Stimulation of Vibratory Urticaria-Associated Adhesion-GPCR, EMR2/ADGRE2, Triggers the NLRP3 Inflammasome Activation Signal in Human Monocytes

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EMR2/ADGRE2 is an adhesion G protein-coupled receptor differentially expressed by human myeloid cells. It modulates diverse cellular functions of innate immune cells and a missense EMR2 variant is directly responsible for vibratory urticaria. Recently, EMR2 was found to activate NLRP3 inflammasome in monocytes via interaction with FHR1, a regulatory protein of complement Factor H. However, the functional involvement of EMR2 activation and its signaling mechanisms in eliciting NLRP3 inflammasome activation remain elusive. In this study, we show that EMR2-mediated signaling plays a critical role in triggering the activation (2nd) signal for the NLRP3 inflammasome in both THP-1 monocytic cell line and primary monocytes. Stimulation of EMR2 by its agonistic 2A1 monoclonal antibody elicits a Gα16- dependent PLC-β activation pathway, inducing the activity of downstream Akt, MAPK, NF-κB, and Ca2+ mobilization, eventually leading to K+ efflux. These results identify EMR2 and its associated signaling intermediates as potential intervention targets of NLRP3 inflammasome activation in inflammatory disorders.

Keywords: adhesion G protein-coupled receptor, inflammasome, NLRP3, signaling, pathogen-associated molecular patterns

Abbreviations: AAV, anti-neutrophil cytoplasmic antibody-associated vasculitis; aGPCR, adhesion GPCR; ASC, apoptosis-associated speck-like protein containing a CARD; DAG, diacylglycerol; DAMP, damage-associated molecular pattern; DC, dendritic cell; EGF, epidermal growth factor; DS, dermatan sulphate; EMR2, EGF-like module-containing mucin-like hormone receptor-like 2; FHR1, complement Factor H-related protein 1; GAIN, GPCR autoproteolysis-inducing; GPCR, G protein-coupled receptor; GPS, GPCR proteolysis site; IP3, inositol triphosphate; LPS, lipopolysaccharide; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MSU, monosodium urate; Mj, macrophage; NHS, normal human serum; NLRP3, NOD-, LRR-, and pyrin domain-containing protein 3; Nø, neutrophil; PAMP, pathogen-associated molecular pattern; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; PRR, pattern-recognition receptor; RA, rheumatoid arthritis; ROS, reactive oxygen species; SIRS, systemic inflammatory response syndrome; VU, vibratory urticaria.
INTRODUCTION

Proteolytic conversion of procaspase-1 to active caspase-1 and the subsequent processing and secretion of inflammatory IL-1β and IL-18 are the hallmarks of inflammasome activation (1, 2). Although it is a critical immune response to microbial infection and tissue injury, dysregulated inflammasome activities are often linked to pathological manifestations such as autoimmune, autoinflammatory, and metabolic disorders as well as cancer (3–5). The NLRP3 inflammasome is a large assembly of multimeric NLRP3, ASC and procaspase-1, and represents the principal mechanism of cytokine release in immune cells. Although it is a critical immune response to microbial infection and tissue injury, dysregulated inflammasome activation plays a potential role in recruiting monocytes to the inflamed synovium in rheumatoid arthritis (RA) (23). Interaction plays a potential role in recruiting monocytes to the inflamed synovium in rheumatoid arthritis (RA) (23). Interaction plays a potential role in recruiting monocytes to the inflamed synovium in rheumatoid arthritis (RA) (23). Interaction plays a potential role in recruiting monocytes to the inflamed synovium in rheumatoid arthritis (RA) (23). Interaction plays a potential role in recruiting monocytes to the inflamed synovium in rheumatoid arthritis (RA) (23).

EMR2/ADGRE2 is a human-specific paralogue of the well-known mouse tissue macrophage (Mψ)-specific F4/80 antigen (Ag) (11). Both EMR2 and F4/80 belong to the ADGRE/EGF-TM7 subfamily of adhesion GPCRs (aGPCRs) that is distinguished by multiple extracellular epidermal growth factor (EGF)-like domains (12–14). Unlike F4/80, however, EMR2 is more widely expressed and differentially regulated in cells of the myeloid lineage including monocytes, Mψ, neutrophils (Nψ), and myeloid dendritic cells (DCs) (13, 15). EMR2 expression is up-regulated restrictedly within inflamed tissues and its levels on Nψ are positively correlated with the severity and overall mortality of patients of systemic inflammatory response syndrome (SIRS) and liver cirrhosis (16, 17). These findings indicated an immune regulatory function for EMR2. Indeed, EMR2 activation has been shown to potentiate adhesion, migration and anti-microbial activities of Nψ in response to diverse inflammatory stimuli (18–20). A missense EMR2-pC492Y variant was identified recently as the cause of familial vibratory urticaria (VU), a rare autosomal dominant dermal allergic disorder resulting from vibration-induced mast cell activation (21).

