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The Novel Activated Microglia/Macrophage WAP Domain Protein, AMWAP, Acts as a Counter-Regulator of Proinflammatory Response

Marcus Karlstetter,* Yana Walczak,* Karin Weigelt,*,† Stefanie Ebert,* Jan Van den Brulle,‡ Heinz Schwer,‡ Rudolf Fuchshofer,§ and Thomas Langmann*

Microgliosis is a common phenomenon in neurodegenerative disorders, including retinal dystrophies. To identify candidate genes involved in microglial activation, we used DNA-microarray analysis of retinal microglia from wild-type and retinoschisin-deficient (Rs1h<sup>−/−</sup>) mice, a prototypic model for inherited retinal degeneration. Thereby, we cloned a novel 76 aa protein encoding a microglia/macrophage-restricted whey acidic protein (WAP) termed activated microglia/macrophage WAP domain protein (AMWAP). The gene consists of three exons and is located on mouse chromosome 11 in proximity to a chemokine gene cluster. mRNA expression of AMWAP was detected in microglia from Rs1h<sup>−/−</sup> retinas, brain microglia, and other tissue macrophages. AMWAP transcription was rapidly induced in BV-2 microglia upon stimulation with multiple TLR ligands and IFN-γ. The TLR-dependent expression of AMWAP was dependent on NF-κB, whereas its microglia/macrophage-specific transcription was regulated by PU.1. Functional characterization showed that AMWAP overexpression reduced the proinflammatory cytokines IL-6 and IL-1β and concomitantly increased expression of the alternative activation markers arginase 1 and Cd206. Conversely, small interfering RNA knockdown of AMWAP lead to higher IL-6, IL-1β, and Ccl2 transcript levels, whereas diminishing arginase 1 and Cd206 expression. Moreover, AMWAP expressing cells had less migratory capacity and showed increased adhesion in a trypsin-protection assay indicating antiserine protease activity. In agreement with findings from other WAP proteins, micro-molar concentrations of recombinant AMWAP exhibited significant growth inhibitory activity against a trypsin-protection assay indicating antiserine protease activity. In agreement with findings from other WAP proteins, micro-molar concentrations of recombinant AMWAP exhibited significant growth inhibitory activity against a trypsin-protection assay indicating antiserine protease activity.

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icroglial cells, the resident phagocyte population of the nervous system, exert several important functions in immune surveillance (1, 2) and neuronal homeostasis (3, 4). In the healthy brain and the retina, ramified microglia serve as highly motile patrolling cells constantly surveying their microenvironment (5). There is an ongoing debate whether resident microglia in the CNS and the retina are replenished by in situ proliferation and/or recruitment of myeloid cells from the bloodstream (6–8). Accumulating evidence now suggests that experimental confounds including alterations of the blood-retina barrier could have biased earlier results from bone marrow chimeras (9).

Thus, parabiosis studies demonstrated that microglia in the healthy or experimentally damaged brain are poorly replenished from the bloodstream (10). Moreover, Mildner et al. (11) showed that irradiation is essential for infiltration of circulating microglial precursors into the brain in chimerism experiments.

Microglia communicate with other glial cells and neurons that regulate their activation status, their capacity for phagocytosis of cellular debris, and secretion of neurotrophic factors (12). Key regulatory signals from neurons and glia are transmitted to microglia cells through various soluble factors, including nucleotides (13), the chemokine fractalkine (14), TGF-β (15), and nerve growth factor (16). Control of microglial activation and function is further regulated by direct neuron-microglia crosstalk, including Cd200/Cd200 receptor interaction (17) and complex formation of triggering receptors expressed on myeloid cells with different ligands on neurons (18).

Activated microglia can elicit both protective and deleterious functions. In the early phase of neurodegeneration, microglia evoked potent tissue remodeling programs (4) and initiated repair mechanisms, such as glial scar formation (19). However, excessive or prolonged microglial activation in the CNS and the retina can lead to chronic inflammation with severe pathological side effects often resulting in irreversible neuronal loss (2, 20, 21). In eye research, various animal models have demonstrated that microglial activation is tightly associated with and mostly precedes retinal degeneration and photoreceptor apoptosis (22–24). Functional and phenotypic characterization of microglia populations in the diseased retina could be of high relevance to reveal signaling events that trigger their activation. However, like other tissue macrophages (25), act-
activated microglia comprised a continuum of diverse functional phenotypes with a broad spectrum of activation markers (26).

Our laboratory has used large-scale transcriptional phenotyping to identify characteristic gene signatures of LPS and chondroitin sulfate proteoglycan-disaccharide (CSPG-DS) stimulated BV-2 microglia as clearly polarized cell populations (27). Moreover, we have profiled primary retinal microglia from wild-type (wt) and retinoschisin-deficient (Rs1h<sup>-/-</sup>) mice (28), a prototypic model for rapid retinal apoptosis and degeneration (29). In contrast to LPS- and CSPG-DS–treated microglia, expression profiles of retinal Rs1h<sup>-/-</sup> microglia indicated overlapping transcript clusters reminiscent of both pro- and anti-inflammatory macrophage activation (28, 30, 31). Finally, several transcripts previously not linked to microglial activation have been identified in these genome-wide expression studies, including high levels of the adaptor protein STAP-1 (32) and the uncharacterized whey acidic protein (WAP) motif-bearing protein AMWAP.

