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Article

Human mast cells synthesize and release angiogenin, a member of the ribonuclease A (RNase A) superfamily

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ABSTRACT

ANG is a plasma protein with angiogenic and ribonucleolytic activity implicated in tumor growth, heart failure, wound healing, asthma, and the composition of the adult gut microflora. Human mast cells (HuMC) are similarly associated with modulation of vascular permeability, angiogenic processes, wound healing, and asthma. We hypothesized that HuMC express and secrete ANG in response to divergent stimuli. ANG expression was evaluated in the LAD2 HMC, the HMC-1, and CD34$^+$-derived HuMC, following exposure to live *Escherichia coli*, TLR ligands, or neuropeptides and following FcεRI aggregation. Expression and production of ANG were determined by microarray analysis, qRT-PCR, confocal microscopy, and ELISA. Microarray analysis showed that ANG is up-regulated by LAD2 cells exposed to live *E. coli*. qRT-PCR analysis revealed that LAD2, HMC-1, and HuMC constitutively expressed *ANG* mRNA and that it was up-regulated by exposure to *E. coli*. Activation of HuMC by FcεRI aggregation resulted in release of small amounts of ANG (<100 pg/mL), whereas compound 48/80, NGF, LPS, PGN, and flagellin activated HuMC to secrete >160 pg/mL ANG. These observations demonstrate that HuMC store and secrete ANG to a variety of stimuli and suggest that MC-derived ANG is available in the subsequent inflammatory response. *J. Leukoc. Biol.* 86: 1217–1226; 2009.

Introduction

ANG (RNase 5) was isolated originally based on its ability to stimulate vasculogenesis in the chick chorioallantoic membrane [1] and is one of the most potent inducers of neovascularization in experimental models in vivo [2]. The 14.1-kDa ANG protein has 35% aa sequence identity with human pancreatic RNase and displays ribonucleolytic activity [3]. ANG expression and its physiological role in human disease are not fully understood. Mice have four *ANG* genes clustered together on chromosome 14; humans, nonhuman primates, and rats have only a single *ANG* gene. ANG was isolated as a tumor angiogenic factor based solely on its angiogenic activity, and subsequent studies have often focused on its angiogenic capacity. However, several recent reports have implicated ANG in rRNA transcription in cancer cells [4], chronic heart failure [5], wound healing [6], and asthma [7–10]. ANG may also have antimicrobial activity, although data are not consistent in this regard [11, 12].

MCs have been similarly related to innate immune responses, wound healing, and asthmatic inflammation through production of proinflammatory cytokines, leukotrienes, and chemokines. MCs also release vascular endothelial growth factor, which can modulate vascular permeability [13] and contribute to tumor growth [14]. Because of these associations, we questioned whether MCs express and secrete ANG [2]. As will be shown, we found that HuMC express, store, and secrete ANG, which was released in response to FcεRI aggregation, TLR ligands, and G-protein-coupled receptor activation.

MATERIALS AND METHODS

HuMC culture

LAD2 MCs [15] were cultured in serum-free media (StemPro-34 SFM, Life Technologies, Gaithersburg, MD, USA), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 50 μg/ml streptomycin, and 100 ng/ml SCF. The cell suspensions were seeded at a density of 10^5 cells/ml and maintained at 37°C and 5% CO_2. Cells were fed by hemi-depletion of media once/week. HMC-1 MCs [16] were cultured in Iscove's medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Biosource International, Rockville, MD, USA) in a humidified atmosphere of 5% CO_2 in air at 37°C.

Human peripheral blood-derived CD34$^+$ cells were cultured in StemPro-34 SFM, supplemented with 2 mM L-glutamine, 50 μg/ml streptomycin, 100

Abbreviations: ANG=angiogenin, CGRP=calcitonin gene-related peptide, C_t=threshold cycle, h=human, HMC-1=human mast cell line-1, HuMC=human cultured mast cell(s), LAD=laboratory of allergic disease mast cell line, MC=mast cell, NGF=nerve growth factor, NIAID=National Institute of Allergy and Infectious Diseases, PGN=peptidoglycan, qRT-PCR=quantitative RT-PCR, SCF=stem cell factor, SFM=serum- and feeder-free medium

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IU/ml penicillin, 100 ng/ml SCF, and 100 ng/ml rhIL-6 (PeproTech, Inc., Rocky Hill, NJ, USA). rhIL-3 (30 ng/ml) was added for the first week. Half of the culture medium was replaced every 7 days. Cultures at 8–10 weeks consisted of >99% HuMC [17]. In cases where ANG expression was measured, MCs were grown in StemPro-34 media lacking the supplement component.

