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Angiopoietin-1 Elicits Pro-Inflammatory Responses in Monocytes and Differentiating Macrophages

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The angiopoietin/Tie2 system is an important regulator of angiogenesis and inflammation. In addition to its functions in endothelial cells, Tie2 expression on non-endothelial cells allows for angiopoietin ligands to stimulate the cells. Although Ang1 is a strong Tie2 receptor agonist, little is known regarding the effect of Ang1 on non-endothelial cells, such as monocytes and macrophages. In this study, we found that Ang1 functionally binds to and stimulates monocytes via p38 and Erk1/2 phosphorylation. Ang1-mediated monocyte stimulation is associated with pro-inflammatory cytokine TNF-α expression. We also determined that Ang1 switched macrophage differentiation toward a pro-inflammatory phenotype, even in the presence of an anti-inflammatory mediator. These findings suggest that Ang1 plays a role in stimulating pro-inflammatory responses and could provide a new strategy by which to manage inflammatory responses.

INTRODUCTION

Monocytes and macrophages generate a variety of cytokines in their environment, which are associated with inflammatory responses. A growing body of evidence reveals that monocytes can be differentially activated depending on the microenvironment (Ruffell et al., 2012). Upon activation, classically activated macrophages (also known as M1 macrophages) produce pro-inflammatory cytokines and kill invading pathogens, whereas alternatively activated macrophages (M2 macrophages) are involved in immunosuppressive and tissue repair functions (Mantovani et al., 2004). Tissue macrophages respond to environmental changes by tailoring their polarization status; therefore, M1 and M2 macrophages are the extremes of a variety of macrophage functional states (Mantovani et al., 2004; Mosser and Edwards, 2008).

Angiopoietins (Angs) are important regulators of vascular remodeling and maturation that act through the Tie2 receptor in endothelial cells (Thomas and Augustin, 2009). Among the different Ang isoforms, Ang1 and Ang2 attract a great deal of attention because of their involvement in angiogenesis and inflammation (Fiedler and Augustin, 2006). In terms of their inflammatory activity, Ang1 protects endothelial cells from inflammatory insults by blunting cell adhesion protein and tissue factor expression (Hwang et al., 2009; Kim et al., 2001; 2002). In contrast, non-endothelial Tie2 expression on leukocytes (De Palma et al., 2005; Lemieux et al., 2005) and hematopoietic cells (Arai et al., 2004) makes the cells Ang-responsive. Recent studies have demonstrated that Tie2-expressing monocytes, which are a subset of circulating monocytes, respond to Ang2 to promote tumor growth by augmenting pro-angiogenic and anti-inflammatory activity (Coffelt et al., 2010). Tie2 blocking studies demonstrated reduced tumor growth (Jeong et al., 2005; Melani et al., 2004); however, the specific role of Ang1 and Ang2 ligands on angiogenesis and inflammation in the tumors could not be discriminated. Given that pro-inflammatory M1-polarized macrophages acquire anti-tumoral activity by promoting immune responses (Guiducci et al., 2005; Saccani et al., 2006), the ability to modulate macrophage differentiation into pro-inflammatory M1 macrophages is of interest.

There is no evidence that Ang1 directly induces an inflammatory response, although Ang1 stimulated inflammatory cytokine production in an explant aortic ring (Aplin et al., 2006). In addition, Angs promote neutrophil and monocyte recruitment using the Tie2 receptor (De Palma et al., 2005; Lemieux et al., 2005); however, the precise mechanism and the cellular response to Angs have not been studied. Given the potency of Ang1 on Tie2 activation in endothelial cells, it is probable that Ang1 activates Tie2 on monocytes and macrophages and plays a role in regulating monocyte and macrophage function. However, whether Ang1 regulates monocyte and macrophage activity is unknown. In this study, we investigated the influence of Ang1 on monocytes and macrophages by determining activation marker and cytokine expression in human monocytes and macrophages.