WAP domain proteins have originally been described as low m.w. proteins with “defensin-like” properties involved in immune homeostasis (33). Their counter-regulatory role on inflammatory mediators is mainly ascribed to the antiprotease and antimicrobial activities of the WAP domain (34–36). This 40–50 aa motif contains eight conserved cysteins that form four defined disulfide bonds. Secretory leukocyte protease inhibitor (SLPI) and elafin are the best studied WAP proteins in humans and mice (37). SLPI is constitutively produced at many mucosal surfaces and is also produced by lung epithelial cells, neutrophils, and macrophages (38). SLPI expression in macrophages is induced by bacterial endotoxin leading to the suppression of NO and TNF secretion (39). Elafin is mainly present in epithelia of the skin, oral cavity, vagina, and lung to fulfill distinct antimicrobial and immunomodulatory functions (40).

In this study, we identified a novel microglia/macrophage-restricted WAP domain protein, AMWAP, in activated primary retinal microglia. AMWAP expression is rapidly induced by ligands for TLR2, -4, and -9 and IFN-γ in the BV-2 cell line. AMWAP overexpression reduces proinflammatory cytokine expression and concurrently induces markers for alternative macrophage activation. We therefore propose that AMWAP is a novel modulator of microglial activation in neurodegenerative disorders.

Materials and Methods

Animals

Retinoschisin knockout (Rs1h<sup>-/-</sup>) mice have been described previously (29) and C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Mice were kept in an air-conditioned barrier environment at constant temperature of 20°C–22°C on a 12-h light-dark schedule, and had free access to food and water. The health of the animals was regularly monitored, and all procedures were approved by the University of Regensburg animal rights committee and complied with the German Law on Animal Protection and the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals, 1999.

Isolation and culture of primary cells

Retinal microglia were isolated and cultured as described earlier (28). Briefly, retinal tissue from wt and Rs1h<sup>-/-</sup> mice at postnatal days 14 and 18 was isolated from eye bulbs and purified from contaminating vitreous body and retinal pigment epithelium/choriocapillaris. Pools of four to ten retinas each were cut into small pieces and incubated for 40 min at 37°C in 1 ml PBS with 1 mg/ml collagenase type I (Sigma-Aldrich, St. Louis, MO), 0.3 mg/ml DNase I (Roche, Basel, Switzerland), and 0.2 mg/ml hyaluronidase (Sigma-Aldrich). The cell suspension was filtered through a 70-μm cell strainer (Becton Dickinson, Heidelberg, Germany). Cells were washed twice with 10 ml DMEM/10% FCS and finally subjected to Ficoll density gradient centrifugation. Cell pellets were collected after 20 min at 2000 rpm (690 g, with a break of 5 min) in a Heracell centrifuge for the isolation of mononuclear cells. The interphase was carefully removed and washed with 10 ml DMEM/FCS. The cells were cultured for 11 d in 75-cm<sup>2</sup> flasks containing DMEM/10% FCS supplemented with 50 ng/ml recombinant human M-CSF (R&D Systems, Minneapolis, MN) and phase-contrast micrographs were taken with a Nikon Eclipse TE2000-S microscope.

Brain microglia were isolated from the brains of 4-wk-old wt mice. Each brain was dissected, cut into small pieces in 520 μl PBS, and incubated for 40 min at 37°C with vigorous shaking (800 rpm) containing the same dissolving solution described previously. The single-cell suspension was filtered through a 70-μm cell strainer (Becton Dickinson), washed twice with 10 ml DMEM/10% FCS, followed by centrifugation at 1600 rpm for 5 min at room temperature. The pellet was resuspended in 10 ml DMEM/10% FCS supplemented with 50 ng/ml recombinant human M-CSF (R&D Systems) and cultured in 75 cm<sup>2</sup> cell culture flasks. Nonadherent cells were removed after 4 d by washing with culture medium. After 11 d of culture, 100 ng/ml LPS was added where indicated and total RNA was isolated after 24 h of stimulation.

Bone marrow macrophages were isolated from bone marrow of adult wt animals. Femurs and tibias were dissected from the surrounding muscle tissue and both ends were cut. Bone marrow was flushed with 2 ml DMEM/10% FCS with a 27 G syringe and cell clumps were separated by pipetting. The cell suspension was centrifuged for 10 min at 1200 rpm at room temperature. The supernatant was discarded and the pellet was dissolved in 2 ml RBC lysis buffer (Sigma-Aldrich) and incubated for 7 min at room temperature. Then incubation was stopped with 5 ml DMEM/10% FCS, followed by centrifugation. The cell pellet was resuspended in 10 ml DMEM/10% FCS supplemented with 50 ng/ml recombinant M-CSF (R&D Systems). Cell culture medium was removed at days 4 and 8. After 10 d in culture, bone marrow macrophages were stimulated with 100 ng/ml LPS where indicated and total RNA was isolated after 24 h.

Dissected spleen tissue of adult wt animals was disintegrated by scissors in 520 μl PBS, passed through a wire mesh, and washed with 10 ml DMEM/10% FCS. Cells were filtered through a 40-μm cell strainer and processed as described previously for bone marrow macrophage isolation.

Immature bone marrow dendritic cells (DCs) were isolated as described previously (41). For maturation, DCs were collected after 10 d, and resuspended in culture medium containing 100 U/ml rmGM-CSF and 1 μg/ml LPS. Total RNA was collected after 24 h of maturation.