We have previously identified dermatan sulphate (DS) as an endogenous glycosaminoglycan ligand of EMR2 (22). DS-EMR2 interaction plays a potential role in recruiting monocytes to the inflamed synovium in rheumatoid arthritis (RA) (23). Interaction of the VU-inducing EMR2-pC492Y variant with DS or a specific 2A1 monoclonal antibody (mAb) sensitizes mast cells for hyper-degranulation upon vibratory stimulation, indicating a mechanosensing role for EMR2 (21). Our earlier results revealed that 2A1-induced EMR2 activation in monocytes lead to Mψ-like phenotypes and enhanced production of IL-8 and TNF via a Gα16-dependent pathway activating the downstream effectors, including phospholipase C (PLC)-β, PI3K, Akt, MAPK, and NF-κB (24). Interestingly, 2A1-induced EMR2 signaling occurred only when the mAb was immobilized on the culture plates (24). In a recent study, complement Factor H-related protein 1 (FHR1) was shown to function both as a sensor of necrotic cells and a specific serum ligand of EMR2 (25). Consequently, FHR1 was shown to mark necrotic regions of vasculopathies such as anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV) and atherosclerosis, and concomitantly triggered EMR2-mediated NLRP3 inflammasome activation in monocytes via a Gβγ-dependent PLC signaling pathway. Peculiarly, the FHR1-induced EMR2 activation was induced only in the presence of normal human serum (NHS) and immobilized FHR1 (25). Taken together, EMR2 is a human myeloid-restricted aGPCR whose activation and signaling is involved in distinct innate immune functions including NLRP3 inflammasome activation.

In this study, we show that 2A1-induced EMR2 signaling plays a critical role in triggering the NLRP3 inflammasome activation (2nd) signal, due to the intracellular K⁺ efflux evoked via a Gα16-dependent PLC-β activation and Ca²⁺ mobilization. EMR2 and its downstream signaling effector molecules hence represent novel GPCR-associated targets for intervention of NLRP3 inflammasome activation in relevant inflammatory disorders.

MATERIALS AND METHODS

Reagents and Antibodies

All chemicals and reagents were purchased from Sigma (St. Louis, MO, USA) and Invitrogen (CA, USA) unless specified otherwise. ATP, N-acetyl-L-cysteine (NAC), diphenyleneiodonium chloride (DPI), U73122, BAPTA-AM, Glyburide, Bay 11-7082, and sp600125 were all from Sigma. Monosodium urate (MSU) was from Invitrogen. Galenin was from Tocris Bioscience (Bristol UK). U0126 was from Promega (Madison, WI, USA). SB203580 was from Cayman Chemical (Ann Arbor, MI, USA). z-YVAD-fmk (ALX-260-074) and Ac-YVAD-CHO (ALX-260-027) were from Enzo Life Sciences (Farmingdale, NY, USA). Muramyl dipeptide (MDP), Pam3CSK4, FLA-ST, and LPS-B5 Ultrapure were obtained from InvivoGen (San Diego, CA, USA). AZD9056 was from MedChemExpress (Princeton, NJ, USA). Monoclonal antibodies (mAbs) used for Western blotting included: anti-caspase-1 (D3U3E) and anti-human IL-1β (D7F10) were obtained from Cell Signaling Technology (Beverly, MA, USA); anti-ASC (AL177) was from AdipoGen (San Diego, CA, USA); anti-NLRP3 (ALX-804-819) was from Enzo Life Sciences; Anti-β-actin mAb was purchased from BD Biosciences (San Jose, CA, USA). Anti-GAPDH mAb was from Proteintech (IL, USA). Abs used for cell stimulation (2A1 and mouse IgG₁ control) and for the detection of signaling molecules have been described previously (24).

Cell Culture and Primary Cell Isolation

THP-1 (ATCC®-TIB-202™), as well as THP-1-defNLRP3 and THP-1-defASC monocytic cell lines (Invitrogen) were cultured in RPMI 1640 medium supplemented with 10% Fetal bovine...
serum (FBS) (Thermo HyClone), 1% L-glutamate, 1% penicillin, 1% streptomycin and 100 μg/ml Normocin (Invitrogen). For THP-1-defNLRP3 and THP-1-defASC cells, Hygromycin B Gold (10 mg/ml)(Invitrogen) was added to culture medium. All cells were cultured in a 5% CO₂ incubator at 37°C. Ficoll-Plague PLUS (Amersham Bioscience, Ltd) gradient centrifugation was used to purify peripheral blood mononuclear cells (PBMCs) from venous blood of healthy donors as described previously (24). All procedures were approved by the Chang Gung Memorial Hospital Ethics Committee (CGMH IRB No: 201700390B0 and 202001020B0) and performed according to their guidelines. Monocytes were isolated from PBMCs by immune-magnetic separation using human CD14 MicroBeads MACS cell separation kit (Miltenyi Biotec, Inc) and cultured in complete RPMI 1640 medium. Unless otherwise specified, 12- or 6-well cell culture plates were pre-coated with appropriate PBMCs (usually 10 μg/ml) in 1× PBS at 4°C for 24 h. Cells (5x10⁵-2x10⁶ cells/ml) were treated without or with lipopolysaccharide (LPS)(50 μg/ml) in 1× PBS at 4°C for 24 h. NHS was derived from fresh human peripheral blood left to clot at room temperature for 30 min, followed by centrifugation at 1,500 x g for 10 min at 4°C. NHS was kept frozen in aliquots at −80°C and used at a final concentration of 5%.