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MATERIALS AND METHODS

Reagents

Recombinant human Ang1 and Ang2 proteins were purchased from R&D Systems (USA). Human macrophage colony stimulating factor (MCSF) and granulocyte macrophage colony stimulating factor (GMCSF) proteins were purchased from Prospec (Israel). Anti-Tie2 antibodies were purchased from Santa Cruz Biotechnology (USA), anti-vWF antibodies were purchased from DAKO (USA), and FITC- and Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch (USA). PE-conjugated anti-Tie2 antibody was purchased from R&D systems and PerCP-Cy5.5-conju-gated anti-human CD14, PE-conjugated anti-human CD163, and APC-conjugated anti-human CD11b antibodies were purchased from eBioscience (USA). All of the primary antibodies for Western blot analysis were purchased from Cell Signaling Technology (USA), and peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology. Culture media, fetal bovine serum (FBS) and other chemicals were purchased from Sigma-Aldrich (USA).

Cell culture

THP-1 monocytes were purchased from American Type Culture Collection (ATCC; USA), and human umbilical cord vein endothelial cells (HUVEC) were purchased from Lonza (USA). THP-1 monocytes were cultured in Dulbecco's minimal essential media containing 10% FBS, and HUVEC were cultured in M199 media containing 20% FBS supplemented with antibiotics.

Human peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats of normal donors on a Ficoll-Paque PLUS (GE Healthcare, USA) gradient in accordance with standard procedures. The Seoul National University Institutional Review Board approved these procedures. Monocytes were purified from PBMCs by magnetic bead sorting using a human monocyte isolation kit II (Miltenyi Biotec, Germany). Monocytes (> 90% $CD14^+$ cells) were cultured at a density of 10^6 cells/mL in RPMI media supplemented with 10% FBS and antibiotics.

RT-PCR and quantitative RT-PCR analysis

Total cellular RNA was extracted using RNeasy (Qiagen, USA) in accordance with the manufacturer's instructions. Equal amounts of total RNA were reverse transcribed using oligo(dT) primers and SuperScript reverse transcriptase (Invitrogen). The synthesized cDNA was used for the PCR reaction template. The PCR products were analyzed using 1.5% agarose gel electrophoresis and ethidium bromide staining. The PCR primer sequences are summarized in Table 1.

A SYBR Green PCR kit with Taq DNA polymerase (Invitrogen) and an i-Cycler PCR thermocycler (Bio-Rad, USA) were used for quantitative RT-PCR (qRT-PCR). The samples containing 20 ng cDNA were analyzed in triplicate. The values were normalized to β-actin expression levels and were expressed as the fold-increase relative to control values.

Immunohistochemistry

For immunocytochemistry, HUVEC and THP-1 monocytes were co-cultured on coverslips to approximately 70-80% confluence and fixed with 4% paraformaldehyde for 10 min. The cells were incubated with anti-Tie2 and von Willibrand Factor (vWF) antibodies followed by incubation with FITC- and Cy3-conjugated secondary antibodies. The nuclei were stained using 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, USA). The processed slides were mounted in mounting media and the signals were examined using confocal microscopy (FV1000, Olympus, Japan).

Flow cytometric analysis

The cells were incubated with His-tagged Ang1 (500 ng/ml × 10^5 cells) or bovine serum albumin (500 ng/ml × 10^5 cells) for 1 h at 4°C, then washed twice and incubated with anti-His antibody or mouse IgG for 1 h at 4°C. The cells were washed and incubated with FITC-conjugated secondary antibodies. The cells were incubated with fluorescence-conjugated anti-human CD14, CD163, and CD11b antibodies to detect macrophage marker expression followed by flow cytometry, which was performed using a FACScan flow cytometer with CELLQuest software (Becton Dickinson, UK). For detecting Tie2 expression, the cells were incubated with PE-conjugated Tie2 antibody for 1 h at 4°C followed by flow cytometry.

Monocyte differentiation

The PBMC-derived monocytes were differentiated using media containing 20 ng/ml MCSF or 25 ng/ml GMCSF to generate the mature macrophages. The macrophages were activated for 24 h with lipopolysaccharide (LPS, 100 ng/ml; *Salmonella enterica*), Ang1, and Ang2, as indicated.

Enzyme-linked immunosorbent assay (ELISA)

After the incubating the cells with Ang1 (300 ng/ml) for 24 h, the culture supernatants were harvested and centrifuged at 13,000 rpm to remove cell debris. TNF-α and IL-6 protein levels were measured using a Bio-Plex Pro™ Assay kit (Bio-Rad Laboratories, USA) according to the manufacturer's protocol.