Cell lines and reagents

RAW264.7 cells were received from the American Type Culture Collection (Manassas, VA) and BV-2 cells were kindly provided by Professor Ralph Lucius (Clinic of Neurology, Christian Albrechts University, Kiel, Germany). RAW264.7 macrophages were kept in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. BV-2 cells were cultured in RPMI/5% FCS supplemented with 2 mM L-glutamine and 195 mM β-mercaptoethanol. Culture and treatment of tamoxifen-inducible PUER cells has been described elsewhere (42). IFN-γ, IL-1β, and cycloheximide (CHX) were purchased from Sigma-Aldrich. PAM3CSK4 was purchased from Invitrogen. The phosphorothioate CpG oligonucleotide (5′-tctaggtcgcttcgct-3′) and control oligonucleotide (5′-tctaggtcgcttcgc-3′) were synthesized by Metabion ( Martinsried, Germany). Caffeic acid phenethyl ester (CAPE) was purchased from Tocris Bioscience (Ellisville, MO). For activation experiments, cells were treated with LPS, IFN-γ, and CHX where indicated. LPS, IFN-γ, and the 5′-RLM-RACE primer 9TTC TTT GCA GAC ATG ACC ACA GC-3 and the inner 5′-RLM-RACE primer 9TCC CATG ACAC ATG TCC-3 were synthesized by Metabion (Martinsried, Germany). Immature bone marrow dendritic cells (DCs) were cultured for 7 d in culture medium containing 100 U/ml rmGM-CSF and 1 μg/ml LPS. Total RNA was collected after 24 h of maturation.

RNA isolation, reverse transcription, and 5′-RLM-RACE

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). The purity and integrity of the RNA was assessed on the Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip reagent kit (Agilent Technologies, Palo Alto, CA). The RNA was quantified spectrophotometrically and stored at −80°C. cDNAs were generated using the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, St. Leon-Rot, Germany).

To identify and clone full-length AMWAP transcripts, 5′ RNLigase mediated RACE-PCR was carried out using RNA from RAW264.7 cells stimulated with 100 ng/ml LPS for 4 h. cDNA was prepared from four to ten 4-h stimulation cDNA samples and the 5′ RLM-RACE kit according to the manufacturer’s instructions. AMWAP-specific primer pairs were designed on the incomplete database entry Mm.24097. The first 5′ RLM-RACE PCR was carried out with the AMWAP-specific reverse primer 5′-GCG GAG CAT GAC CCT-3′ and the 5′ RACE outer primer 5′-GCT GAT GGC CAT GAA TCA ATG CTC-3′. The nested 5′ RLM-RACE PCR was conducted with the AMWAP-specific reverse primer 5′-TGT TGC GAT GAC ATC AGC TTT-3′ and the 5′-CCG GGA TCC GAA CAC TGC GGT TGC TGG CTT CTA TGC-3′ PCR products were analyzed on 2% agarose gels and individual bands were extracted, cloned into the pCR2.1 Topo TA vector (Invitrogen, Carlsbad, CA).
Quantitative real-time RT-PCR

Relative transcript levels were assessed by amplifications of 50 ng cDNA in a 7900 HT real-time PCR detection system (Invitrogen Life Technologies, San Diego, CA). The 20 μl reaction volumes contained 1× TaqMan Gene Expression Master Mix (Invitrogen Life Technologies), 200 nM primers (Supplemental Table I) and 0.25 μl dual-labeled probe (Roche Universal Probe Library). The PCR reaction parameters were as follows: as 2 min 50˚C hold, 30 min 60˚C hold, and 5 min 95˚C hold, followed by 45 cycles of 20 s 94˚C melt and 1 min 60˚C anneal/extension. The results were analyzed using the ΔΔCt method for relative quantification (43).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments of RAW264.7 cells coupled to Affymetrix Promoter Arrays have been described elsewhere (44). For ChIP-PCR with microgiglia, 10 million BV-2 cells were treated with 1% formaldehyde for 15 min and lysed with SDS, Empigen, and NP-40 (supplemented with 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). The nuclear pellet was homogenized by sonication twice at 325 pulse amplitude for 10 s. Immunoprecipitation was performed on the lysate with 2.5 μg of anti–PU.1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) anti–di-acetylated histone H3 (Upstate Biotechnology, Lake Placid, NY), anti–p300 clone RW128 (Upstate Biotechnology), anti–CBP (Upstate Biotechnology), or anti–IgG Ab (Santa Cruz Biotechnology). After washing and elution steps, cross-links were reversed at 65˚C overnight. The immunoprecipitated DNA was purified using the QIAquick PCR purification kit (Qiagen) and analyzed by PCR using the forward primer 5′-CTT GCC TCT GAG GAA AAA GGA ACC TGG TTG-3′ and the reverse primer 5′-CCC AAG CTT AAA CAC CGG GGT TTT GC-3′ specific for the AMWAP proximal promoter region —114/+68.

Overexpression of AMWAP in BV-2 cells

The full-length coding sequence of mouse AMWAP was RT-PCR amplified from RAW264.7 RNA with primer pair forward 5′-CCC AGT CAG CCA CA-3′ and reverse 5′-CCC CTC GAG CA T GCT ACA GAA-3′. PCR products were sequenced and cloned into the HindIII/XhoI site of a pCEP1.4 vector inframe with a C-terminal rhodopsin (Rho)-1D4 tag and analyzed by PCR using the forward primer 5′-CTC CTC GAG CA T GCT ACA GAA-3′ and reverse primer 5′-CCC GAT ATC TAA AGA CAG GAG TTT TGC AGA C-3′. A fusion protein of milk fat globule protein 8 with a C-terminal Rho-1D4 tag (pMFG-1D4) was obtained from the 5′-CCC CAT ATG ACC TAT GTG GTG TCC TGT CC-3′ and antisense primer 5′-CCC AAG CTT AAA CAC CGG GGT TTT GC-3′.