Escherichia coli

The *E. coli* K12 strain purchased from American Type Culture Collection (Manassas, VA, USA) was cultured in 8 g/l Trypton (Fisher Scientific, Fair Lawn, NJ, USA) and 0.5 g/l NaCl (Sigma-Aldrich, St. Louis, MO, USA). This media (20 mL), in a sterilized, 50-ml tube, were inoculated with *E. coli*, incubated at 37°C for 16 h with constant shaking at 250 rpm and spread on a 1.5% agar plate containing Luria-Bertani broth (Molecular Biologicals, Inc., Columbia, MD, USA) using a platinum loop. Single colonies of bacteria were picked and placed in 50 ml sterilized tubes containing 20 ml bacteria culture media. These cultures were incubated at 37°C for 16 h and then centrifuged at 3000 rpm for 15 min. The supernatant was discarded, and the *E. coli* pellet was resuspended in 20 ml PBS and centrifuged at 3000 rpm for 15 min. The supernatant was discarded, and the concentration of *E. coli* was adjusted to 5×10^9 bacteria/ml with PBS. To measure *E. coli* concentration in liquid culture, the turbidity of the culture was measured as light absorbance readings on a spectrophotometer at 600 nm. A standard curve of *E. coli* CFU was established and used to determine the number of *E. coli* CFU/mL culture. CFU measurements were performed by making tenfold serial dilutions of the liquid culture, which was seeded onto agar plates, and the numbers of colonies/plate were determined subsequently. Additionally, the Alexa Fluor 488-labeled *E. coli* K12 strain (Molecular Probes, Inc., Eugene, OR, USA) was used for flow cytometry and in some confocal experiments.

E. coli internalization by MCs

LAD or CD34-derived HuMC cultures were centrifuged at 1000 rpm for 5 min, the supernatant discarded, and the cells resuspended in cell culture media adjusted to 5×10^5 cells/ml. The cell suspension (5 ml) was then added to a 25-cm^2 culture flask, incubated for 30 min at 37°C in a 5%-CO$_2$ incubator, and 5 μl 5×10^9 bacteria/ml added (bacteria:cell ratio of 10:1). The cell-bacteria suspension was then maintained at 37°C in a 5%-CO$_2$ incubator for times specified. After incubation, the cell-bacteria suspension was centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. The pellet was resuspended in 20 ml cell culture media containing antibiotics and centrifuged at 1000 rpm for 5 min. The procedure was repeated and the pellet resuspended in 2.5 ml culture media containing antibiotics and incubated for times specified.

MTT assay

The MTT assay was performed to determine cell viability. Briefly, MTT (Sigma-Aldrich) was dissolved at 5 mg/ml in PBS and stored in the dark at 4°C. MCs were cocultured with live *E. coli* for 24 h at 37°C in complete StemPro media in a 96-well plate. A 10-μl aliquot of MTT (5 mg/ml in PBS) was added to each well, and the plate was incubated at 37°C for 5 h. The plate was spun at 400 *g* for 10 min, and the media were removed. Solubilizing solution [100 μl; 20% (w/v) SDS and 50% (v/v) N,N-dimethyl formamide)] in deionized water] was added to each well, and the plate was incubated overnight at 37°C. OD of the formazan crystals was measured at 570 nm.