Western blot analysis

THP-1 monocytes were incubated with 300 ng/ml of Ang1 protein for the indicated times. The cells were then lysed in a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail. The lysates were separated on 10% SDS-PAGE gels (50 μg per lane) and were transferred to nitrocellulose membranes. The membranes were incubated with the appropriate primary antibodies, i.e., phospho-p38, p-38, phospho-Erk1/2, Erk1/2, phospho-Akt, and Akt. Horseradish peroxidase-conjugated species-specific IgGs were used as secondary antibodies. An Enhanced Chemiluminescence Detection kit (Pierce, USA) was used to detect and visualize the bands.

Statistical analysis

The data are presented as the mean ± SEM. The statistical analyses were performed using Prism 5.0 software (GraphPad Software, Inc., USA) and Student's *t*-test was used to compare the differences between the groups; $P < 0.05$ was considered to be statistically significant.

RESULTS

Ang1 activates MAP kinase signaling in Tie2-expressing monocytes

To determine whether Ang1 can stimulate monocytes, Tie2 expression was analyzed in human monocytes by RT-PCR (Fig. 1A). Both peripheral blood mononuclear cells (PBMC) and THP-1 monocytes expressed the Tie2 transcript (Fig. 1A). To compare Tie2 expression levels between endothelial cells and monocytes, we determined Tie2 expression in co-cultured hu-

Table 1. Experimental PCR primer sequences

PCR targets	Forward primers (5′ to 3′)	Reverse primers (5′ to 3′)
Tie2	TGTTCCTGTGCCACAGGCTG	CACTGTCCCATCCGGCTTCA
IL-1ra	GTGTCAAGTCTGGTGATGAG	CACATGGAACAGAACTACCC
iNOS	ACAACAAATTCAGGTACGCTGTG	TCTGATCAATGTCATGAGCAAAGG
MRC1	GGCGGTGACCTCACAAGTAT	ACGAAGCCATTTGGTAAACG
β-actin	GGGAAATCGTGCGTGACATT	AGTTTCGTGGATGCCACAGG

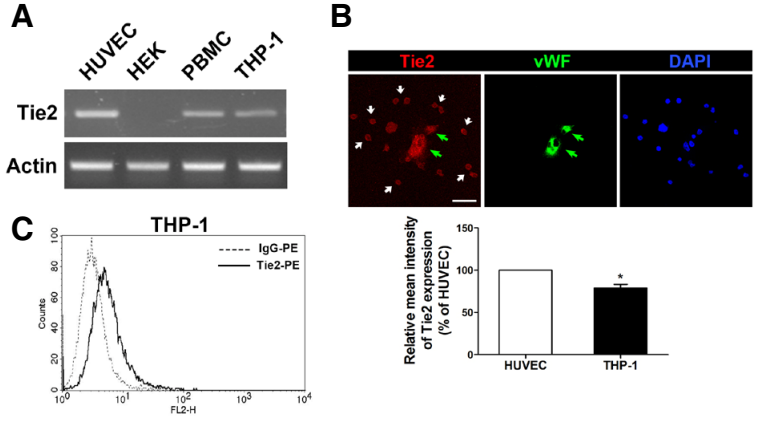

Fig. 1. Monocyte Tie2 receptor level determination. (A) Tie2 expression was analyzed in human endothelial cells (HUVEC), human peripheral blood mononuclear cells (PBMC), human monocytes (THP-1), and HEK293 cells (HEK) using conventional RT-PCR. Tie2 is expressed in both endothelial cells and monocytes. (B) HUVEC/THP-1 cell co-cultures were immunostained with anti-Tie2 (red) and anti-vWF (green) antibodies. The THP-1 monocytes were only positive for Tie2 (white arrows), whereas the HUVEC were positive for both Tie2 and vWF (green arrows). The relative mean Tie2 expression intensity was quantified using image analysis. The columns indicate the mean fluorescence intensity of each cell compared to that of HUVEC, and the bars indicate ± SEM (*P < 0.05 *vs.* HUVEC, N = 5). The scale bar represents 50 μm. (C) Cell surface expression of Tie2 was determined by flow cytometry using PE-conjugated anti-Tie2 and IgG antibodies in THP-1 monocytes. FL2-H indicates PE fluorescence intensity, and counts indicate the number of cells. Representative histogram from three independent experiments.

man umbilical vein endothelial cells (HUVEC) and THP-1 monocytes by immunocytochemistry. As expected, the THP-1 monocytes expressed the Tie2 receptor, and Tie2 expression levels were reduced in monocytes compared with HUVECs (Fig. 1B). Consistently, homogenous Tie2 expression in monocytes was detected by flow cytometry using anti-Tie2 antibody (Fig. 1C).