Trypsin protease protection assay

Stably pAMWAP-GFP and pGFP expressing BV-2 cells were grown in 24-well plates at 80% confluent monolayers and were wounded with a sterile pipette tip. Cell migration into the open scar was documented with microphotographs at time points 0 h and 24 h after wounding. As an independent method, the Costar Transwell System (8-μm pore size polycarbonate membrane) was used to evaluate cell migration. BV-2 cells (1 × 107 cells in 1.5 ml serum-free medium) were added to the upper well, and 2.6 ml serum-free medium was added to the lower chamber. A total of 50 ng/ml LPS or ethanol as solvent control were added to the lower chamber medium. At the end of the 24 h incubation, cells on the top of the membrane were removed by swiping with a damp cotton swab, and cells that had migrated to the lower surface were fixed in methanol for 15 min at room temperature and stained with 1% crystal violet. The migration activity was quantified by counting the migrated cells on the lower surface of the membrane using light microscopy.

Cell luciferase reporter assay

The murine Ccl2 promoter (−988/+113) was PCR amplified from genomic DNA using the primers forward 5′-CCC CTC GAG CAT GCT ACA GAA AGC CCA AAA-3′ and reverse 5′-CCC AGT GAG TGC CCA GAA GCA GCA TGA CAG-3′. The PCR product was cut with restriction enzymes XhoI and BglII and cloned into the pGL4.10 vector (Promega, Madison, WI). BV-2 cells were cotransfected with the luciferase construct and a pTK-Hyg vector (Promega). Cells were harvested along with the pGL4.10 vector (Promega, Madison, WI). BV-2 cells were transfected with 2 μg of pAMWAP-GFP or pGFP vectors. Transfected cells were harvested after 24 h and luciferase activity was determined with the Luciferase assay system (Promega) on a FLUOstar Optima plate reader (BMG Labtech, Offenburg, Germany).

Bacterial expression and purification of recombinant AMWAP

The AMWAP cDNA sequence was codon-optimized and de novo synthesized for recombinant expression in Escherichia coli (Sloning Biotechnology, Puchheim, Germany), resulting in an increased codon adaptation index from 0.7 to 0.91 (Supplemental Fig. 1) (46). Signal-sequence lacking and codon-optimized AMWAP was PCR amplified from pShLO1.0-AMWAP with forward primer 5′-CCC CAT ATG ACC TAT GTG GTG TCC TGT CC-3′ and reverse primer 5′-CCC AAG CTT AAA CAC CGG GGT TTT GC-3′. The PCR product was inserted with NdeI and HincII into the 3′-terminal vector (Novagen, Madison, WI) inframe with a N-terminal His-tag sequence.

E. coli BL21α-DE3 cells transformed with pAMWAP-His were grown in 500 ml LB-medium until an OD600 of 0.7 was reached. Isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM and culture was continued at 37˚C for 4 h. Cells were harvested, the cell pellet was suspended in 7 ml lysis buffer (300 mM NaCl, 50 mM sodium phosphate, pH 8.0) containing 7 mg lysozyme, and the sample was incubated for 30 min at 37˚C. Cells were homogenized by sonication twice at 30% amplitude for 10 s. The crude lysate was centrifuged at 12,000 × g for 15 min. The supernatant was added to Protopro
Ni-TED Columns (Macherey-Nagel, Düren, Germany). Three fractions were eluted in buffer containing 250 mM imidazole and concentrated in Amicon Ultra-4 Ultracel 5 kDa tubes (Millipore, Bedford, MA) by centrifugation. The protein was dialyzed four times against PBS, the concentration of each fraction was determined by Bradford assay and purified recombinant protein was stored at −80°C.

Antimicrobial assay

The bacterial strains E.coli (ATCC25922), Pseudomonas aeruginosa (ATCC27853), and Bacillus subtilis (ATCC60633) were grown in LB medium at 37°C to an OD_{600} of 1.0. Bacteria were then diluted in MT-LB medium (16 mM disodium hydrogen phosphate, 5 mM sodium dihydrogen phosphate, 150 mM sodium chloride, 5 mM sodium phosphate, 150 mM sodium chloride, and 1% LB medium) to a concentration of 1 × 10^8 CFU/ml. The 50 μl bacterial suspensions were incubated with PBS as control or 10 μM, 20 μM, or 30 μM AMWAP-His protein for 2 h at 37°C. Subsequently, appropriate serial dilutions were plated on LB agar plates, incubated overnight at 37°C and CFUs were determined.

Statistical analyses

The Student t test or Mann-Whitney U test were used for the comparison of experimental groups as indicated. p < 0.05 was considered significant.

Microarray datasets used in this study

The microarray datasets cited in this study are available at the National Center for Biotechnology Information Gene Expression Omnibus as series records GSE5581 (Rs1h^{−/−} versus wt retinas), GSE9011 (PU.1 ChIP-Chip), and GSE13125 (PUER cells) at (www.ncbi.nlm.nih.gov/geo/).

Results

AMWAP is strongly induced in activated retinal microglia

To identify novel genes involved in the activation of microglia, we have previously performed DNA-microarray analysis of isolated retinal microglia from degenerating Rs1h^{−/−} and wt retinas (24, 28, 30). Nine previously uncharacterized transcripts showed a significantly different expression pattern in activated Rs1h^{−/−} versus nonactivated wt microglia (data not shown). Among these, a more than 7-fold induction of a predicted gene with the UniGene entry Mm.24097 was detected in activated retinal microglia. In this study, we confirmed these microarray findings by quantitative real-time RT-PCR assays with RNA samples from independently isolated retinal microglia from Rs1h^{−/−} and wt mice. AMWAP transcript levels were increased in microglia from postnatal day 14 (P14) Rs1h^{−/−} retinas and were further substantially induced in P18 Rs1h^{−/−} microglia (Fig. 1A). We have previously shown that microglia activation starts at P14 in the retina of Rs1h^{−/−} mice, a time point where neuronal apoptosis and degeneration is not yet evident (24).

