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Angiopoietin-like protein 2 induces proinflammatory responses in peritoneal cells

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Abstract

Monocytes and macrophages are important effectors and regulators of inflammation, and both their differentiation and activation are regulated strictly in response to environmental cues. Angiopoietin-like protein 2 (Angptl2) is a multifaceted protein, displaying many physiological and pathological functions in inflammation, angiogenesis, hematopoiesis, and tumor development. Although recent studies implicate Angptl2 in chronic inflammation, the mechanisms of inflammation caused by Angptl2 remain unclear. The purpose of the present study was to elucidate the role of Angptl2 in inflammation by understanding the effects of Angptl2 on monocytes/macrophages. We showed that Angptl2 directly activates resident murine peritoneal monocytes and macrophages and induces a drastic upregulation of the transcription of several inflammatory genes including nitric oxide synthase 2 and prostaglandin-endoperoxide synthase 2, and several proinflammatory cytokine genes such as interleukin (IL)-1β, IL-6, TNFα, and CSF2, along with activation of ERK, JNK, p38, and nuclear factor kappa B signaling pathways. Concordantly, proinflammatory cytokines IL-1β, IL-6, TNFα, and GM-CSF, were rapidly elavated from murine peritoneal monocytes and macrophages. These results demonstrate a novel role for Angptl2 in inflammation via the direct activation of peritoneal monocytes and macrophages.

INTRODUCTION

Monocytes and macrophages play a central role in inflammation [1]. Monocytes are immune effector cells that are continuously generated in the bone marrow from hematopoietic stem

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Conflict of interest

The authors declare no conflict of interest.

cells (HSCs) via macrophage and dendritic cell (DC) progenitors [2]. Upon tissue damage or infection, monocytes are rapidly recruited to the tissue, differentiating into tissue macrophages and DCs. Macrophages are tissue-resident professional phagocytes [3]. During inflammation, macrophages also modulate inflammatory response through the production of various cytokines and growth factors. Two different functional subpopulations of macrophages, M1 and M2 macrophages, have been identified [1]. Classical M1 macrophages are induced by inflammatory stimulations such as Toll-like receptor ligands and IFN-γ, and express numerous proinflammatory mediators, including tumor necrosis factor (TNF) α, IL-1, IL-6, and reactive nitrogen oxide intermediates, which have strong microbiocidal and tumoricidal activities. Alternatively activated M2 macrophages are induced in the presence of IL-4/IL-13 [3], and express signature molecules, including arginase I (ArgI), chitinase 3-like 3 (also known as YM1), IL-10, Fizz1, and mannose receptor Mrc1, which are supposed to be involved in parasite infestation, tissue remodeling, and tumor progression. By strict regulation of these functions, monocytes/macrophages play a critical role in the initiation, maintenance, and resolution of inflammation to avoid excessive tissue damage.

Angiopoietin-like proteins (Angptls) are a family of 7 secreted glycoproteins that play important roles in lipid metabolism, angiogenesis, atherogenesis, hematopoiesis, and inflammation [4, 5]. We demonstrated that Angptl2 inhibits differentiation and promotes *ex vivo* expansion of HSCs [6,7,8,9]. Angptl2 was also reported to play a critical role in chronic inflammation of adipose tissue via inflammatory signaling in endothelial cells [10]. Transgenic overexpression of Angptl2 in mouse skin induces inflammation characterized by abundant attachment of leukocytes to the vessel walls and increased vascular permeability. The expression of Angptl2 is elevated particularly in inflammatory diseases, and its knockdown reduces inflammation and leukocyte infiltration to inflammation sites [10]. These findings suggest that Angptl2 plays an important role as a mediator of the inflammatory process. It remains unclear how secreted Angptl2 induces inflammation and which types of cells are target cells.

Here we studied the effects of Angptl2 on monocyte/macrophage-related cells. We demonstrated a novel role for Angptl2 in inflammation by direct activation of tissue-resident monocytes/macrophages, resulting in proinflammatory cytokine production.

MATERIALS AND METHODS

Animals

C57BL/6J mice (8–16weeks) were purchased from CREA-Japan, Inc. (Tokyo, Japan). The animal experimentation protocols were approved by the Animal Care and Use Committee of University of the Ryukyus (Okinawa, Japan).