We next examined whether Tie2 expression accompanied functional Ang1 ligand binding to THP-1 monocytes. Ang1 binding was detectable on THP-1 monocytes, although there was less binding on THP-1 cells than on HUVECs (Fig. 2A). Because Ang1 binding-mediated fluorescence intensity can vary by cell size or protein affinity to the binding site, we quantified the binding by calculating the mean fluorescence intensity difference between control and Ang1-treated cells (Fig. 2B). To determine whether Ang1 binding to THP-1 monocytes can trigger intracellular signaling pathways, we examined the effect of Ang1 treatment on signaling pathway phosphorylation in THP-1 monocytes. Ang1 treatment evoked a rapid and sustained increase in p38 phosphorylation within 15 min (Figs. 2C and 2D). Ang1 treatment also elicited Erk1/2 phosphorylation, which peaked at 30 min and decreased thereafter (Figs. 2C and 2D). However, Akt pathway activation was not detected following both Ang1 and Ang2 treatment (Fig. 2C). In contrast, Ang2 treatment provoked slower and weaker p38 and Erk1/2 phosphorylation in monocytes (Figs. 2C and 2D). These data suggest that Ang1 is a more potent activator of the p38 and Erk1/2 pathways in THP-1 monocytes than Ang2.

Ang1 induces pro-inflammatory monocyte activation

Given that monocyte activation causes cytokine production (Mosser and Edwards, 2008), we examined whether Ang1 can also induce TNF-α expression in THP-1 monocytes using quantitative RT-PCR and ELISA. After a 24 h treatment, Ang1 significantly increased TNF-α mRNA and protein expression (Figs. 3A and 3B). When THP-1 monocytes were pre-treated with pathway-specific inhibitors including the Erk1/2 inhibitor PD98059 (10 μM) and the p38 inhibitor SB203580 (10 μM), the Ang1-induced increase in TNF-α mRNA expression was abolished (Fig. 3C). We also determined whether Ang1 had any effect on pro-inflammatory marker expression in THP-1 monocytes. Incubation of THP-1 monocytes with Ang1 for 24 h led to a modest up-regulation of inducible nitric oxide synthase (iNOS), an M1 marker gene, and down-regulation of mannose receptor 1 (MRC1), an M2 marker gene (Fig. 3D) (Mantovani et al., 2004). These findings demonstrate that Ang1 can enhance pro-inflammatory cytokine and marker expression in monocytes.

Ang1 is involved in PBMC monocyte differentiation to pro-inflammatory macrophages

To investigate the effect of Ang1 on monocyte to macrophage differentiation, PBMC monocytes were incubated with Ang1 in the presence of the differentiation inducers MCSF or GMCSF. All of the populations examined were CD11b-positive, which is a mature macrophage marker (Fig. 4A, right column). The scatter plots demonstrated that MCSF successfully induced M2 macrophage differentiation by illustrating CD14/CD163 double-positive populations (Q2) (Fig. 4A). Similar to LPS (a well-known M1 inducer) treatment with MCSF to PBMC monocytes, when Ang1 was added together with MCSF to PBMC monocytes, M2 phenotype acquisition was impaired (Figs. 4B and 4C), suggesting that Ang1 treatment switched the macrophage differentiation toward the pro-inflammatory M1 phenotype.