For a more precise refinement of the temporal expression profile of AMWAP in relation to the inflammatory process, mRNA levels were quantified in retinal tissue of postnatal stages (P)11, 12, 14, 18, 21, 24, and 28 (Fig. 1B). The highest AMWAP expression was noted...
at P18 and P21 and thereafter declined to intermediate levels (Fig. 1B). The AMWAP transcript profile is in good accordance with the time kinetics of the known early activation markers lysozyme (Lyzs) (Fig. 1C) and secreted phosphoprotein 1 (SPP1, alias osteopontin) (Fig. 1D). This implicates that high AMWAP expression is already present at the very early stage of retinal microglial activation and declines in the chronic and resolution phase of neuroinflammation. The deduced protein sequence contains a WAP domain and together with the data on its specific expression pattern, this gene was named AMWAP for activated microglia/Macrophage WAP domain protein.

**AMWAP is exclusively expressed in activated microglia and macrophages**

We next addressed the question whether AMWAP is also expressed in brain microglia and macrophages outside of the CNS. Primary brain microglia, spleen macrophages, bone marrow-derived macrophages, and bone marrow-derived DCs were isolated and stimulated with LPS to induce a proinflammatory state. AMWAP transcripts were already present in the four different types of macrophages but stimulation with LPS further upregulated AMWAP gene expression (Fig. 2). Examination of AMWAP transcript levels in public domain Affymetrix microarray data (BioGPS, http://biogps.gnf.org and http://macrophages.com) further revealed a macrophage-restricted expression of probeset 1436530_at, which specifically and exclusively targets the AMWAP mRNA (Supplemental Fig. 2). In the BioGPS database, high abundance of AMWAP transcripts was detected in microglia, bone marrow-derived macrophages, peritoneal macrophages, and osteoclasts, but not in any of the other studied mouse tissues or cell types (Supplemental Fig. 2). Consistent with our quantitative RT-PCR (qRT-PCR) data, these comprehensive expression profiles indicate that AMWAP is a microglia/macroage-specific transcript induced by proinflammatory activation.

**FIGURE 3.** AMWAP is upregulated by multiple TLR ligands and IFN-γ in BV-2 microglia in a time- and dose-dependent manner. Time-course (A, C, E, G) and dose-response (B, D, F, H) of BV-2 cells treated with the indicated concentrations for the given time periods of LPS (A, B), CpG oligonucleotides or control GG oligonucleotides (C, D), PAM3CSK4 (E, F), or IFN-γ (G, H). qRT-PCR was performed to determine AMWAP levels. Values represent the means ± SD of three independent cell cultures analyzed in triplicates. *p < 0.05; **p < 0.01; ***p < 0.001; Student t test.
Multiple TLR ligands and IFN-γ rapidly trigger AMWAP transcription in microglia

We next analyzed AMWAP expression and regulation in the BV-2 microglial cell line as an independent in vitro model system. AMWAP mRNA was present in BV-2 cells, but transcript levels were markedly elevated after proinflammatory stimulation with TLR ligands and IFN-γ (Fig. 3). Specifically, treatment of BV-2 cells with LPS as TLR4 ligand (Fig. 3A, 3B), CpG oligonucleotides as TLR9 ligand (Fig. 3C, 3D), PAM3SCK4 as TLR2 ligand (Fig. 3E, 3F), and IFN-γ (Fig. 3G, 3H) evenly upregulated AMWAP expression in a time- and dose-dependent manner. The shortest time of treatment (1 h) and the lowest concentration of each compound were sufficient to induce significant increases in AMWAP levels. These data suggest that AMWAP transcription is very sensitive to proinflammatory stimulation of BV-2 microglial cells.

We then examined whether AMWAP induction by TLR ligands depends on the transcription factor NF-κB. BV-2 cells were pretreated with the specific NF-κB inhibitor CAPE before exposure to LPS, PAM3SCK4, or CpG oligonucleotides. CAPE completely prevented TLR-dependent AMWAP upregulation in all three stimulatory conditions (Fig. 4A), indicating that NF-κB is absolutely required for AMWAP induction. Next, we pretreated BV-2 cells with CHX, a protein synthesis inhibitor, to study whether newly synthesized proteins may be involved in this process. We found that TLR-dependent expression of AMWAP was diminished by CHX pretreatment (Fig. 4B). This suggests that new protein synthesis is necessary and that additional factors may be relevant for AMWAP induction by TLR ligands. As a positive control, the LPS-induced expression of IL-1β was critically dependent on NF-κB, but required no protein synthesis (Fig. 4C).

Cloning and genomic structure of AMWAP

The previous database entry of Mm.24097 (GenBank Accession no. BC089618) was complete at the 5′-end including the polyA tail, but contained only nine nucleotides upstream of the start codon. We therefore performed 5′-RLM-RACE with RNA from LPS-stimulated RAW264.7 cells (Fig. 5A). Three major 5′-RLM-RACE-PCR products were amplified and sequencing after cloning of the individual products identified four different transcripts with the longest variant extending 68 bp further upstream (Fig. 5A). The 526-bp full-length AMWAP cDNA encodes for a 76 aa polypeptide. After predicted cleavage of the 19 aa signal sequence, the mature AMWAP polypeptide should consist of a 58 aa single WAP motif (Fig. 5B). BLAST analysis against DNA databases resulted in the identification of the AMWAP gene on mouse chromosome 11, consisting of three exons and two introns (Fig. 5C). The N-terminal signal peptide is encoded by exon 1, whereas exon 2 completely codes for the C-terminal WAP domain with eight cysteines presumably forming four disulfide bonds (Fig. 5C). The four-disulfide core domain and its genomic structure as single exon are characteristic features of all WAP proteins (47).