Cells and cell culture

HEK293T and its derivative were cultured in DMEM with 10% FCS. HEK293T cells stably expressing Angptl2-flag were generated by pantropic retrovirus packaging system (Takara Bio) with pQCX-GFP-humanAngptl2-Flag plasmid. GFP expressing cells were selected and

cultured to secrete Angptl2-Flag protein in the medium. Murine primary peritoneal macrophages were harvested from C57BI/6J mice (8–16 weeks) as described [11]. M-BMDM and GM-BMDM were differentiated from bone marrow cells isolated from C57BI/6J mice (8–16 weeks). Bone marrow cells were cultured in RPMI-1640, supplemented with FCS, 10 ng/ml of murine M-CSF (PeproTech) or GM-CSF (PeprotTech) respectively, for 5 to 7 days. Murine macrophage-like cell lines RAW264.7 and J774.1 and human THP-1 cells were cultured in RPMI-1640 and FCS.

Reagents and protein

HEK293T cells stably expressing Angptl2-flag or HEK293T cells were seeded at a density of 6.0×10^6 cells in 24ml culture medium in 14 cm dishes. After 4 days of culture, secreted Angptl2-flag protein was purified from cultured medium using Anti-FLAG M2 Affinity Gel (Sigma-aldrich). 100 μg/ml of 1xFLAG peptide (Sigma-aldrich) in PBS was used to elute proteins. The control solution was prepared in parallel with recombinant Angptl2-flag protein purification from the culture medium of HEK293T cells. Purified Angptl2 protein and control solution were stored in deep freezer at −80°C until use. LPS was purchased from Sigma-aldrich.

ELISA

Cells (3.0×10^5 cells) were cultured in a 24-well flat-bottomed culture plate (BD Falcon) with control solution, Angptl2 or LPS. Cultured medium were collected at indicated time and IL-6, TNFα, IL-1β, IL-10, and GM-CSF were measured by ELISA kit (Biolegend).

Quantitative RT-PCR

5.0×10^6 murine peritoneal cells were seeded in 6 cm culture dishes. Following 2 h incubation, cells were treated with equal volume of control solution or Angptl2-Flag (0.5μg/ml) for 3 h. Total RNA was isolated and cDNA was synthesized from 0.5 μg total RNA using PrimeScript RT reagents (Takara Bio). Quantitative real-time polymerase chain reaction (qPCR) were performed using SYBR Premix EX Taq II kit (Takara Bio) with a StepOnePlus Real-Time PCR System (Applied Biosystems). Oligonucleotide primers used for PCR are listed in Supplemental Table.

Flow cytometry analysis and fluorescence activated cell sorting (FACS)

Flow cytometry were performed as described [8,12]. Peritoneal cells were scraped off and pretreated with Fc blocker (BD Bioscience) followed by staining with anti-mouse F4/80-PE, anti-mouse CD80-APC, anti-mouse CD11b-PerCP/Cy5.5, and anti-muse CD11c-PE/Cy7. Intercellular IL-1β staining (biotinylated anti-mouse IL-1β and FITC-streptavidin) was conducted using BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit with BD GolgiPlug (BD Bioscience). Antibodies were from Biolegend. Analysis was performed on FACS Verse system (BD Bioscience). F4/80-positive or -negative cells were sorted using Cell Sorter SH800 (Sony).

Immunoblot

3.0×10^6 murine primary peritoneal cells were seeded in 2 ml culture medium in 3.5 cm culture dishes. Following 5 h incubation, cells were treated with equal volume of control solution, Angptl2-Flag (0.5 μg/ml) or LPS (0.1 μg/ml) for indicated time. Whole adherent cells were washed with 2 ml of ice cold PBS, then lysed with 250 μl of Laemmli sample buffer containing phosphatase inhibitor cocktail (Nakarai Tesque). 15 μl of each sample were subjected to western blot analysis as described [13]. All antibodies were purchased from Cell Signaling Technology.

Immunostaining

Murine peritoneal cells were allowed to adhere to 35mm culture dish (Nalgene) for 2 h. M-BMDM and GM-BMDM were differentiated in 35mm culture dish (Nalgene) for 5 days. Cells were stimulated with equal volume of control solution, Angptl2-Flag (0.5 μg/ml), or LPS (0.1 μg/ml) for 2 h. After washing with PBS, cells were stained with anti-NF-B p65 antibody (Rockland) followed by Alexa 488-conjugated goat anti-rabbit IgG (Life technologies), DAPI (Life technologies).

Statistics

Data are expressed as mean ± SEM. A two-tailed Student's *t*-test was performed to evaluate the significance between experimental groups.