siRNA-Mediated Gene Silencing
All siRNAs used were purchased from Invitrogen. Briefly, 200 nM of gene-specific siRNAs were transfected into THP-1 cells using DharmaFECT-2 transfection reagent (GE Dharmacon) and incubated for 48 h as suggested by the manufacturer. The siRNA sequence information is listed below: NLRP3-siRNA #1: 5’-ACCCGCGGUGAUCUCUUCUUCUUU-3’, NLRP3-siRNA #2: 5’-GGAAUGUGAAGAAACGCAAUGCUAA-3’, NLRP3-siRNA #3: 5’-UCCACCGAGAUGUGGACCAUUGGUU-3’, ASC-siRNA #1: 5’-GGCGUGCGGUAUCUUCUCGUGGAA-3’, ASC-siRNA #2 ASC-siRNA: 5’-ACCCAGCAAGAUGCGGAAACGUCUUCU-3’, ASC-siRNA #3: 5’-GGCCUGG AACUGGACCGAAGGACU-3’. EMR2-specific, Gto-1c-specific, and scramble control siRNAs were used as described previously (24).

Detection of ASC Oligomerization
THP-1 cells (4 × 10⁶ cells) were pelleted by centrifugation and resuspended in 300 μl ice-cold buffer 1 (20 mM HEPES-KOH, pH 7.5, 150 mM KCl, 320 mM sucrose, 0.01 μg/ml Aprotinin, 10 mM AEBSF, 20 mM Levamisole, 0.1 mM sodium orthovanadate (Na₃VO₄) and protease inhibitor mixture) and lysed by vortex vigorously for 10 s. Cell lysates were centrifuged at 520× g for 8 min at 4°C and pellets removed. Supernatants were diluted with 300 μl Buffer 2 (20 mM HEPES-KOH, 5 mM MgCl₂, 0.5 mM EDTA, 0.1% CHAPS) and centrifuged at 4000× g for 8 min to pellet the ASC pyroptosome (26). Next, the pellets were resuspended in 300 ml Buffer 3 (20 mM HEPES-KOH, 2 mM DSS, 5 mM MgCl₂, 0.5 mM EDTA), incubated at room temperature for 30 min, and pelleted again by centrifugation at 4000× g for 10 min. The cross-linked pellets were resuspended in SDS sample buffer (30 μl), separated in 12% SDS-PAGE by gel electrophoresis, and blotted using anti-ASC mAb.

Cytokine ELISA Assay
Unless specified otherwise, cells (2 × 10⁶ cells/well) were seeded into 6-well plates pre-coated with or without 2A1 mAb in the absence or presence of various PAMPs, then incubated at 37°C for 24 h. Conditioned medium was collected by centrifugation at 1000× g for 5 min at 4°C and transferred into new 1.5 ml eppendorf tubes. The levels of human IL-8, IL-1β, and IL-18 were measured by DuoSet® ELISA Development Systems (R&D System) according to the protocols suggested by the manufacturer.

Western Blot Analysis
Cell lysates for Western blot analysis were collected at specific time points as indicated. In brief, cells were harvested by centrifugation at 1500 rpm for 5 min at 4°C, washed once with ice-cold 1× HBSS, and lysed in 100 μl ice-cold modified cell lysis buffer as described previously (24). Proteins were quantified using Bicinchoninic acid (BCA) protein assay kit (PIERCE, Rockford, USA). Proteins were denatured and separated by electrophoresis in SDS-polyacrylamide gels, and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). The blotted PVDF membranes were incubated in blocking buffer (5% of BSA in washing buffer) for 1 h with agitation, followed by incubation with the indicated 1st Ab (2–4 μg/ml in blocking buffer) for 1 h. The membranes were washed extensively and then incubated with appropriate horseradish peroxidase (HRP)-conjugated 2nd Ab (1:2000-1:5000 in blocking buffer). Following final extensive washes, bound 2nd Ab was detected by chemiluminescence (ECL, Amersham Life Science Ltd or SuperSignal West Pico Plus, Pierce).

Statistical Analysis
Quantitative analysis was conducted on results of at least three independent experiments unless indicated otherwise. Data were analyzed using one-way ANOVA analysis by Prism 5 software (GraphPad Software Inc., La Jolla, California, USA) and shown as means ± standard error of the mean (SEM). The statistical significance of p value was set at *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

RESULTS
Ligation and Activation of EMR2 Receptor in Human Monocytic Cells Enhance IL-1β and IL-18 Production in the Presence of PAMPs
To investigate the role of EMR2-induced signaling in inflammasome activation, THP-1 monocytic cells were cultured...
on plates coated with EMR2-specific 2A1 mAb in the absence or presence of various PAMPs. Low but significant levels of IL-1β were noted when cells were stimulated with 2A1 or LPS individually. However, much higher IL-1β levels were detected when cells were treated simultaneously with 2A1 and LPS, indicating a synergistic effect (Figure 1A). By contrast, neither the isotype control nor the soluble 2A1 mAb had any effect on IL-1β production (Supplementary Figure 1A). Importantly, the specificity of 2A1-induced EMR2 activation in up-regulating IL-1β was verified in cells transfected with EMR2-specific siRNAs (Figure 1B and Supplementary Figure 1B). These results were in line with our previous data and hence all following experiments were performed using immobilized 2A1 mAb (24). Similar 2A1-induced IL-1β up-regulation was obtained in cells treated with conventional or ultrapure LPS, and this effect was both concentration- and time-dependent; ranging from 5–10 μg/ml of 2A1 mAb and starting as early as 4 h of incubation (Figures 1A, C and Supplementary Figure 1C). In parallel, significantly higher IL-18 levels were produced by cells co-stimulated with 2A1 plus LPS or 2A1 plus Pam3CSK4 (TLR2/TLR1 ligand) than those treated with the stimulus alone (Figure 1D). Finally, enhanced levels of IL-1β were also identified in cells co-stimulated with 2A1 and various TLR and NLR ligands including Pam3CSK4, FLA-ST (TLR5 ligand) and MDP (NOD2 ligand) (Figure 1E). These results suggest that EMR2 activation can collaborate with the signaling of diverse PRRs to up-regulate IL-1β and IL-18 production in mononuclear cells.