Microglia/macrophage-specific AMWAP expression depends on PU.1 binding to its proximal promoter

Because of the microglia/macrophage-restricted expression of AMWAP, we sought to investigate the relevant cis-regulatory elements. A comparison of mouse and rat AMWAP promoter sequences and their closest rodent homologs Exp1 revealed a highly conserved TATA-box, proximal PU.1 sites, a STAT motif and an overlapping IRF/PU.1 sequence (Fig. 6A). Our information from 5′-RLM-RACE identified the existence of four alternative transcription start sites (Fig. 6A, arrows). Because the longest AMWAP transcript includes the TATA-motif and extends 40 bp upstream, a nearby PU.1 site could function in the formation of the transcription preinitiation complex and thus regulate AMWAP gene activity (Fig. 6A).
AMWAP promoter region harboring the conserved PU.1 site was specifically enriched in PU.1-precipitated DNA compared with the IgG control (Fig. 6C, upper panel). Moreover, ChIP-PCR products were obtained with precipitated DNA from Abs directed against the transcriptional coactivators p300 and Cbp (Fig. 6C, lower panel), factors known to be involved in chromatin relaxation via histone acetyltransferase activity and RNA polymerase II recruitment to transcription start sites. The 2114/+68 AMWAP promoter DNA was also enriched in anti-AcH3 Ab precipitated DNA (Fig. 6C, lower panel), indicating open chromatin and active AMWAP gene expression in BV-2 cells.

We then aimed to validate the microarray findings of increased AMWAP mRNA expression in differentiated PUER cells by real-time qRT-PCR. In this tuneable system the PU.1-estrogen receptor binding domain fusion protein PUER is conditionally activated in myeloid progenitors upon addition of 4-hydroxy-tamoxifen (OHT), leading to rapid cell cycle arrest and differentiation into macrophages (42, 48). qRT-PCR analysis of independent RNAs from a time series of OHT-treated PUER cells showed very low AMWAP mRNA levels in PU.1−/− progenitors and a steep increase of AMWAP transcripts in OHT-differentiated cells (Fig. 6D). These data strongly support the hypothesis that AMWAP expression in macrophages as well as in microglia is critically dependent on PU.1 binding to its proximal promoter region.

FIGURE 5. AMWAP full-length cDNA, genomic structure, and protein domains. A. Distinct 5′-RLM-RACE-PCR products generated with template cDNA from LPS-activated RAW264.7 cells and a reverse primer located in the second exon. Specific bands are indicated by arrows and the asterisk denotes a double band. B. Full-length AMWAP cDNA sequence deposited in GenBank under Accession no. FJ007372. The open reading frame and translated amino acids are given in bold letters. The potential signal sequence is shaded in light gray and the WAP domain is marked in dark gray. The position of the stop codon is indicated by an asterisk and the polyadenylation signal is underlined. C. Genomic organization of the AMWAP gene (top), predicted protein domains (middle panel), and eight cysteine-residues forming four putative disulfide bonds (bottom).

AMWAP reduces proinflammatory gene expression and triggers alternative activation markers in microglia

To further investigate the immunological functions of AMWAP in microglia cells, the full-length open reading frame was overexpressed in BV-2 cells. A C-terminally 1D4-Rho-epitope-tagged variant of AMWAP was transiently expressed in BV-2 cells. The Rho epitope is not endogenously expressed in BV-2 cells, as shown by the absence of a Western blot band using a monoclonal anti-1D4 Ab (Fig. 7A). A specific band of 10 kDa was detected in cells transfected with the pAMWAP-1D4 construct, demonstrating successful overexpression of AMWAP in BV-2 cells (Fig. 7A). A milk fat globule protein-1D4 fusion protein was used as positive control and produced the expected band size of 15 kDa (Fig. 7A). To verify these findings and to visualize AMWAP within cells, BV-2 transformants expressing AMWAP-GFP or only GFP as control were generated. GFP-immunofluorescence
confirmed that recombinant AMWAP was expressed in BV-2 cells (Fig. 7B). AMWAP-GFP was localized in perinuclear structures and in some cells in dome-shaped structures with a polarity that resembled the Golgi apparatus (Fig. 7B, arrows in left panel). In contrast, GFP alone was broadly expressed in the cytoplasmic region (Fig. 7B, right panel). This finding is consistent with proteins that are processed through the endoplasmic reticulum and Golgi for extracellular transport.

Because AMWAP transcription was regulated by proinflammatory activation and the related WAP protein SLPI modifies inflammatory responses (49, 50), the effect of AMWAP on macrophage/microglia activation marker expression was studied. The immune-regulatory potential of AMWAP was addressed under basal and LPS-treated conditions. Enhanced AMWAP-GFP expression in nonstimulated and LPS-treated BV-2 cells reduced transcripts for IL1-β, IL-6, and Ccl2, three typical markers for proinflammatory activation of macrophages (Fig. 7C–E). In the same cells, the alternative macrophage activation markers arginase 1 and Cd206 levels as alternative activation markers (F, G) in AMWAP-GFP versus GFP overexpressing BV-2 cells under basal and LPS-stimulated conditions. H, Luciferase assays of BV-2 microglia containing a stable Ccl2-promoter–luciferase-construct. BV-2 cells stably expressing the Ccl2 reporter were transiently transfected with either AMWAP-GFP or GFP control vector. Twenty-four hours after transfection, luciferase activity was determined. Bar graphs represent means ± SD of three independent cell cultures analyzed in triplicates. *p < 0.05; **p < 0.01; Student t test.