RESULTS

Angptl2 induces morphological changes of murine peritoneal cells

To prepare functional recombinant Angptl2 protein, we generated HEK293T stably expressing Flag-tagged human Angptl2 (Angptl2-flag). The Flag-tagged protein was purified by immunoaffinity chromatography from cultured medium collected 4 days after the cells reached subconfluence. SDS-PAGE of Angptl2-flag showed 2 major bands: full-length protein (60 kDa) and cleaved protein (28 kDa), as described before [6]. The control solution prepared by the same procedure from the original HEK293T culture medium did not show any appreciable bands (Fig. 1A and B).

Angptl2-flag is active to stimulate *ex vivo* expansion of mouse HSCs [6]. We examined the effects of Angptl2 on murine peritoneal cells, M-CSF-induced bone marrow-derived macrophages (M-BMDMs), GM-CSF-induced bone marrow-derived macrophages (GM-BMDMs), and macrophage cell lines RAW164.7 and J774. Cells incubated with the control solution for 24 h did not show any morphological changes (Fig. 1C, left panels), whereas Angptl2 treatment (0.5 μg/ml) induced spreading of adherent cells in murine peritoneal cells but not in M-BMDMs, GM-BMDMs (Fig. 1C, center panels), or RAW164.7 and J774 cell lines (Supplemental Fig. 1). LPS treatment induced cell spreading and extension in all cell types tested (Fig. 1C, right panels). These results suggest that Angptl2 activates primary murine peritoneal cells.

Angptl2 upregulates pro-inflammatory cytokines in murine peritoneal cells

Next, the production of proinflammatory cytokines such as IL-6 and TNFα was examined in the culture medium of murine peritoneal cells, M-BMDMs, and GM-BMDMs after Angptl2 stimulation. Secreted IL-6 and TNFα were detected only in the culture medium of mouse peritoneal cells after 6 and 24 h of Angptl2 treatment (IL-6: 3.7 ng/ml at 6 h and 15.0 ng/ml at 24 h, TNFα: 370.7 pg/ml at 6 h and 447.6 pg/ml at 24 h) by ELISA (Fig. 1D, left panels). In contrast, IL-6 and TNFα were not detected in the medium of Angptl2-treated M-BMDMs, GM-BMDMs, RAW264.7 cells, J774.1 cells, or murine splenic macrophages (Fig. 1D and supplemental Fig. 2). LPS induced IL-6 and TNFα secretion from all cell types tested (Fig. 1D and supplemental Fig. 2). The anti-inflammatory cytokine IL-10 was not detected in the medium of Angptl2-treated murine peritoneal cells (data not shown). We further demonstrated that Angptl2-stimulated murine peritoneal cells produced IL-6 and TNFα in a dose-dependent manner, reaching the maximal induction level to the same extent by 100 ng/ml LPS stimulation (Fig. 2A). Furthermore, heat-denatured Angptl2 (a condition under which LPS endotoxin cannot be denatured) lost its ability to induce IL-6 (Fig. 2B), indicating that the observed activity was not due to endotoxin contamination. These results indicate that Angptl2 induces production of pro-inflammatory cytokines in primary murine peritoneal cells.

Angptl2 upregulates pro-inflammatory cytokines in monocytes/macrophages

We sought to identify the cellular source of pro-inflammatory cytokines in mouse primary peritoneal cells. Adherent cells and non-adherent peritoneal cells were sorted to determine IL-6 concentration in the culture medium by ELISA at 24 h after Angptl2 stimulation. Adherent cells consisting of 2 phenotypes of cells—spreading cells and rounded cells—produced IL-6 to 59% of original cell population in response to Angptl2 (Fig. 2C). In contrast, non-adherent cells did not secrete IL-6. LPS stimulation also induced IL-6 production only in adherent cells (96% of original cells; data not shown).

Adherent cells in peritoneal cells consist mostly of monocytes and macrophages. 54% and 43% of peritoneal cells were F4/80^{+} and F4/80^{-} respectively. As expected, F4/80^{+} cells secreted IL-6 by Angptl2 stimulation, while F4/80^{-} cells did not (Fig. 2D).

Angptl2 induces pro-inflammatory gene expression

We further investigated the mRNA expression pattern of inflammation-related genes and alternative macrophage activation marker genes of Angptl2-treated murine peritoneal cells. qPCR analysis revealed that the expression of inflammation-related genes TNFα, IL-6, IL-1β, Ptgs2, Nos2, and CSF2 was greatly upregulated in peritoneal cells 3 h after Angptl2 stimulation compared to controls (17.8-, 126.7-, 153.1-, 120.5-, 203.4- and 850.7-fold, respectively) (Fig. 2E). In contrast, the expression of alternative activation markers arginase-1 (ARG1) and YM1 was unchanged, although the anti-inflammatory cytokine IL-10 was slightly upregulated (3.4-fold) (Fig. 2F). Together, Angptl2 strongly induces the inflammatory (classical) activation of macrophages.

