cytometry analyses of C5aR1+ cells and C5aR1+/F4.80+ cells in the populations isolated from spleens (C-D), livers (E-F) or lungs (G-H) of *LysM-Cre:BVR$^{fl/fl}$* and *BVR$^{fl/fl}$* mice. Data are representative for n=6–10 mice. *p<0.0, **p<0.01, ***p<0.001 (ANOVA, Tukey post-hoc test).

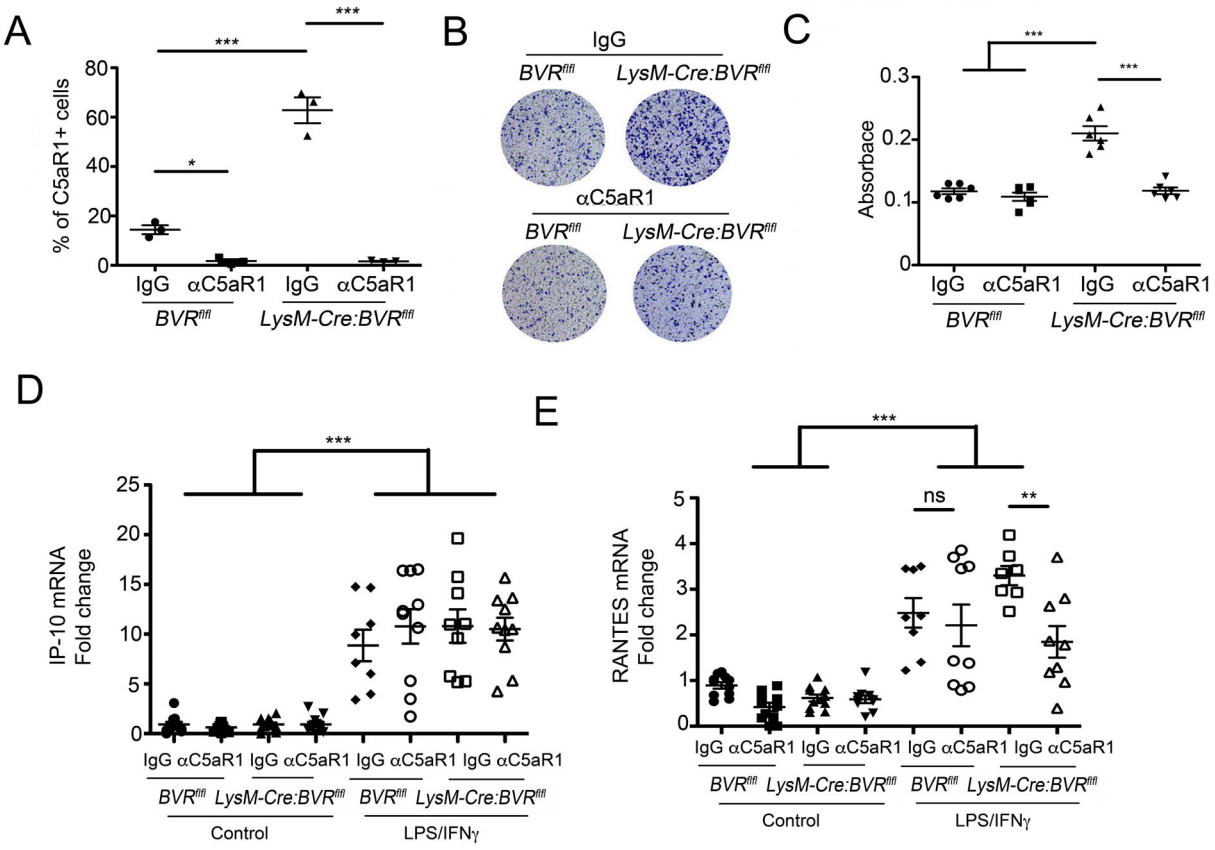

Figure 6. BVR effects on chemotaxis and chemokine expression are in part dependent on C5aR1.

A. BMDM from *BVR*$^{fl/fl}$ and *LysM-Cre:BVR*$^{fl/fl}$ mice incubated with anti-mouse IgG or C5aR1 for 30 min and cell surface expression of C5aR1 was analysed by flow cytometry. Results are representative of three independent experiments (n = 3/group). *p<0.05, ***p<0.001 (ANOVA, post-hoc Tukey test). B-**C.** Representative images (**B**) and absorbance of BMDM (**C**) from *BVR*$^{fl/fl}$ and *LysM-Cre:BVR*$^{fl/fl}$ mice that migrated through to the lower part of transwell chamber in response to C5a after 24 hours incubation in the presence or absence of anti-mouse IgG or anti-mouse C5aR1. Data are expressed mean ± S.E. of three independent experiments (n = 3/group). ***p<0.001 (ANOVA, post-hoc Tukey test). **D-E**. BMDM isolated from *LysM-Cre:BVR*$^{fl/fl}$ or *BVR*$^{fl/fl}$ mice were incubated with anti-mouse IgG or C5aR1 for 30 min and then LPS/IFNγ (M1) was added for 24 hours. Expression of IP-10 (**D**), RANTES (**E**) was measure by RT-PCR. Data are representative of three independent experiments (n = 4–6 mice per group). *p<0.05, **p<0.01 (ANOVA with Tukey post-hoc test).