Along with the time-dependent IL-1β up-regulation, increased levels of pro-IL-1β and active caspase-1 were detected in the cell lysate and conditioned medium, respectively, of cells co-treated with LPS and 2A1 than those treated individually with 2A1 or LPS (Figures 2A, B). Conversely, EMR2-induced IL-8 levels remained unchanged in the presence of LPS, indicating a specific synergistic effect of EMR2 activation on IL-1β up-regulation (Figure 2A). Critically, EMR2-induced IL-1β and IL-18 up-regulation was markedly diminished in cells pre-treated with an irreversible caspase-1 inhibitor, z-YVAD-fmk, confirming the essential role of active caspase-1 in EMR2-promoted IL-1β and IL-18 production (Figures 2C, D). Taken together, activation of EMR2 in THP-1 cells by immobilized, but not soluble, 2A1 mAb significantly up-regulates IL-1β and IL-18 production in the presence of PAMPs, most likely due to inflammasome activation.

### EMR2 Stimulation Activates the NLRP3 Inflammasome

The NLRP3 inflammasome is the most common inflammasome activated by diverse PAMPs in monocytes and was reported to be elicited by FHR1-EMR2 interaction (25). We hence examined the role of 2A1-induced EMR2 ligation in NLRP3 inflammasome activation by first performing gene-specific siRNA knock down targeting ASC and NLRP3. As shown, up-regulated IL-1β production induced by the combined LPS and 2A1 treatment was greatly attenuated in THP-1 cells transfected with the ASC-
or NLRP3-specific siRNAs, but not the scrambled control siRNA. Concomitantly, reduced levels of active caspase-1 were found in ASC- and NLRP3-knock down cells (Figures 3A, B).

Thereafter, we employed commercially available ASC-deficient (def-ASC) and NLRP3-deficient (def-NLRP3) THP-1 cell lines to clarify the role of EMR2-induced signaling in activating the NLRP3 inflammasome. Comparable EMR2 expression levels were detected in all three THP-1 cell lines, suggesting no direct roles for ASC and NLRP3 in regulating EMR2 expression (Supplementary Figure 1D). As expected, the enhanced IL-1β levels induced by 2A1 plus LPS in parental THP-1 cells were greatly diminished in def-ASC and def-NLRP3 cells (Figure 3C). Likewise, notably reduced levels of IL-1β and active caspase-1 were detected in the conditioned medium of def-ASC and def-NLRP3 cells (Figure 3D). Conversely, comparable IL-8 levels were induced in all three cell lines treated with 2A1, LPS, or 2A1 plus LPS, again supporting the specific role of ASC and NLRP3 in EMR2-promoted IL-1β production (Figure 3C). Collectively, these results establish a role for EMR2-mediated signaling in promoting NLRP3 inflammasome activation.

**EMR2 Ligation Triggers the Activation Signal for NLRP3 Inflammasome Activation**

In accordance with the canonical two-signal activation mechanism of NLRP3 inflammasome (8, 9), our results suggested that 2A1-activated EMR2 most likely elicited the activation (2nd) signal. To validate this, we incubated THP-1 cells with 2A1 in the absence or presence of exogenous ATP or monosodium urate (MSU) crystal, two typical triggers of the activation signal. As reported elsewhere, ATP and MSU treatments lead to significant IL-1β up-regulation in the presence, but not in the absence, of LPS. However, no apparent IL-1β enhancement was detected in cells co-stimulated with 2A1 plus ATP or MSU versus cells treated with 2A1 alone (Figure 4A). Additionally, ASC oligomerization, a signature feature of NLRP3 inflammasome assembly and activation, was more readily identified in cells co-treated with LPS and 2A1 than those treated singly with LPS or 2A1 (Figure 4B, Supplementary Figure 2). These results indicate strongly that 2A1-induced EMR2 activation predominantly provides the activation signal for NLRP3 inflammasome activation.

Unlike murine myeloid cells, human primary monocytes promptly secrete mature IL-1β in response to the PAMP-triggered priming signal alone. This is mainly due to the heightened responsiveness of human primary monocytes, leading to the release of endogenous ATP or activation of the “alternative” inflammasome activation pathway following PAMP stimulation (27–30). To attest to the role of EMR2 ligation in inducing the NLRP3 inflammasome activation signal, primary CD14+ monocytes were isolated and incubated with 2A1 in the absence or presence of LPS. In line with previous reports, significant IL-1β production was induced in cells receiving LPS treatment alone. Importantly, cells treated with LPS plus 2A1 produced markedly higher IL-1β levels, while those stimulated with 2A1 alone generated only basal IL-1β levels as did control...
cells (Figures 5A–D). Again, the enhanced IL-1β induced by LPS or LPS plus 2A1 in primary monocytes was significantly attenuated in the presence of a selective inhibitor of caspase-1, Ac-YVAD-CHO (Figures 5C, D).