Flow cytometry

E. coli internalization by MCs was evaluated by incubating cells with or without cytochalasin D or mannose for 30 min in a CO$_2$ incubator. Cells were next incubated with or without Alexa Fluor 488-labeled *E. coli* for 30 min in a CO$_2$ incubator. The suspension was centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. The pellet was resuspended in 1 ml PBS and centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. This procedure was repeated, and the pellet was then suspended in 0.25 ml PBS. The cells were analyzed by FACScan (BD Biosciences, Mountain View, CA, USA) after addition of 0.75 ml 0.4% trypan blue solution (Sigma-Aldrich) to a 0.25-ml cell suspension to quench fluorescence associated with bacteria on the cell surface.

Microarray analysis

The array chips are custom-made by NIAID (human sequence chip series "sa") and consist of 13,971 oligonucleotides, each of which represents a unit gene cluster. All of these elements are 70-mer oligonucleotides synthesized by Qiagen Operon Inc. (Valencia, CA, USA), which can hybridize human cDNA synthesized from a human mRNA library.

Total RNA was extracted from LAD3 HuMC incubated with or without *E. coli* for the times specified. RNA was then purified using an RNeasy mini kit (Qiagen Operon Inc.). For probe generation, RNA was converted to dscDNA by RT and Cy3- or Cy5-labeled. Oligo dT 20-mer was first annealed to the RNA, and then reverse transcription was performed using Superscript II RT (Invitrogen Corp., Carlsbad, CA, USA). Cy3-labeled dUTP (Amersham Biosciences AB, Uppsala, Sweden) was added along with unlabeled dNTPs to make a Cy3-labeled probe for *E. coli*-nonexposed samples (control), and Cy5-labeled dUTP (Amersham Biosciences AB) was added along with unlabeled dNTPs to make a Cy5-labeled probe for *E. coli*-exposed samples. The probes were then purified using a Vivaspin 30K centrifugal filter device (Vivascience AG, Hannova, Germany) in Tris-EDTA buffer. Probes were quantitated at 550 nm for Cy3 or at 650 nm for Cy5. The microarray chip was incubated with a blocking mixture that contained 5× SSC, 1% BSA, and 0.1% SDS at 42°C for 1 h, and the hybridization was performed by adding 50 pmol-labeled probes to a reaction mix that included 10 μg human Cot-1 DNA, 1 μg Poly dA40-60, 4 μg yeast transfer-RNA (Invitrogen Corp.), 5× SSC, and 0.1% SDS in 25% formamide solution. The mixture was heated at 98°C for 2 min, applied to a microarray slip after cooling, and incubated overnight at 42°C. The arrays were then washed sequentially in 1× SSC and 0.05% SDS and 0.1× SSC. Next, the arrays were centrifuged for 5 min at 500 rpm for drying and scanned with a GenePix 4000A microarray scanner (Axon Instruments, Inc., Union City, CA, USA). Data were analyzed using National Institutes of Health-designed software called "mAdb" located on the website at http://nciarray.nci.nih.gov/, and the data with a *P* value ≤0.02, compared with control, with a signal-to-noise ratio 2.0 or greater, were selected for further analysis.

qPCR

Total RNA was purified from MCs as described in "Microarray analysis". Contaminating genomic DNA was digested and thus removed by incubating 10 μg total RNA with 2 U DNase (amplification grade; Life Technologies) in DNase buffer (200 mM Tris-HCl, 20 mM MgCl$_2$, 500 mM KCl, pH 8.4; Life Technologies) and RNase-free H$_2$O for 10 min at room temp. RNA was then precipitated with 3 M $C_2H_2O_2Na$ (pH 5.2; Sigma-Aldrich).

Treated RNA (1 μg) was incubated with 0.5 μg oligo(dT) (Life Technologies) at 70°C for 10 min and then added to a mixture containing First-Strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl$_2$, pH 8.3; Life Technologies), 10 mM DTT, 10 mM each dNTP, sterile water (Sigma-Aldrich), and 200 U Moloney murine leukemia virus RT enzyme (Life Technologies). This mixture was incubated at 37°C for 1 h and then at 70°C for 10 min.