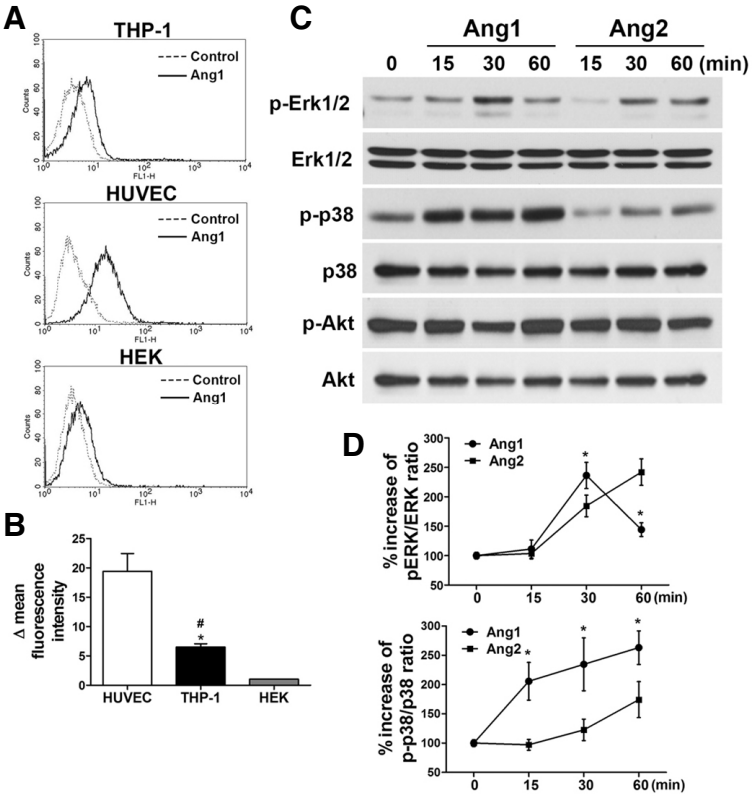

Fig. 2. Ang1 binds to and activates monocyte cellular signaling. (A) Bovine serum albumin (Control, dashed lines) and His-tagged Ang1 (Ang1, lines) were incubated with the cells and stained with fluorescent-conjugated antibodies. The binding was analyzed using flow cytometry. The data are representative of three independent experiments. FL1-H indicates FITC fluorescence intensity, and counts indicate cell number. (B) The mean fluorescence intensity difference was measured from the mean fluorescence intensity of each Ang1-positive cell and subtracted from the control in (A). The columns indicate mean fluorescence intensity difference, and the bars indicate ± SEM (*P < 0.05 *vs.* HUVEC; $^{\#}P$ < 0.05 *vs.* HEK, N = 3). (C) Western blot analysis of MAPK phosphorylation. Serum-starved THP-1 monocytes were treated with 300 ng/ml Ang1 and Ang2 for the indicated times. p38, Erk1/2, and Akt phosphorylation was measured. Ang1 treatment induced rapid and strong p38 and Erk1/2 activation compared with Ang2. Akt pathway activation was not detected by both Ang1 and Ang2 treatment. (D) Densitometric analyses demonstrate the relative ratios of phosphorylated Erk1/2 versus total Erk1/2 and phosphorylated p38 versus total p38. The dots indicate the mean of the relative ratio, and the bars indicate ± SEM (*P < 0.05 *vs.* Ang2 at the indicated times, N = 3).