During the course of this study, Irmscher et al. identified the complement system protein FHR1 as an EMR2-specific ligand and showed that FHR1-EMR2 interaction triggered NLRP3 inflammasome activation in primary monocytes (25). Intriguingly, the FHR1-induced NLRP3 inflammasome activation took place only in the presence of normal human serum (NHS) and when FHR1 was immobilized (25). These findings are somewhat similar to ours and prompted us to investigate the effect of NHS on 2A1-induced EMR2 activation.

We conclude that the major role of 2A1-elicited EMR2 signaling is to trigger the activation (2nd) signal for NLRP3 inflammasome activation.

EMR2-Induced NLRP3 Inflammasome Activation Signal Is Mediated via the PLC-β/Akt/Ca²⁺/MAPK/NF-κB Axes Downstream of Gα₁₆

Our previous studies have established that 2A1-elicited EMR2 signaling activated the Gα₁₆/PLC-β/P3K/Akt/MAPK/NF-κB axes (24). On the other hand, FHR1-induced EMR2 activation involved a Gβγ-dependent PLC-sensitive pathway (25). Gα₁₆ belongs to the Gαq subfamily that is known to activate PLC-β.
isoforms, leading to the generation of diacylglycerol (DAG) and inositol triphosphate (IP₃), which then triggered MAPK and NF-kB activation as well as intracellular calcium ion (Ca²⁺) mobilization, respectively (31). Similarly, the Gβγ subunits are also able to signal independently via PLC-β isoforms when dissociated from the active Gα subunit (32). To dissect the 2A1-induced EMR2 signaling cascades that triggered the NLRP3 inflammasome activation signal, we performed biochemical analyses using gene-specific siRNAs and diverse signaling inhibitors in THP-1 cells and monocytes.

FIGURE 4 | EMR2 ligation triggered the activation signal for NLRP3 inflammasome activation in THP-1 cells. (A) ELISA analysis of IL-1β in the supernatants of THP-1 cells cultured on plates coated without or with 2A1 mAb in the absence or presence of LPS for 24 h. Cells were either treated without (lane 1) or with additional stimuli (lane 2, 5 mM ATP; lane 3, 100 μg/ml MSU), or 10 μg/ml mouse IgG1 (lane 4) as a negative control (n=3, mean ± SEM, one-way ANOVA. *p<0.05, ***p<0.001). (B) THP-1 cells were cultured on plates coated without or with 2A1 mAb in the absence or presence of LPS for 24 h. The ASC oligomers and relevant components of NLRP3 inflammasome in the cell lysate and pellets were analyzed by Western blotting as indicated.

FIGURE 5 | EMR2 ligation triggers the activation signal for NLRP3 inflammasome activation in human primary monocytes. (A–F) Isolated CD14⁺ primary monocytes (2 x 10⁶ cells/well) were incubated in 6-well plates coated without or with 2A1 mAb in the absence or presence of LPS for 24 h (A–D). When necessary, cells were pretreated with Ac-YVAD-CHO (50 μM) for 1 h before treatment with LPS and 2A1 (C, D) or cultured in medium containing 5% NHS or ATP (5 mM) as described (E, F). Cell supernatants were collected and analyzed for IL-1β by ELISA (A, C, E, F). Cell lysates were analyzed by WB analysis for relevant proteins as indicated (B, D). Data are means ± SEM of three independent experiments performed in triplicate using cells from three different donors (E, F). Data were analyzed by one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus the control group. ns, non significant.
As expected, 2A1-enhanced IL-1β levels were reduced in THP-1 cells transfected with Gα16-specific siRNAs but not scrambled siRNA controls (Supplementary Figure 4A). Intriguingly, no significant effects on IL-1β production were observed by Gβγ inhibition both in THP-1 cells and monocytes, whereas the use of PLC inhibitor (U73122) and cell-permeable Ca2+ chelator (BAPTA-AM) attenuated 2A1-enhanced IL-1β release markedly (Figures 6A, B). In parallel, 2A1-induced IL-1β up-regulation was diminished following the blocking of cellular K+ efflux in THP-1 cells and monocytes (Figures 6C, D). These results indicate that 2A1-induced EMR2 activation in monocytes stimulates a Gα16-dependent (but Gβγ-independent) Ca2+ mobilization signaling activity leading to intracellular K+ efflux, which is generally considered a common trigger of the NLRP3 inflammasome activation signal (confirmed in Supplementary Figure 4B).