Angptl2 modulates IL-1β production and cell surface markers of murine primary peritoneal monocytes/macrophages

While the expression of IL-1β mRNA was induced immediately, IL-1β protein was not detected in the extracellular medium of peritoneal cells after Angptl2 treatment (data not shown). Flow cytometry analysis showed that most F4/80$^+$ cells stimulated by Angptl2 accumulated more IL-1β protein intracellularly compared to controls, similar to LPS-stimulated cells (Fig. 3A, 3B and 3C, left). Western blot analysis revealed that IL-1β accumulated in an unprocessed precursor form (47 kDa) in the Angptl2- or LPS-stimulated cells (Fig. 3C, right), suggesting that Angptl2 induced the expression of IL-1β but did not induce activated inflammasomes, which are required for the generation of mature IL-1β.

We analyzed whether Angptl2 treatment resulted in alteration of surface markers of peritoneal cells. The fluorescence intensity of CD80, a maturation/activation marker of macrophages and DCs, increased 3.4-fold on F4/80$^+$ cells. The macrophage markers F4/80 and CD11b and the DC marker CD11c remained unaffected (Fig. 3D).

Angptl2 activates ERK, JNK, p38, and NF-κB signaling pathways in murine monocytes/macrophages

The release of pro-inflammatory cytokines from macrophages, such as IL-6, TNFα, and IL-1β, in response to LPS is mediated by the activation of signaling pathways of 3 major groups of MAP kinases (ERK, JNK, p38) and nuclear factor kappa B (NF-κB) [14,15]. Angptl2 induced the phosphorylation of all 3 MAPKs within 15 min, with a peak of ERK phosphorylation at 30 min and that of JNK and p38 phosphorylation at 15 min, declining until 120 min (Fig. 3E). Stat3 phosphorylation was observed at 120 min (Fig. 3E). The phosphorylation of IκB was also observed within 15 min, and continued until 2 h. The induction of phosphorylation of MAPKs and IκB by Angptl2 was weaker than those by LPS. Although LPS stimulation significantly decreased the total amount of intercellular IκB, Angptl2 stimulation reduced IκB slightly (Fig. 3E). It has been reported that ERK1/2 and Akt are phosphorylated by Angptl2 treatment in human umbilical vein endothelial cells (HUVECs) [16], but we did not detect Akt phosphorylation by Angptl2 stimulation in murine peritoneal cells (Supplemental Fig. 3).

Angptl2 induces nuclear translocation of NF-κB subunit p65 in murine peritoneal monocytes/macrophages and M-BMDMs

Phosphorylation of IκB leads to its ubiquitination and degradation, followed by NF-κB nuclear translocation [15]. Immunostaining of the RelA (p65) subunit of NF-κB showed its presence predominantly in the cytoplasm under basal conditions (data not shown) and the parallel control experiments of peritoneal adherent spreading macrophages (Fig. 4A, upper panel). Angptl2 and LPS increased the nuclear translocation of p65 moderately and robustly respectively (Fig. 4A, middle and lower panel).

Interestingly, Angptl2 clearly induced the nuclear translocation of p65 in M-BMDMs, similar to that in murine peritoneal cells (Fig. 4B). This translocation was not found in GM-BMDMs, RAW264.7 cells, or J774.1 cells, although it was strongly induced by LPS stimulation (Fig. 4C, and supplemental Fig. 4).

DISCUSSION

Here we showed that Angptl2 activates peritoneal monocyte/macrophages and strongly induced pro-inflammatory cytokine production. This specific effect of Angptl2 on peritoneal cells but not on BMDM suggests that peritoneal monocytes/macrophages are Angptl2 target cells and Angptl2 induces inflammation by directly regulating the activity of these cells.

Angptl2 was reported to play an important role as a mediator of the inflammatory process [10,17,18]. HSCs, endothelial cells, adipocytes, synovial-hyperplastic lining cells, lymphoid follicle cells, fibroblast-like and macrophage-like synoviocytes in the rheumatoid arthritic synovium, endothelial cells, and retinal cells were reported as Angptl2-producing cells. Endothelial cells (HUVECs and HACEs) and monocytes (THP-1 cells, synovial fluid-derived monocytes) were proposed as the target cells [10,17,18].