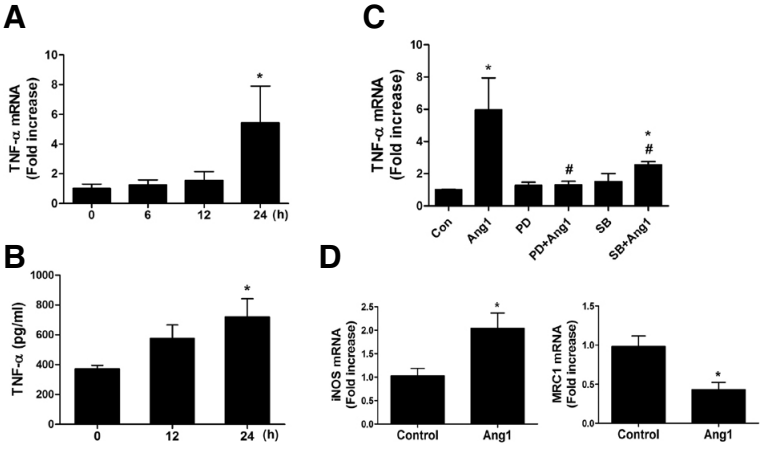

Fig. 3. Ang1 treatment elicits a pro-inflammatory response in monocytes. (A) TNF-α gene expression in THP-1 monocytes was analyzed by qRT-PCR. The cells were incubated with Ang1 (300 ng/ml) for the indicated times, and the total RNA was isolated. The columns indicate the mean of the fold change, and the bars indicate ± SEM (*P < 0.05 *vs.* 0 h, N = 3). (B) TNF-α protein expression in cell culture supernatants was assessed using ELISA. After incubating THP-1 monocytes with Ang1 for 24 h, the supernatant was harvested. The columns indicate the mean protein amount, and the bars indicate ± SEM (*P < 0.05 *vs.* 0 h, N = 3). (C) Pathway-specific inhibition of Ang1-induced TNF-α gene expression in monocytes. THP-1 monocytes were pretreated with Erk1/2 (PD; PD58059, 10 μM) and p38 (SB; SB203580, 10 μM) inhibitors for 30 min before Ang1 treatment for 24 h. TNF-α gene expression was assessed using qRT-PCR. The columns indicate the mean of the fold change, and the bars indicate ± SEM (*P < 0.05 *vs.* control; $^{\#}P$ < 0.05 *vs.* Ang1, N = 3). (D) The effect of Ang1 treatment on iNOS and MRC1 gene expression. THP-1 monocytes were treated with Ang1 for 24 h, and iNOS and MRC1 gene expression levels were determined using qRT-PCR. The columns indicate the mean of the fold change, and the bars indicate ± SEM (*P < 0.05 *vs.* control; $^{\#}P$ < 0.05 *vs.* Ang1, N = 3).

To determine whether Ang1 also modulates macrophage cytokine expression during differentiation, we measured cyto-kine levels in the cell culture supernatants by ELISA. As expec-ted, Ang1 markedly increased macrophage pro-inflammatory TNF-α secretion during MCSF stimulation, whereas Ang2 did not affect TNF-α secretion (Fig. 5A). In addition, both Ang1 and Ang2 treatment slightly increased macrophage interleukin-6 (IL-6) secretion (Fig. 5A). We next examined whether the Ang1-mediated pro-inflammatory macrophage polarization was accompanied by changes in M2-related gene expression. RT-PCR analysis revealed that Ang1 treatment down-regulated the mRNA expression of MRC1 and IL-1 receptor antagonist (IL-1ra) (Fig. 5B), which are M2 differentiation markers. These results suggest that Ang1 prevents macrophages from acquiring the M2 phenotype and promotes the M1 phenotype during MCSF stimulation.