Duplex qPCR amplification of the ANG and β-actin genes in each sample was performed using qPCR master mix containing AmpliTaq Gold® DNA Polymerase Ultra Pure, Uracil-DNA glycosylase, dTNPs with dUTP, and optimized buffer components (Applied Biosystems, Foster City, CA, USA). cDNA (100 ng) was used in each qPCR assay, and primers were designed using Primer Express software (Applied Biosystems). All reactions were performed in triplicate for 40 cycles. Samples were normalized using the C_t of the internal control gene (β-actin) and target gene using the $\Delta\Delta C_t$ method, according to the formula $\Delta\Delta C_t = \Delta C_{tsample} - \Delta C_{tcontrol}$, where the controls are untreated cells. Each "n" represents an experiment executed independently, representing a different RNA sample.

TABLE 1. Antimicrobial Gene Expression by HuMC following Exposure to *E. coli*

Gene	Description	Fold induction at 4 hr	Fold induction at 8 hr	Fold induction at 24 hr
ANG	angiogenin-RNase family	**2.36**	**2.33**	**3.81**
DEFA1	defensin alpha 1	**7.85**	**3.84**	**6.57**
DEFA4	defensin alpha 4	1.26	1.42	1.18
DEFA5	defensin alpha 5	**3.76**	0.92	1.22
DEFA6	defensin alpha 6	0.81	0.87	1.12
DEFB1	defensin beta 1	**3.70**	1.57	1.55
DEFB2	defensin beta 2	1.69	1.68	1.19
CAMP	cathelicidin	**2.08**	1.72	1.50

Confocal microscopy

After 4 or 8 weeks of culture, CD34+-derived HuMC were washed with PBS, and cytospin slides were prepared. Slides were incubated in 2% paraformaldehyde in PBS (pH 7.4) for 15 min, rinsed with PBS, and incubated with 0.1% saponin in PBS for 15 min. Slides were washed once with PBS and incubated for 1 h with TBS containing 5 μg/ml mouse mAb for human ANG (Sigma-Aldrich) or rabbit mAb for human tryptase (Calbiochem, San Diego, CA, USA). Primary antibody-binding was detected using 20 μg/ml Texas Red-conjugated goat anti-mouse antibody (Abcam, Cambridge, UK) or Alexa Fluor 488-conjugated anti-rabbit IgG (Molecular Probes Inc.). Rabbit and mouse IgG (5 μg/ml) was used as an isotype control (R&D Systems, Minneapolis, MN, USA). Slides were examined using a ×100 objective under a TCS-NT/SP laser-scanning confocal microscope (Leica, Heidelberg, Germany), as described.

Statistical analysis

Each experiment was performed at least three times, and values represent mean of $n = 3 \pm$ SEM. *P* values were determined by Student's *t*-test (between groups) or one-way ANOVA (comparing more than two groups).

RESULTS

HuMC express angiogenin following exposure to *E. coli*

An important consequence of HuMC exposure to *E. coli* would be the production of molecules that would promote bacterial killing and promote vascularization to clear these pathogenic organisms. The expression of some antimicrobial genes, such as defensins, following *E. coli* exposure for 4, 8, and 24 h, was confirmed [18] by microarray analysis We found that *E. coli* exposure up-regulated members of the defensin family (α1, α5, and β1), cathelicidin, as well as ANG (**Table 1**). As *ANG* expression had not been examined heretofore in MCs, we selected this gene for