We next studied the role of physiological AMWAP levels in the modulation of microglial activation. Knockdown experiments with specific AMWAP siRNA or control siRNA were performed and marker transcripts were determined in transiently transfected BV-2 cells. A 30% knockdown of AMWAP expression was achieved in both the basal and LPS-activated state (Fig. 8A). AMWAP silencing resulted in significantly derepressed transcript levels of IL1-β, IL-6, and Ccl2 (Fig. 8B–D) and markedly reduced arginase 1 and Cd206 expression (Fig. 8E, 8F). These data suggest that AMWAP acts as negative regulator of proinflammatory gene expression and may support alternative activation of microglial cells.

AMWAP blocks microglial migration and acts as serine-protease inhibitor

To assess whether AMWAP influences the motility of microglia as a functional parameter, in vitro wound-healing assays and transwell migration experiments were performed. A cell-free zone was created in confluent monolayers of stably transfected BV-2 cells by scraping cells off with a pipette tip. The repopulation of the cell-free zone was then monitored by microscopy. In GFP control cells, migration into the wounded area was completed after 24 h (Fig. 9A, left panels). In contrast, AMWAP-GFP expressing cells showed a clearly reduced capacity to repopulate the cleared area, indicating diminished migratory potential (Fig. 9A, right panels).

To rule out the possibility that the reduced microglial migration in the scratch assays was due to different proliferation rates of
stably transfected BV-2 cells, we tested their LPS-induced chemotactic migration in transwell chambers. In this system, stable AMWAP overexpression reduced the LPS-induced transwell migration of BV-2 cells to 50% compared with GFP expressing cells (Fig. 9B). In contrast, transient siRNA knockdown of AMWAP significantly promoted chemotactic migration of BV-2 microglial

![FIGURE 8. AMWAP knockdown leads to increased proinflammatory gene expression and impairs expression of alternative activation markers in BV-2 microglia. BV-2 cells were transiently transfected with 33 nM of each AMWAP-specific siRNA or nontargeting (scrambled) siRNA for 24 h. Thereafter, cells were left untreated or were further stimulated with 5 ng/ml LPS for 24 h. mRNA levels for AMWAP (A), IL-1β (B), IL-6 (C), Ccl2 (D), arginase 1 (E), and Cd206 (F) were determined by qRT-PCR. Bar graphs represent means ± SD of three independent cell cultures analyzed in triplicates. *p < 0.05; **p < 0.01; ***p < 0.001; Student t test.](http://www.jimmunol.org/)

![FIGURE 9. AMWAP attenuates microglial migration and exerts antiserine protease activity. A, Wound-healing assay in BV-2 microglia stably expressing AMWAP-GFP or GFP as control. AMWAP-GFP and GFP expressing BV-2 microglia were grown to comparable cell density, wounded with a pipette tip and microphotographs from wounded areas were taken immediately and after 24 h. B, Relative LPS-induced transwell chamber migration of stably transfected AMWAP-GFP versus GFP expressing BV-2 microglia. C, BV-2 microglia were transiently transfected with AMWAP-specific siRNA or nontargeting (scrambled) siRNA for 24 h. Thereafter, the lower wells of transwell chambers were treated with LPS for further 24 h and the absolute numbers of migrating cells were counted. D, Trypsin protease protection assay. Stable AMWAP-GFP and GFP expressing BV-2 microglia were treated with 0.25% trypsin for the indicated time points. Residing attached cells were stained with crystal violet, washed with PBS and photomicrographs were taken. E, Quantitative analysis of anti-protease activity in AMWAP-GFP and GFP expressing BV-2 microglia. Optical densities were normalized to untreated cells. Bars represent the percentage of adherent cells after different time points of trypsin incubation. Data are expressed as mean ± SD of duplicate measurements from three independent biological replicates. *p < 0.05; **p < 0.01; ***p < 0.001; Student t test.](http://www.jimmunol.org/)
cells compared with cells transfected with nontargeting control siRNA (Fig. 9C). These findings implicate that modulation of AMWAP expression levels in microglia directly influences their migration capacity.

Because SLPI and other WAP proteins are potent serine protease inhibitors (51), we investigated a similar function of AMWAP in a trypsin-protection assay. In this test system, the adhesive capacity of cells is monitored in the presence of trypsin in a time course (52). The rate of adherent cells after trypsinization for 5 min was significantly higher in AMWAP-expressing cultures than GFP control cells (Fig. 9D, 9E). This difference persisted at all time points analyzed up to 25 min trypsin incubation, indicating that AMWAP confers protease resistance and increases microglial adhesion. In agreement with the wound-healing assays, the transwell chemotaxis tests and the Ccl2 expression data, these results indicate an immunomodulatory role of AMWAP by limiting microglial migration.

**Recombinant AMWAP has antibacterial activity**

As antibacterial activity is a major feature of WAP domain proteins, we studied the effect of recombinant AMWAP on different bacterial strains. Initially, we only achieved low recombinant expression of AMWAP in *E. coli* (data not shown). Therefore, codon-optimized AMWAP (Supplemental Fig. 1) was synthesized, expressed in *E. coli*, and purified via an N-terminal His-tag sequence by affinity chromatography. Western blot analysis of the recombinant protein via an anti-His Ab detected a single specific band at the expected molecular mass of ~10 kDa (Fig. 10A). To study the antibacterial potential of AMWAP, the target bacteria *E. coli* (ATCC25922, Gram-negative rod), *P. aeruginosa* (ATCC27853, Gram-negative rod), and *B. subtilis* (ATCC6633, Gram-positive rod) were incubated in the presence of recombinant AMWAP for 2 h. After overnight growth on plates, CFUs were counted to assess the number of viable bacteria. AMWAP showed a relatively weak (IC50 of 30 μM) but significant bactericidal activity against *E. coli* (Fig. 10B). Growth of *P. aeruginosa* (Fig. 10C) and *B. subtilis* (Fig. 10D) was significantly inhibited at lower micromolar concentrations (IC50 of 16 μM). Thus, the antibacterial potential of AMWAP increased dose dependently and reached similar IC50 values as those of other WAP motif proteins, including SLPI (34) and elafin (53).