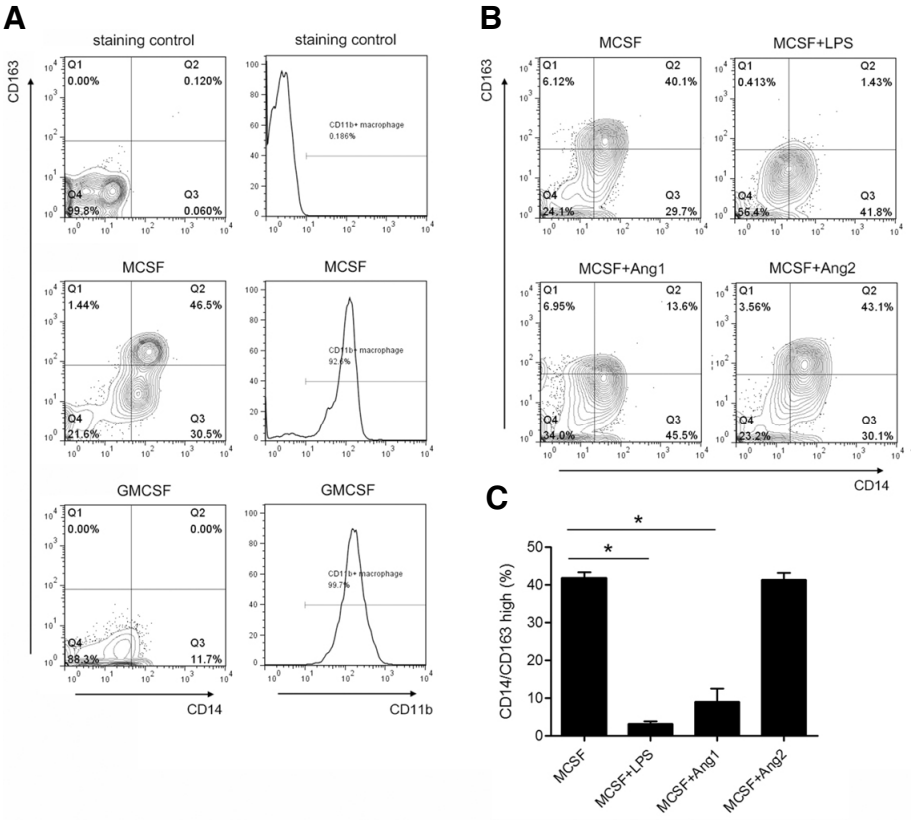

Fig. 4. Ang1 switches macrophages toward a pro-inflammatory phenotype during differentiation. (A) Representative scatter plots of primary macrophages that were generated from PBMC monocytes. The dot plots indicate the CD14- and CD163-expressing macrophage population (left column). The histograms indicate CD11b expression, which is a marker of mature macrophages (right column). (B) Representative dot plots indicating CD14/CD163 expression during differentiation in the presence of LPS, Ang1 and Ang2. The stimulants were added 24 h before examination. (C) Quantitative analysis of the proportion CD14/CD163 double-positive cells in (B); the columns indicate the mean of the CD14/CD163 double-positive population, and the bars indicate ± SEM (**P* < 0.05 *vs.* MCSF, N = 4).

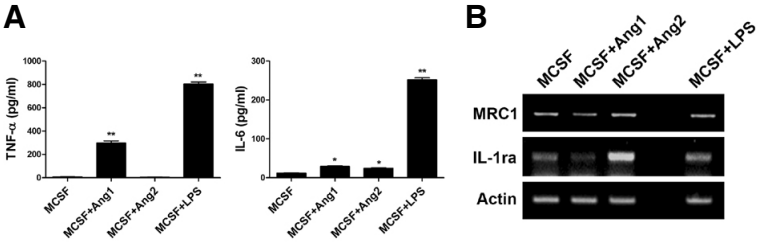

Fig. 5. The effects of Ang1 treatment on cytokine and marker expression in differentiating macrophages. (A) MCSF-induced differentiating macrophages were incubated with LPS, Ang1 and Ang2 for 24 h. Cytokine levels, including TNF-α and IL-6, were measured using ELISAs. The columns indicate the mean cytokine concentrations (pg/ml), and the bars indicate ± SEM (**P* < 0.05 *vs.* MCSF; ***P* < 0.01 *vs.* MCSF, N = 3). (B) RT-PCR analysis of macrophage marker expression. Total RNAs were extracted from the cell lysates from (a), and the cDNA was analyzed. Ang1 treatment markedly down-regulated MRC1 and IL-1ra gene expression. The results are representative of three independent experiments.

DISCUSSION

In this study, we investigated the influence of Ang1 treatment on monocyte and macrophage cellular responses. We found that monocyte expresses Tie2 and that Ang1 binds to and functionally activates p38 and Erk1/2 to induce pro-inflammatory TNF-α in monocytes. We also determined that Ang1 promotes pro-inflammatory macrophage differentiation.

It is interesting that Ang1 treatment switches macrophage differentiation to a pro-inflammatory state because several studies have suggested that Ang1 plays an anti-inflammatory role, especially in endothelial cells. Ang1 potentiates endothelial integrity and dampens inflammation-induced vascular leakage *in vivo* (Baffert et al., 2006; Thurston et al., 2000; 2005). Conversely, other studies have suggested that Ang1 may be pro-inflammatory; for example, Ang1 treatment stimulates inflammatory cytokine production in an aortic ring explant culture (Aplin et al., 2006), Ang1 and Ang2 promotes leukocyte recruitment (Coffelt et al., 2010; De Palma et al., 2005; Lemieux et al., 2005), and Ang1 and Tie2 proteins are elevated in inflammatory diseases such as rheumatoid arthritis and kidney disease (Gravallese et al., 2003; Long et al., 2008). The reason for the controversy among studies is unclear; however, it appears to stem from the Ang1 target cells. Although most studies investigating the anti-inflammatory actions of Ang1 have focused on endothelial cells, the effect of Ang1 on the leukocyte itself has received less attention. In this regard, our results demonstrated that Ang1 binds to and functionally activates cellular signaling to induce inflammatory cytokine production in monocytes, supporting the concept that Ang1 directly contributes to pro-inflammatory responses in monocytes.