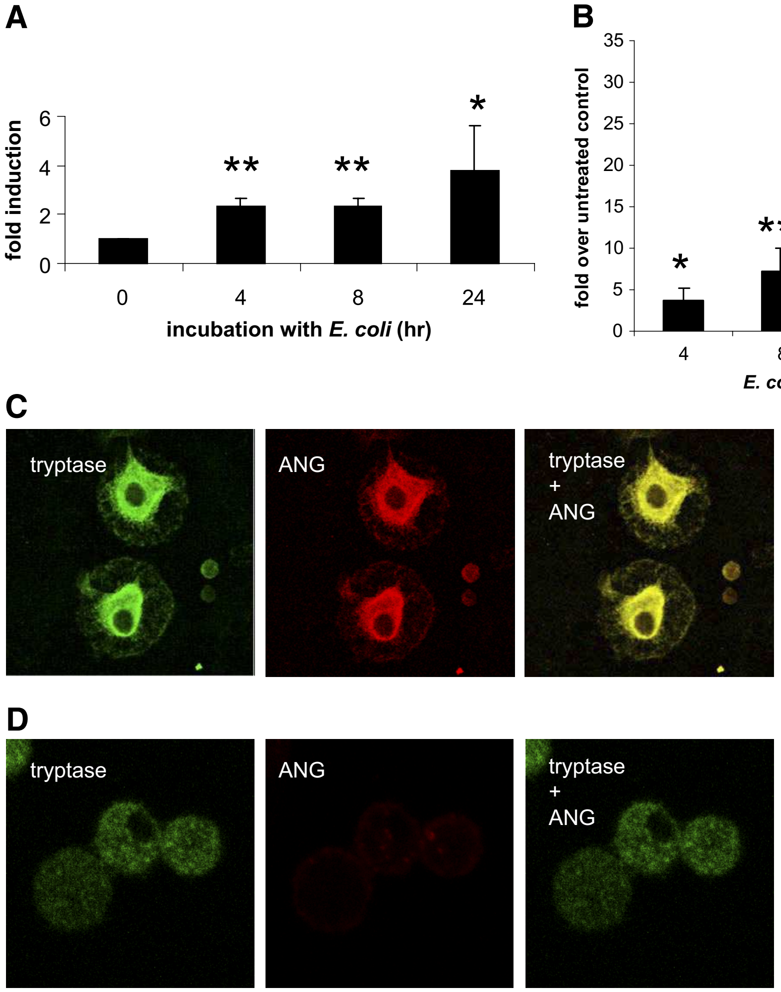

Figure 1. Microarray and qPCR analysis of ANG expression by HuMC (LAD2) coincubated with *E. coli*. (A) Microarray data showing the expression of *ANG* by LAD2 cells exposed to live *E. coli* ($n=3$; $P<0.01$). (B) qPCR analysis of *ANG* and β-actin expression by HuMC following coincubation with *E. coli*. RNA isolated from untreated HuMC was used as a control ($n=5$). *, Significance of $P < 0.05$; **, significance of $P < 0.01$. (C) Confocal analysis of ANG and tryptase expression in mature (8-week-old) HuMC showing partial colocalization with tryptase. Cells were prepared as described in Materials and Methods. (D) Confocal analysis of ANG and tryptase expression in immature (4-week-old) HuMC showing small amounts of tryptase expression and no detectable ANG expression.

further study. Microarray analysis showed that *ANG* expression was up-regulated more than twofold at all time-points analyzed but with the greatest up-regulation at 24 h (**Fig. 1A**). qPCR analysis confirmed that *E. coli* up-regulated *ANG* mRNA expression in CD34$^+$-derived HuMC after 24 h of exposure (Fig. 1B). To determine if exposure to *E. coli* induced cell death, HuMC were exposed to live *E. coli*, and cell viability was analyzed by the MTT assay. HuMC showed 72 ± 12% viability after exposure to *E. coli* for 24 h compared with untreated cells (94±4%). HuMC showed 96 ± 2% viability after exposure to *E. coli* for 16 h compared with untreated cells (94±4%).

To determine whether HuMC stored ANG and whether it was localized to their granules, we analyzed ANG expression by confocal microscopy using antibodies to ANG and tryptase, and tryptase expression was used as a granule marker. As shown in Figure 1C, this imaging was consistent with the conclusion that HuMC store ANG intracellularly, localized to tryptase-positive granules. In our analysis, the granules surrounded the nucleus, attributed to the cytospin process in these mature cells. Immature (4-week-old) HuMC did not express ANG, although they expressed low levels of tryptase in the cytoplasm (Fig. 1D). The appearance of aggregation of granules around the nucleus was not observed, likely as the granules are not yet as complex and dense as in the mature 8-week-old HuMC.