We discovered that Angptl2 activates mouse tissue-resident monocytes/macrophages and dramatically upregulated the gene expression of proinflammatory cytokines - TNFα (17.8-fold), IL-6 (126.7-fold), IL-1β (153.1-fold), and CSF2 (850.7-fold) - about half of the levels by LPS stimulation (Supplemental Fig. 5). By contrast, the expression of M2 macrophage-specific genes ArgI and Ym1 were unchanged by Angptl2 treatment, and IL-10 expression was induced slightly (3.45-fold), weaker than that by LPS stimulation (Supplemental Fig. 5). We also showed that Angptl2 induced TNFα, IL-6, and GM-CSF secretion in a dose-dependent manner, and the amount of secreted proteins treated by 1.0 μg/ml Angptl2 reached almost the same level as that by 0.1 μg/ml LPS stimulation (Fig. 1D and 2A). Even with the highest concentration of Angptl2, chemotactic activities of THP-1 cells and murine peritoneal cells were not observed in the present experiments (Supplemental Fig. 6). We therefore surmise that Angptl2 causes tissue inflammation through proinflammatory cytokine induction on tissue-resident monocytes/macrophages rather than promoting cell adhesion and cell migration.

Several groups have reported that Angptl2 induces IL-6 gene expression in mouse peritoneal macrophages [19], human ligamentum flavum fibroblasts [20], and the human keratinocyte cell line HaCaT [21]. The induction level of IL-6 and Angptl2 concentration in those experiments were only 1.7-fold with 5 mg/ml, 6-fold with 5 μg/ml, and 8-fold with 10 μg/ml, respectively. These induction levels, however, are much lower than that determined in this study (126.7-fold by 0.5 μg/ml Angptl2). These discrepancies might have arisen from the differences in the quality of recombinant protein used in individual reports, although the origins of recombinant Angptl2 protein have not been fully described in [19,21]. Endogenous Angptl2 is glycosylated, and posttranslational modification appears to be important for its physiological activities [6]. To obtain active Angptl2, we purified recombinant Angptl2 from the culture medium of Angptl2-Flag-expressing HEK293T cells (Fig. 1A), and confirmed that the same quality of recombinant protein supported HSC expansion.

Another potential caveat of the use of recombinant Angptl2 *in vitro* is the unspecific activity caused by osmotic stress. It is well established that osmotic stress acts as a stimulator for cytokine production [22]. Osmotic conditions are bound to change during stimulation with

high concentrations of Angptl2, because the ratio of protein in the total system increases. Unspecific activation leading to IL-6 gene expression might thus occur when 5–20 μg/ml Angptl2 is used. It is unclear whether such unspecific activation was occurred in these previous studies in which the results of control experiments were not described [19,20,21]. We strictly performed experiments, with carefully prepared control solutions, and confirmed that the nonspecific effects were practically negligible in our study.

We showed that treatment with Angptl2, similar to LPS, activates ERK, JNK, p38, and NF-κB in murine peritoneal monocytes/macrophages. It is important to elucidate the mechanisms responsible for such signaling activation in monocytes/macrophages. Recently we reported that Angptl2 inhibits the differentiation and promotes the repopulation of HSCs through its putative receptor, human leukocyte immunoglobulin-like receptor B2 and its mouse ortholog paired Ig-like receptor [8]. GST-Angptl2 protein generated by an *in vitro* translation system, as well as Angptl2-Flag recombinant protein from culture medium showed *ex vivo* HSC expansion activities. Meanwhile, we could not detect any effects of GST-Angptl2 on the peritoneal monocytes/macrophages (data not shown). It is reasonable to suppose that Angptl2 activates peritoneal monocytes/macrophages through a specific molecular system different from that of HSCs.

Other studies have suggested that Angptl2 exerts its effect through integrin family proteins; monocytes/macrophages, through integrin α4 or β2; and HUVECs, human LF fibroblasts, and human keratinocytes, through integrin α5β1 [10,20,21]. However, we could not observe the inhibitory effect of the integrin α4- or β2-neutralizing antibody on the binding of Angptl2 to THP-1 cells (data not shown). Thus, it remains unclear whether integrin family proteins mediates Angptl2's effects in the peritoneal cells.