In line with our earlier findings, western blot analyses revealed time-dependent phosphorylation and/or activation of Akt, ERK, and IκB following 2A1-induced EMR2 signaling (24). Critically, these signaling activities were further enhanced in cells co-treated with 2A1 and LPS (Figure 7A and Supplementary Figure 5A). Concomitantly, EMR2-induced IL-1β up-regulation was mitigated significantly in cells pre-treated with selective inhibitors of ERK (U0126), JNK (SP600125), and IκB kinase (IKK)(Bay 11-7082), but not the p38 inhibitor (SB203580) (Figures 7B, C). Similarly, IL-8 production induced by EMR2 activation was also reduced by the inhibition of ERK, JNK and IKK but not p38, reconfirming our earlier results (24). Further extensive western blot analyses of 2A1-activated THP-1 cells treated with various signaling inhibitors revealed intricate signaling regulation and potential cross-talk among selective signaling molecules. As such, inhibition of PLC-β (with U73122), PI3K (with Wortmannin and LY294002), and NF-κB activation (with TPCA-1 and Bay11-7082) did not only inactivate Akt, but also ERK phosphorylation. By contrast, inhibition of ERK (with U0126) had no impact on Akt phosphorylation, while inhibition of reactive oxygen species (ROS) production (with NAC) specifically inactivated JNK but...
not ERK (Figure 8A, Supplementary Figure 5B) Therefore, inhibition of Akt (with LY294002) and ROS (with NAC and DPI) both lead to diminished IL-1β and IL-8 production in 2A1-activated THP-1 cells (Figures 8B, C and Supplementary Figure 5C). These results prompted us to propose a Gα16-mediated PLC-β dependent signaling network evoked by 2A1-elicited EMR2 activation that worked collectively to induce K⁺ efflux and hence the NLRP3 inflammasome activation signal (Figure 9).

**DISCUSSION**

EMR2/ADGRE2 is a human myeloid-restricted aGPCR strongly associated with diverse inflammatory pathologies such as RA, SIRS, and VU (20, 33). However, EMR2-mediated signaling events and their functional significance remained to be fully elucidated. In this study, we have unraveled the signaling mechanisms of EMR2 in inducing NLRP3 inflammasome activation in monocytes. The conclusion that EMR2-mediated signaling triggers the activation (2nd) signal for NLRP3 inflammasome is reminiscent of other inflammasome-activating GPCRs such as the calcium sensing receptor (CaSR) and its closely-related GPRC6A protein (10). Our findings hence confirm and mark EMR2 as one of a limited number of GPCRs known to activate NLRP3 inflammasome in human myeloid cells.

EMR2 is a typical aGPCR that undergoes autoproteolytic processing at the extracellular GPCR proteolysis site (GPS) and is expressed as a bipartite complex containing the extracellular N-terminal fragment (NTF) and the seven-transmembrane (7TM) C-terminal fragment (CTF) (34, 35). One of the aGPCR activation mechanisms is the tethered-ligand activation model, in which an aGPCR is activated when the NTF dissociates from CTF following the binding of its specific extracellular ligand(s),
possibly with the help of mechanical cues (36, 37). This allows the exposure of the most N-terminal region of the CTF, called the Stachel peptide, which then acts as a tethered ligand to interact and activate its own 7TM region. Indeed, the EMR2-pC492Y variant has been shown to function as a mechanosensor in mast cells of VU patients and is prone to shed its NTF in response to vibratory challenge (21). As such, it is of great interest to note the variant has been shown to function as a mechanosensor in mast cells (21).

It is noteworthy to mention that the EMR2-pC492Y variant on mast cells is activated by the 2A1 mAb as well as its FHR1, remain to be characterized. Whether EMR2-NTF is shed and, if so, the extent of shedding upon the binding of EMR2 to immobilized 2A1 and FHR1, remain to be characterized.

The identification of a G\(\alpha_{16}\)-dependent PLC-\(\beta\) activation pathway induced by 2A1-activated EMR2 is consistent with our earlier findings (24). Importantly, EMR2 coupling to G\(\alpha_{16}\) and PLC-\(\beta\) activation was also demonstrated recently by Bhudia et al. who showed that HEK-293T cells co-expressing G\(\alpha_{16}\) and EMR2-CTF resulted in inositol monophosphate (IP1) accumulation as well as a strong increase of the NFAT-luciferase activity, two critical indicators of the activated G\(\alpha_{16}/\)PLC-\(\beta\)/Ca\(^{2+}\) signaling axis (43). While these results reiterate the specific and strong coupling of EMR2 with the G\(\alpha_{16}\) protein, we failed to find the involvement of G\(\beta\gamma\) in 2A1-induced EMR2 signaling, unlike that of FHR1-EMR2 interaction. Interestingly, a recent study by Naranjo et al. similarly revealed that vibratory interaction of DS and EMR2-pC492Y in mast cells induced a G\(\beta\gamma\)-, G\(\alpha_{11}\)-, and G\(\alpha_{16}\)-independent mechanism leading to specific activation of PLC-\(\beta\), PI3K, ERK1/2 and a transient cytosolic calcium increase (38). One possibility for these differential signaling activities might be due to the potential biased signaling mediated by EMR2 in different cell types (monocyte vs. mast cell) in response to different stimuli (2A1 vs. FHR1 plus NHS vs. DS and vibration). This possibility is further supported by the fact that we did not find a role for NHS in 2A1-induced EMR2 activation (Figure 5), while it is absolutely required for EMR2 activation elicited by FHR1. The exact role of NHS in FHR1-induced NLRP3 inflammasome activation warrants further investigation.