Binding and internalization of *E. coli* by HuMC

Murine and cord blood-derived HuMC have been found to attach to and internalize bacteria [19, 20]. Therefore, the ability of LAD2 HuMC to recognize and internalize bacteria was confirmed by incubating LAD2 with fluorescinated *E. coli*. As can be seen in **Figure 2**, fluorescent intensity increased significantly (histogram shifted to the right in Fig. 2B compared with Fig. 2A; dot plot analysis Fig. 2D vs. Fig. 2E) following exposure of MCs to fluorescinated *E. coli*. Furthermore, there was a dose-response relationship between the number of bacteria added to the cell cultures and the fluorescence intensity that plateaued when the ratio reached approximately 50 bacteria to one MC (Fig. 2C). Pretreatment of the MCs with paraformaldehyde (Fig. 2F) or with cytochalasin D (a specific inhibitor of actin and contractile microfilaments; Fig. 2, G and H) resulted in a decrease in the internalization of fluorescinated bacteria into these cells in a dose-re-

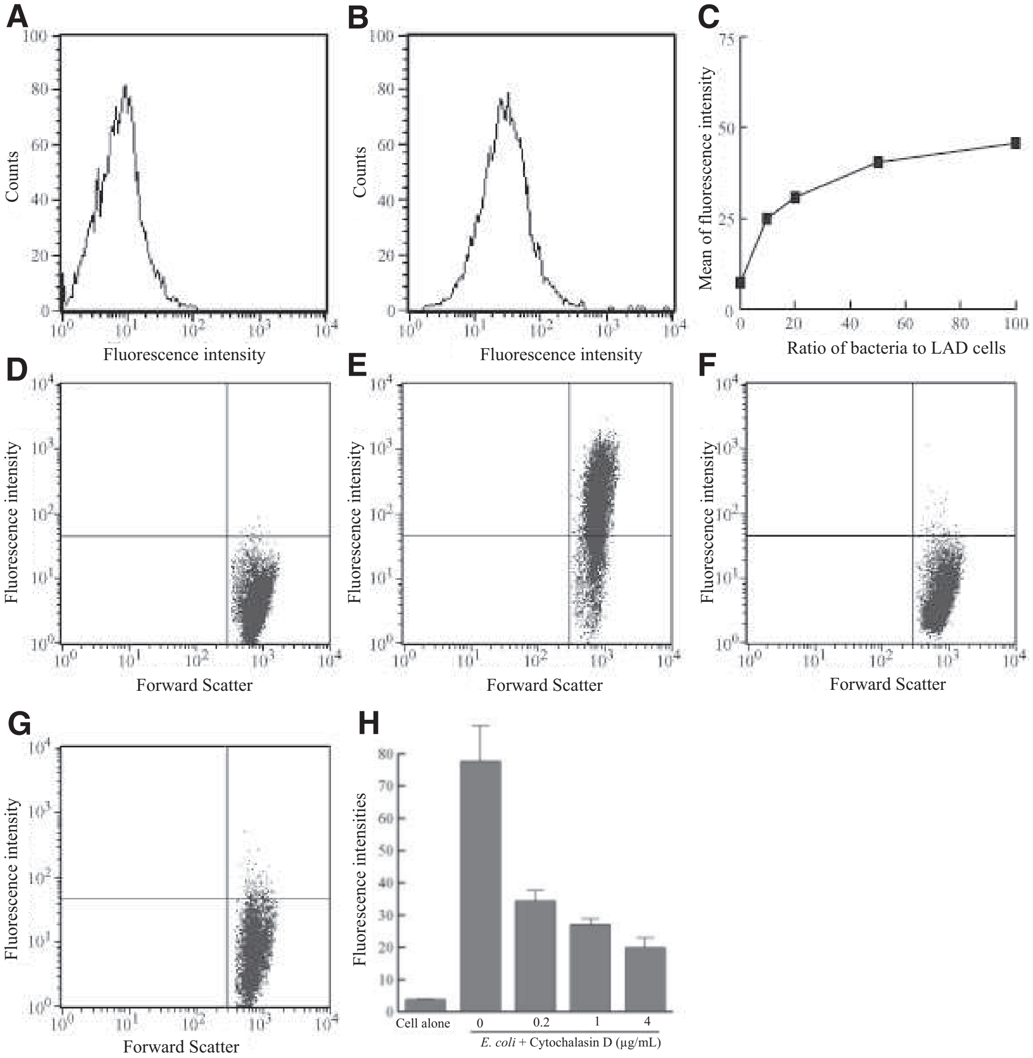

Figure 2. Flow cytometric analysis of phagocytosis of fluorescinated *E. coli* by LAD2 HuMC. (A) No bacteria added to MCs. (B) MCs incubated for 30 min with 50-fold-fluorolabeled *E. coli* particles. (C) Relationship between fluorescence intensity of the MCs and the number of *E. coli* particles added (per one MC). (D) A dot plot presentation of MCs without fluorolabeled *E. coli*. (E) A dot plot of MCs incubated for 30 min with fluorolabeled *E. coli* particles (10 particles/cell). (F) Same conditions as in E except that cells were pretreated with paraformaldehyde. (G) Same conditions as in E except that cells were pretreated with 4 μg/ml cytochalasin D. (H) Dose-dependent effects of cytochalasin D pretreatment on internalization of fluorolabeled *E. coli* by MCs.

sponse manner. Thus, LAD2 HuMC are able to internalize *E. coli* through a process requiring cytoskeletal rearrangement.

Primary cultured HuMC express and store angiogenin in granules