Our data highlight a new immunomodulatory function of Angptl2 biology, including the existence of a yet unidentified molecular mechanism to induce the inflammatory reaction of monocytes/macrophages. This suggests that the suppression of Angptl2 signaling could be a therapeutic strategy for diseases associated with acute and chronic inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. Angptl2 directly activates resident murine peritoneal monocytes and macrophages and induces a drastic upregulation of expression of inflammatory genes in these cells.
2. Angptl2 induces activation of ERK, JNK, p38, and nuclear factor kappa B signaling pathways in murine peritoneal monocytes and macrophages.
3. Angptl2 does not induce upregulation of expression of inflammatory genes in bone marrow derived macrophages or macrophage cell lines.

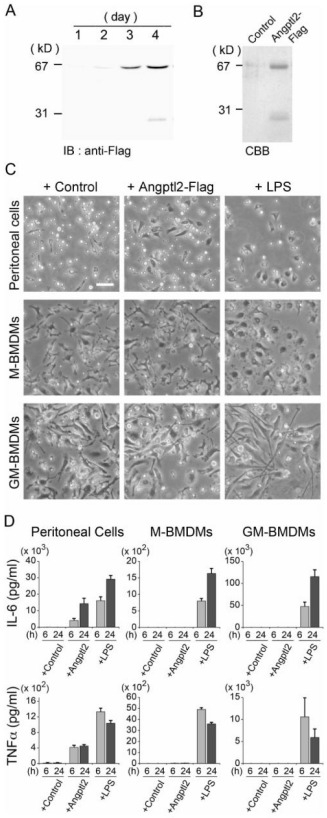

Figure 1. Morphological change and inflammatory cytokine production of mouse peritoneal macrophages by Angptl2

(A) Western blot analysis of Angptl2-flag protein secreted by Angptl2-Flag expressing 293T cells by immunoblotting with M2 α-Flag antibody. (B) Purified Angptl2-Flag and control solution as visualized by Coomassie brilliant blue (CBB) staining.

(C) Morphological changes of cultured murine peritoneal cells, bone marrow derived macrophages differentiated with M-CSF (M-BMDMs), or GM-CSF (GM-BMDMs). Cells were stimulated with equal volume of control solution, Angptl2-Flag (0.5 μg/ml), or LPS (0.05 μg/ml) for 24 h. (Scale bar, 20 μm)

(D) IL-6 and TNFα levels in conditioned medium of murine peritoneal cells, M-BMDMs or GM-BMDMs stimulated with the control solution, Angptl2-Flag (0.5 μg/ml), or LPS (0.1 μg/ml) for 24 h (n=3).