**Discussion**

This study is part of an ongoing effort to characterize known and novel transcripts involved in the activation and regulation of microglia during retinal degeneration. We identified the novel mouse protein AMWAP and investigated its specific expression and function in activated microglia and macrophages. AMWAP attenuates proinflammatory cytokine and chemokine expression, whereas promoting expression of alternative activation markers. AMWAP also regulates microglial migration and chemotaxis and it directly exerts antibacterial activity.

Although initially identified in early activated microglia from Rs1h−/− retinas, induced AMWAP transcript levels were also detected in activated microglia stimulated with multiple TLR ligands, including LPS, CpG oligonucleotides, Pam3CSK4 as well as INF-γ. Upregulation of AMWAP by these proinflammatory stimuli was critically dependent on NF-κB but also involves new protein synthesis. AMWAP mRNA expression was highly enriched in microglia and other tissue macrophages, but transcripts were absent in all other mouse tissues studied so far. These findings on TLR-dependent and microglia/macroage-restricted expression correlate well with the major influence of NF-κB and PU.1 in the transcriptional control of the AMWAP gene. PU.1 is a key transcription factor in myeloid cells, with high expression levels in activated retinal microglia (28, 54). Although the precise mechanisms underlying the activation-dependent induction of AMWAP are not fully understood, we postulate that PU.1 plays a critical role in AMWAP expression by direct binding to the proximal promoter.

Immune-related genes, including cytokines and chemokines, as well as their receptors are often organized in chromosomal clusters or miniclusters (55–57). In agreement with this concept, the murine AMWAP gene is located in close proximity to a cluster of the chemokine genes Ccl3, Ccl4, Ccl5, Ccl6, and Ccl9. In addition, the AMWAP gene is flanked by the WAP protein extracellular peptidase inhibitor precursor (Expi), which shares 83% sequence homology possibly suggesting a gene duplication event. Our ChIP-Chip and ChIP-PCR assays have revealed that the AMWAP promoter, but not the Expi region are bound by PU.1 and the coactivators Cbp and p300. These findings are consistent with microglia/macroage-restricted expression of AMWAP and the ubiquitous expression of Expi (58). Likely, the different expression patterns and regulation of both genes coincides with different cellular functions. In line with this, Expi was identified as a protein overexpressed in nonmetastatic mammary cancer cells (59, 60) and ectopic expression of Expi resulted in induced apoptosis of mammary epithelial cells (58).

Together with SLPI (61), the best studied WAP protein so far, AMWAP is the only other WAP family member expressed and...
regulated in microglia. SLPI transcript levels were markedly increased in experimental autoimmune encephalomyelitis and a role for SLPI in the promotion of tissue repair was discussed (61). SLPI can be secreted by activated macrophages and acts as a suppressor of LPS response (62). The inhibition of LPS-induced NO and TNF production in macrophage cell lines by SLPI is independent of its antiprotease function (39). Intracellular SLPI also competes with NF-κB for binding sites in the promoters of proinflammatory cytokines (50) and blocks LPS-induced IκBα-degradation (63). Both mechanisms may also be relevant for AMWAP function. Support for the hypothesis that AMWAP similar to SLPI interferes with NF-κB signaling comes from our finding that Ccl2 promoter activity was directly repressed by AMWAP.

Two major questions relate to why and how AMWAP expression is induced during microglial activation in retinal degeneration. Because no obvious infection is present in the gene mutation-based model of Rs1h−/− retinas, we speculate that AMWAP is induced by damage-associated molecular patterns (DAMPs) (64) and/or alarmins (65). Our data on the rapid induction of AMWAP in early retinal degeneration and the TLR-dependent upregulation in BV-2 cells support the assumption that AMWAP may respond very sensitive to DAMPs. Innate immune cells are often recruited to injured areas by matrix degradation products (66) or actively secreted alarmin molecules like HMGB1 (67), consequently initiating tissue remodeling and repair. Related to this, a secreted and a nonsecreted intracellular form of SLPI were both induced in mouse macrophages after exposure to apoptotic cells (68), indicating that SLPI may promote clearance of dying cells in the absence of inflammation. AMWAP may have a similar “alarmin-like” function in the degenerating retina and thereby may limit microglial activation. A hallmark of retinal degeneration in the retinoschisin-deficient mouse model is the rapid migration of activated microglia into the photoreceptor and ganglion cell layer (30). Ccl2, an important chemotaxtractant is strongly expressed in Rs1h−/− retinas (24) and we demonstrated that AMWAP overexpression downregulated Ccl2 transcripts in microglia. Furthermore, AMWAP diminished BV-2 cell migration and inhibited the serine protease trypsin. Recent data indicated that Ccl2 is proteolytically activated by the serine protease plasmin and that plasmin-deficient mice were resistant to excitoxic neurodegeneration (69). Thus, in addition to its effect on protease plasmin and that plasmin-deficient mice were resistant to excitoxic neurodegeneration (69). 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AMWAP REGULATES MICROGLIAL ACTIVATION