The expression of *ANG* in LAD2 was compared with other types of MCs such as the HMC-1 and CD34$^+$-derived HuMC. qPCR analysis showed further that the HMC-1 and primary-cultured HuMC derived from peripheral blood also expressed *ANG* (**Fig. 3A**). To determine the kinetics of *ANG* expression during MC development, we cultured CD34$^+$ peripheral blood progenitors in SCF and IL-6 for 8 weeks and analyzed *ANG* expression at 4, 6, 8, and 10 weeks of culture. Immature 4-week-old MCs do not express *ANG*, but at 6 weeks, they begin to express mRNA for *ANG*, and by 10 weeks of culture, MCs express the highest levels of *ANG* mRNA (Fig. 3B). To determine whether the process of *E. coli* ingestion is related to ANG production, HuMC were incubated with *E. coli* in the presence of cytochalasin D, and ANG production was measured. Our data show that cytochalasin D inhibited ANG production by ~50% (50 uM cytochalasin D, Fig. 3C). This suggests that ANG production may be related to the process of *E. coli* internalization.

To explore further the connection between *E. coli* ingestion and ANG production, a kinetic study was performed in which HuMC ingestion of fluorescinated *E. coli* and ANG production was measured simultaneously (Fig. 3D). *E. coli* interaction/ingestion was measured by flow cytometry as in Figure 2, and ANG production from the same samples was measured by ELISA. The data in Figure 3D show that these two events correlate.

HuMC secrete angiogenin upon stimulation

As HuMC were found to store ANG, we determined whether activation of HuMC would induce ANG secretion. First, we activated HuMC with different ratios of *E. coli* for 24 h (**Fig. 4A**). HuMC secreted significant amounts of ANG protein when exposed to *E. coli* bacteria, where the number of bacteria was 10 times or 100 times the number of MCs (Fig. 4A). Maximum release of ANG in response to *E. coli* (1:10) stimulation occurred at 8 h of exposure (Fig. 4B). Activation of HuMC via FcεRI (Fig. 4C) induced similar levels of ANG secretion with those induced by incubation with a 1:10 ratio of *E. coli* (60.2±11.4 pg/mL with *E. coli* and 44.5±4.7 with streptavidin; Fig. 4A). Stimulation of HuMC with 48/80 induced the most significant amount of ANG (Fig. 4D), suggesting that direct activation of degranulation via G-protein activation also induces ANG secretion. It has been shown that *E. coli* interaction with MCs can induce degranulation [21]. To determine whether HuMC similarly degranulate in response to *E. coli* interaction, β-hexosaminidase release in HuMC and *E. coli* cocultures was measured (Fig. 4E). *E. coli* induced HuMC degranulation, but this process was not blocked by mannose.