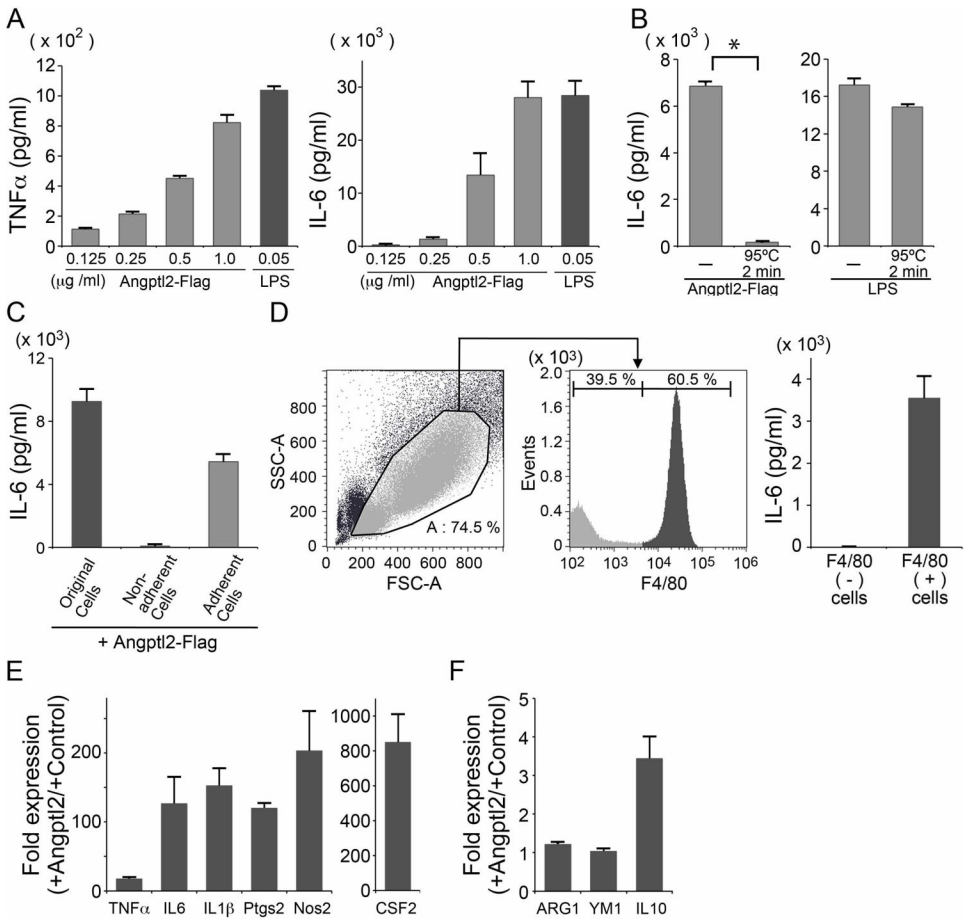

Figure 2. Characterization of inflammatory cytokine production by Angptl2

(A) ELISA analysis of TNFα and IL-6 in the conditioned medium of mouse peritoneal macrophages treated with Angptl2-Flag for 24 h (n=4). (B) ELISA analysis of IL-6 in the conditioned medium of mouse peritoneal macrophages treated with Angptl2-Flag (0.5 μg/ml) or LPS (0.1 μg/ml) that was boiled for 1min at 95°C for 24 h (n=4). * significantly different from control value, $p<0.05$. (C) ELISA analysis of IL-6 in the conditioned medium of mouse peritoneal adherent and non-adherent cells treated with Angptl2-Flag (0.5 μg/ml) for 24 h (n=4). (D) ELISA analysis of IL-6 in the conditioned medium of mouse peritoneal F4/80-positive or -negative cells cultured in RPMI-1640/10%FCS with Angptl2-Flag (0.5 μg/ml) for 24 h (n=3). (E–F) Regulation of pro-inflammatory and anti-inflammatory gene expression in murine peritoneal cells by control or 0.5 μg/ml Angptl2 for 3 h, as determined by qRT-PCR analysis. Fold change (ΔΔCt) of TNFα, IL-6, IL-1β, Ptgs2, Nos2 and CSF2 gene expression (E). Fold change (ΔΔCt) of ARG1, YM-1, and IL-10 gene expression (F). GAPDH was used for internal control (n=3).