In the present study, we found specific binding of Ang1 to THP-1 monocytes. Because Ang1 binding to cells can be varied by cell size or protein affinity to the binding site, it is not

easy to directly compare Ang1 binding affinity to different cells. Nonetheless, the reduced mean fluorescence intensity difference using flow cytometry (Fig. 2B) suggested that there are fewer Ang1 binding sites on THP-1 monocytes compared with HUVECs. Although Ang1 binding to THP-1 monocytes was reduced, its ability to activate cellular signaling pathways was not diminished. We observed that treatment with a similar Ang1 dose (300 ng/ml) as endothelial cells (Harfouche et al., 2003) activated cellular signaling pathways in THP-1 monocytes. We sought to understand that the Ang1-induced monocyte responses are directly and/or solely mediated by Tie2 using a blocking peptide against Tie2 (Ahmad et al., 2010); however, Ang1-induced TNF-α expression was not interfered by the blocking peptide even at sub-millimolar concentration (Supplementary Fig. 1). These results suggest that Ang1-induced TNF-α expression is not directly mediated by Tie2 or Tie2 is not the sole mediator of the response. The latter possibility is supported by a previous study - the blocking peptide against Tie-2 only partially interfered with the Ang1-induced enhancement of HUVEC migration (Ahmad et al., 2010). The incomplete interference of HUVEC migration even at sub-millimolar concentration suggests that the blocking peptide has low affinity or specificity to Tie2. The finding of highly specific inhibitors of Tie2 would enable us to estimate what proportion of the Ang1-induced TNF-α expression is mediated by Tie2.

In endothelial cells, phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPKs Erk1/2 and p38) pathway activation is recognized to be downstream of Ang1-induced cellular signaling (Harfouche et al., 2003). However, little is known regarding Ang1-induced cellular signaling in monocytes and macrophages. Although one report demonstrated that Ang1 inhibits LPS-induced NF-κB activation in mouse macrophages, they did not describe how Ang1 abolished macrophage NF-κB signaling (Gu et al., 2010). We demonstrated that Ang1 induced Erk1/2 and p38 phosphorylation in THP-1 monocytes. Given a previous finding that Erk or p38 MAPK are important for macrophage TNF-α induction (Rutault et al., 2001; Schindler et al., 2007), potent Ang1-mediated MAPK (p38 and Erk1/2) signaling pathway activation corroborates the hypothesis that Ang1 elicits pro-inflammatory responses in monocytes.

A previous study demonstrated that Ang2 converts macrophages to the anti-inflammatory or M2-polarized phenotype (Coffelt et al., 2010). In our experimental settings, we confirmed that Ang2 switches GMCSF-induced pro-inflammatory macrophages to the anti-inflammatory phenotype (Supplementary Fig. 2). Interestingly, Ang1 is a more potent activator of the MAP kinase signaling pathway than Ang2 (Figs. 2C and 2D). Considering that Ang2 possesses a partial agonistic function on the Tie2 receptor (Yuan et al., 2009), there are two possible explanations. One is that Ang2-induced Tie2 activation is not enough to produce the same response as Ang1 does. Another is that other receptors such as integrins (Bezuidenhout et al., 2009; Carlson et al., 2001) could be involved in Ang2-induced responses in monocytes. The underlying mechanism of Ang2-induced activation of monocytes remains to be investigated.

Our results suggest that Ang1 modestly elicits early pro-inflammatory monocyte activation by inducing TNF-α. Given that TNF-α action in the early inflammatory response activates vascular endothelium to induce cell adhesion molecule expression, modest TNF-α induction might be involved in facilitating monocyte adhesion and transmigration into tissues. Thus, Ang1 may play a role in early pro-inflammatory monocyte priming and augmenting the pro-inflammatory functions of committed macrophages.

In summary, our results demonstrate that Ang1 is a potent stimulator of p38 and Erk1/2 MAPKs and induces pro-inflammatory monocyte activation. We also demonstrate that Ang1 plays a role in monocyte differentiation to stimulate a pro-inflammatory M1 phenotype acquisition during differentiation. Therefore, our study could provide a new strategy for modulating inflammatory responses.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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