FIGURE 8 | EMR2-mediated signaling in THP-1 cells induces Akt activation and ROS production, leading to NLRP3 inflammasome activation. (A) Western blotting analyses of EMR2-mediated signaling in THP-1 cells incubated with or without 2A1 and specific signaling inhibitors as indicated for 30 min. Blots were probed to detect phospho-Akt, phospho-ERK and \(\beta\)-actin level. Cells treated with mouse IgG\(_{1}\) and LPS were included as negative and positive controls, respectively. (B, C) Culture supernatants of THP-1 cells treated with indicated conditions for 24 h were collected for the detection of IL-1\(\beta\) by ELISA. Data are means ± SEM of at least three independent experiments performed in triplicate and analyzed by one-way ANOVA. ***p < 0.001, ****p < 0.0001 versus the control group. LY294002, PI3K inhibitor; NAC (N-Acetyl-L-cysteine) and DPI (Diphenyleneiodonium), ROS inhibitors.
Although most of the downstream signaling effectors of Ga16/PLC-β activation, including ERK1/2, JNK, NF-κB, and Akt have been reported in our previous study and re-confirmed here and elsewhere, we further showed that Ca** mobilization, ROS production, and K' efflux are critically involved in the signaling pathways of EMR2-induced NLRP3 inflammasome activation (Figure 9). While both Ca** and ROS are well-known triggers of NLRP3 inflammasome, cellular K' efflux has been considered to be the universal inducer of NLRP3 inflammasome activation, involved in the signaling of almost all NLRP3 stimuli, such as ATP, nigericin, and particulate matter (6, 8). Hence, we conclude that 2A1-induced EMR2 activation is initiated by Ga16 coupling and PLC-β stimulation, followed by the activation of Akt, ERK1/2, JNK, NFκB, Ca** mobilization, and ROS production, likely via the generation/activation of DIG, IP3, and PI3K. The collective actions of these signaling events eventually trigger K' efflux and NLRP3 inflammasome activation (Figure 9). It is of great interest to note that PLC-β, Akt, ERK1/2, NFκB, and Ca** mobilization are consistently identified in several independent studies of EMR2 activation and signaling. These signaling intermediates along with EMR2 itself hence represent potential targets of therapeutic intervention for relevant inflammatory disorders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.
AUTHOR CONTRIBUTIONS

K-YI, W-YT, W-CW, and H-HL performed experiments. K-YI, W-YT, SG, K-FN, and H-HL designed the experiments, analyzed, and interpreted the data. K-YI, W-YT, and H-HL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Lamkanfi M, Dixit VM. Mechanisms and functions of inflammasomes. Cell (2014) 157:1013–22. doi: 10.1016/j.cell.2014.04.007
2. Man SM, Kanneganti TD. Regulation of inflammasome activation. Immunol Rev (2015) 265:6–21. doi: 10.1111/imr.12296
3. Lamkanfi M, Dixit VM. Inflammasomes and their roles in health and disease. Annu Rev Cell Dev Biol (2012) 28:137–61. doi: 10.1146/annurev-cellbio-101011-155745
4. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. Nature (2012) 481:278–86. doi: 10.1038/nature10759
5. Davis BK, Wen H, Ting JP. The inflammasome NLRs in immunity, inflammation, and associated diseases. Annu Rev Immunol (2011) 29:787–35. doi: 10.1146/annurev-immunol-031210-101405
6. He Y, Hara H, Nunez G. Mechanism and Regulation of NLRP3 Inflammasome Activation. Trends Biochem Sci (2016) 41:1012–21. doi: 10.1016/j.tibs.2016.09.002
7. Malik A, Kanneganti TD. Inflammasome activation and assembly at a glance. J Cell Sci (2017) 130:3955–63. doi: 10.1242/jcs.207365
8. Kelley N, Jeltema D, Duan Y, He Y. The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. Int J Mol Sci (2019) 20 (13):3328. doi: 10.3390/ijms20133328
9. Yang Y, Wang H, Koudiar M, Song H, Shi F. Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors. Cell Death Disease (2019) 10:128. doi: 10.1038/s41419-019-1413-8
10. Tang T, Gong T, Jiang W, Zhou R. GPCRs in NLRP3 Inflammasome Activation, Regulation, and Therapeutics. Trends Pharmcol Sci (2018) 39:798–811. doi: 10.1016/j.tips.2018.07.002
11. Lin HH, Stacey M, Hamann J, Gordon S, McKnight AJ. Human EMR2, a novel EGF-TM7 molecule on chromosome 19p13.1, is closely related to pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. Cell Death Differ (2017) 24:528–40. doi: 10.1038/cdd.2016.114
12. Stacey M, Chang GW, Davies JQ, Kwakkenbos MJ, Sanderson RD, Hamann J, et al. The epidermal growth factor-like domain of the human EMR2 receptor mediate cell attachment through chondroitin sulfate glycosaminoglycans. Blood (2003) 102:2916–24. doi: 10.1182/blood-2002-11-3540
13. Kop EN, Kwakkenbos MJ, Teske GJ, Kraan MC, Smeets TJ, Stacey M, et al. Identification of the epidermal growth factor-TM7 receptor EMR2 and its ligand dermatan sulfate in rheumatoid synovial tissue. Arthritis Rheumatism (2005) 52:442–50. doi: 10.1002/art.20788
14. I KY, Huang YS, Hu CH, Tseng WY, Cheng CH, Stacey M, et al. Activation of Adhesion GPCR EMR2/ADGRE2 Induces Macrophage Differentiation and Inflammatory Responses via Galphal6/Akt/MAPK/NF-kappaB Signaling Pathways. Front Immunol (2017) 8:373. doi: 10.3389/fimmu.2017.00373
15. Irmischer S, Brix SR, Zipfel SLH, Halder LD, Muttluk S, Wulf S, et al. Serum FHR1 binding to necrotic-type cells activates monocyte inflammasome and marks necrotic sites in vasculopathies. Nat Communications (2019) 10:2961. doi: 10.1038/s41467-019-10766-0
16. Fernandes-Alnemri T, Wu J, Yu JW, Datta P, Miller B, Jankowski W, et al. The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. Cell Death Differ (2007) 14:1590–604. doi: 10.1038/sj.cdd.4402194
17. Wang H, Mao L, Meng G. The NLRP3 inflammasome activation in human or mouse cells, sensitivity causes puzzle. Protein Cell (2013) 4:565–8. doi: 10.1007/s13238-013-3905-0
18. Netea MG, Nold-Petry CA, Nold MF, Joosten LA, Opitz B, van der Meer JH, et al. Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. Blood (2009) 113:2324–35. doi: 10.1182/blood-2008-03-146720
19. Piccini A, Carta S, Tassi S, Lasiglie D, Fossati G, Rubartelli A. ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1beta and IL-18 secretion in an autocrine way. Proc Natl Acad Sci U States A (2008) 105:8067–72. doi: 10.1073/pnas.0708664105
20. Gaidt MM, Ebert TS, Chauban D, Schmidt T, Schmid-Burgk JL, Rapino F, et al. Human Monocytes Engage an Alternative Inflammasome Pathway. Immunity (2016) 44:833–46. doi: 10.1016/j.immu.2016.01.012
21. Mizuno N, Itoh H. Functions and regulatory mechanisms of Gq-signaling pathways. Neuro Signals (2009) 17:42–54. doi: 10.1159/0001866869
22. Lowry WE, Huang XY. G Protein beta gamma subunits act on the catalytic domains to stimulate Bruton’s agammaglobulinemia tyrosine kinase. J Biol Chem (2002) 277:1488–92. doi: 10.1074/jbc.M110390200