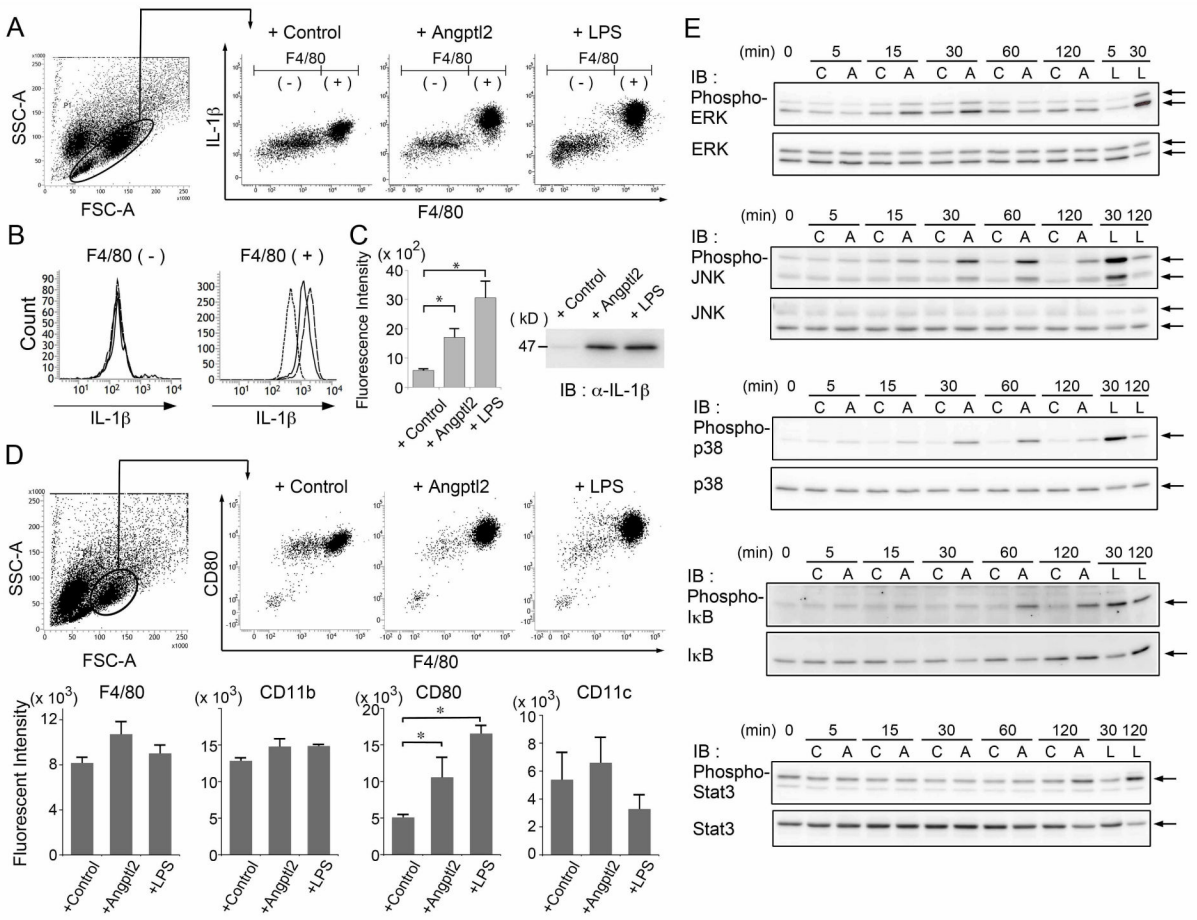

Figure 3. Effects of Angptl2 on markers and signaling pathways of murine peritoneal cells

(A–D) Murine peritoneal cells were treated with control solution, Angptl2-Flag (0.5μg/ml) or LPS (0.1 μg/ml) for 18 h, then scraped off for flow cytometry and western blot analyses. (A) Representative flow cytometry plots of F4/80 and IL-1β on gated cells. (B) Representative flow cytometry analysis of IL-1β protein in F4/80 -negative cells or -positive cells. ----; +Control, —; +Angptl2, – –, +LPS (C) IL-1β fluorescence intensity of F4/80-positive cells (n=6) and western blot analysis for IL-1β. Blot shown is representative of three separate experiments. (D) Representative flow cytometry plots of CD80 and F4/80, and florescence intensity of F4/80, CD11b, CD80, and CD11c on gated cells (n=4). (E) Induction of phospho-ERK, -JNK, -p38, -IκB, or -Stat3 in mouse peritoneal cells by Angptl2 or LPS was shown as representative of three separate experiments (C: Control solution, A: Angptl2, L: LPS). *significantly different from control value, $p<0.05$

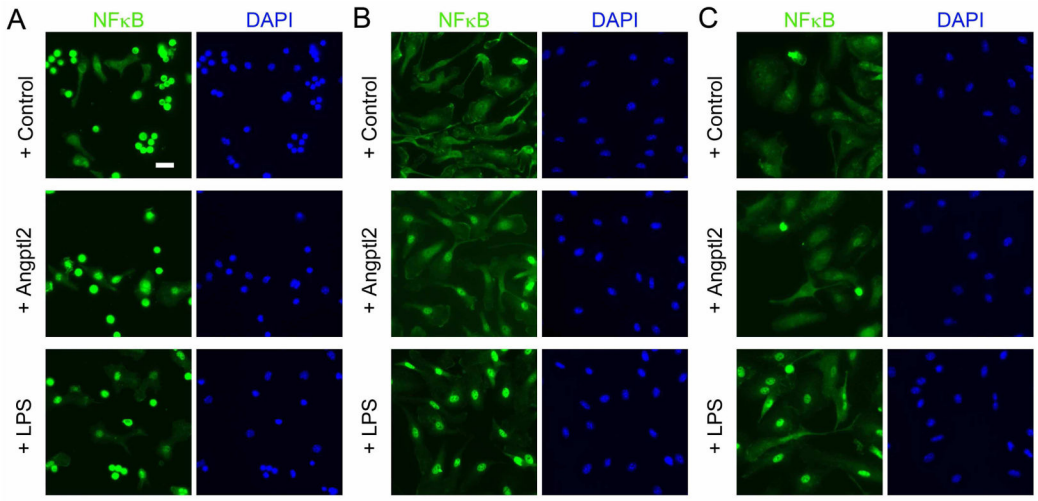

Figure 4. Nuclear translocation of NF-κB subunit p65 by Angptl2

(A) Murine peritoneal cells, (B) M-BMDMs, and (C) GM-BMDMs were stimulated with control solution, Angptl2, or LPS for 2 h and localization of the NF-κB p65 subunit was determined by immunostaining. Nuclei were visualized by DAPI. (Scale bar, 20 μm)