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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 elevated 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.

1. 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 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 microbicidal 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 [6–9]. Angptl2 was also reported to play a critical role in chronic inflammation of adipose tissue via inflammatory signaling in endothelial cells [10].

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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.

2. Materials and methods

2.1. 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).

2.2. 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 5–7 days. Murine macrophage-like cell lines RAW264.7 and J774.1 and human THP-1 cells were cultured in RPMI-1640 and FCS.

2.3. Reagents and protein

HEK293T cells stably expressing Angptl2-flag or HEK293T cells were seeded at a density of 6.0×10^6 cells in 24 ml 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.

2.4. 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).

2.5. 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 (0.5 μg/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.

2.6. 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).

2.7. 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.

2.8. Immunostaining

Murine peritoneal cells were allowed to adhere to 35 mm culture dish (Nalgene) for 2 h. M-BMDM and GM-BMDM were differentiated in 35 mm 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).

2.9. Statistics

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

3. Results

3.1. 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