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.602016/full#supplementary-material
33. Hamann J, Hsiao CC, Lee CS, Ravichandran KS, Lin HH. Adhesion GPCRs as Modulators of Immune Cell Function. *Handb Exp Pharmacol* (2016) 234:329–50. doi: 10.1007/978-3-319-41523-9_15

34. Kwakkenbos MJ, Chang GW, Lin HH, Pouwels W, de Jong EC, van Lier RA, et al. The human EGF-TM7 family member EMR2 is a heterodimeric receptor expressed on myeloid cells. *J Leukocyte Biol* (2002) 71:854–62. doi: 10.1189/jlb.71.5.854

35. Chang GW, Stacey M, Kwakkenbos MJ, Hamann J, Gordon S, Lin HH. Proteolytic cleavage of the EMR2 receptor requires both the extracellular stalk and the GPS motif. *FEBS Lett* (2003) 547:145–50. doi: 10.1016/S0014-5793(03)00693-1

36. Liebscher I, Schon J, Petersen SC, Fischer L, Auerbach N, Demberg LM, et al. A tethered agonist within the ectodomain activates the adhesion G protein-coupled receptors GPR126 and GPR133. *Cell Rep* (2014) 9:2018–26. doi: 10.1016/j.celrep.2014.11.036

37. Stoveken HM, Hajduczok AG, Xu L, Tall GG. Adhesion G protein-coupled receptors are activated by exposure of a cryptic tethered agonist. *Proc Natl Acad Sci U S A* (2015) 112:6194–9. doi: 10.1073/pnas.1421785112

38. Naranjo AN, Bandara G, Bai Y, Smelkinson MG, Tobio A, Komarow HD, et al. Critical Signaling Events in the Mechanoactivation of Human Mast Cells through p.C492Y-ADGRE2. *J Invest Dermatol* (2020) 140(11):2210–20.e5. doi: 10.1016/j.jid.2020.03.936

39. Simon Davis DA, Parish CR. Heparan sulfate: a ubiquitous glycosaminoglycan with multiple roles in immunity. *Front Immunol* (2013) 4:470. doi: 10.3389/fimmu.2013.00470

40. Parker H, Bigger BW. The role of innate immunity in mucopolysaccharide diseases. *J Neurochem* (2019) 148:639–51. doi: 10.1111/jnc.14632

41. Babelova A, Moreth K, Talata-Szegi W, Zeng-Brouwers J, Eickelberg O, Young M, et al. Biglycan, a danger signal that activates the NLRP3 inflammasome via toll-like and P2X receptors. *J Biol Chem* (2009) 284:24035–48. doi: 10.1074/jbc.M109.014266

42. Orlowsky EW, Stabler TV, Montell E, Verges J, Kraus VB. Monosodium urate crystal induced macrophage inflammation is attenuated by chondroitin sulphate: pre-clinical model for gout prophylaxis? *BMC Musculoskeletal Disord* (2014) 15:318. doi: 10.1186/1471-2474-15-318

43. Bhudia N, Desai S, King N, Ancellin N, Grillot D, Barnes AA, et al. G Protein-Coupling of Adhesion GPCRs ADGRE2/EMR2 and ADGRE5/CD97, and Activation of G Protein Signalling by an Anti-EMR2 Antibody. *Sci Rep* (2020) 10:1004. doi: 10.1038/s41598-020-57989-6

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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