HuMC release TNF when activated by *E. coli* [22]. Therefore, the production of cytokines by HuMC incubated with *E. coli* was characterized. Compared with ANG levels shown in Figure 4, HuMC incubated with *E. coli* produced similar amounts of TNF (**Fig. 5A**), IL-10 (Fig. 5B), and IL-1β (Fig. 5C). However, HuMC produced significantly more IL-6 and IL-8 than ANG when incubated with *E. coli*. HuMC did not produce IL-12p70 when incu-

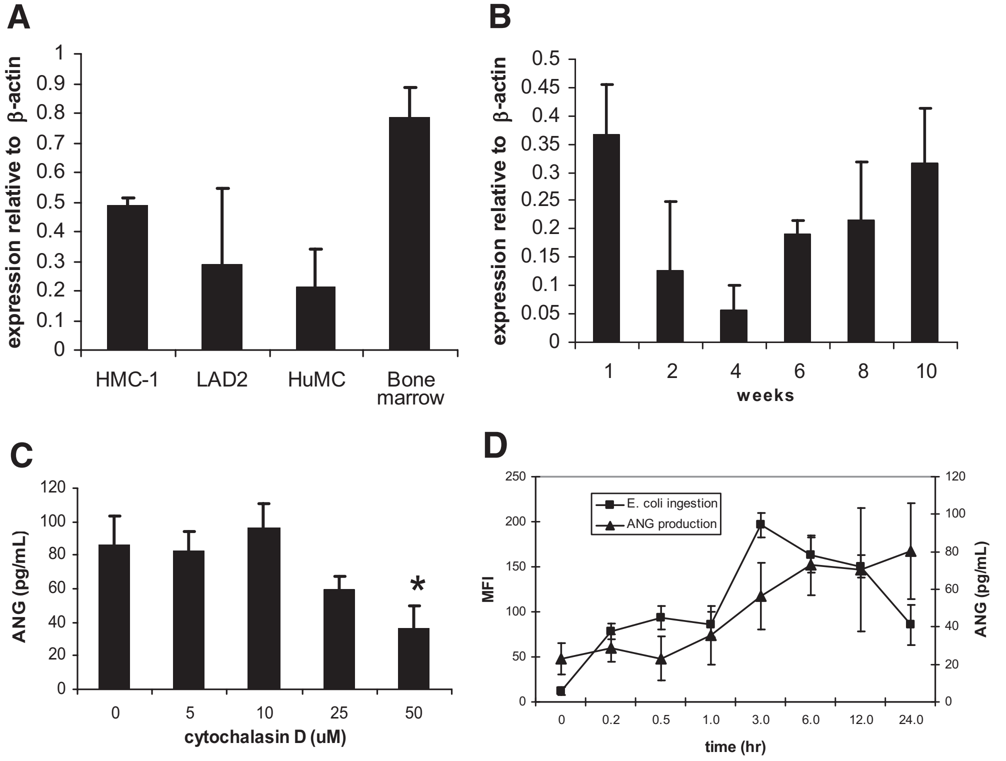

Figure 3. Analysis of ANG expression. (A) HMC, HMC-1, LAD2, and CD34+-derived HuMC expression of ANG and β-actin by qRT-PCR. RNA isolated from untreated HuMC was used as a control. (B) CD34+ HuMC with ANG expression measured during 10 weeks of development. PCR-amplified *ANG* bands from an ethidium bromide-stained gel were excised and sequenced and found to have a sequence corresponding with human *ANG*. (C) Confocal analysis of *ANG* expression in HuMC showing partial colocalization with tryptase. Cells were prepared as described in Materials and Methods. HuMC were incubated with *E. coli* (1:100 HuMC:bacteria ratio) in the presence of cytochalasin D (0, 5, 25, and 50 μM) for 24 h, and ANG production was measured by ELISA. *, $n = 5$; $P < 0.05$. (D) HuMC were incubated with fluorescinated *E. coli* as in Figure 2 for indicated times. *E. coli* particle ingestion was measured by flow cytometry, and supernatants from the same samples were analyzed for ANG production by ELISA ($n=5$; $P<0.01$). MFI=Mean fluorescence intensity.

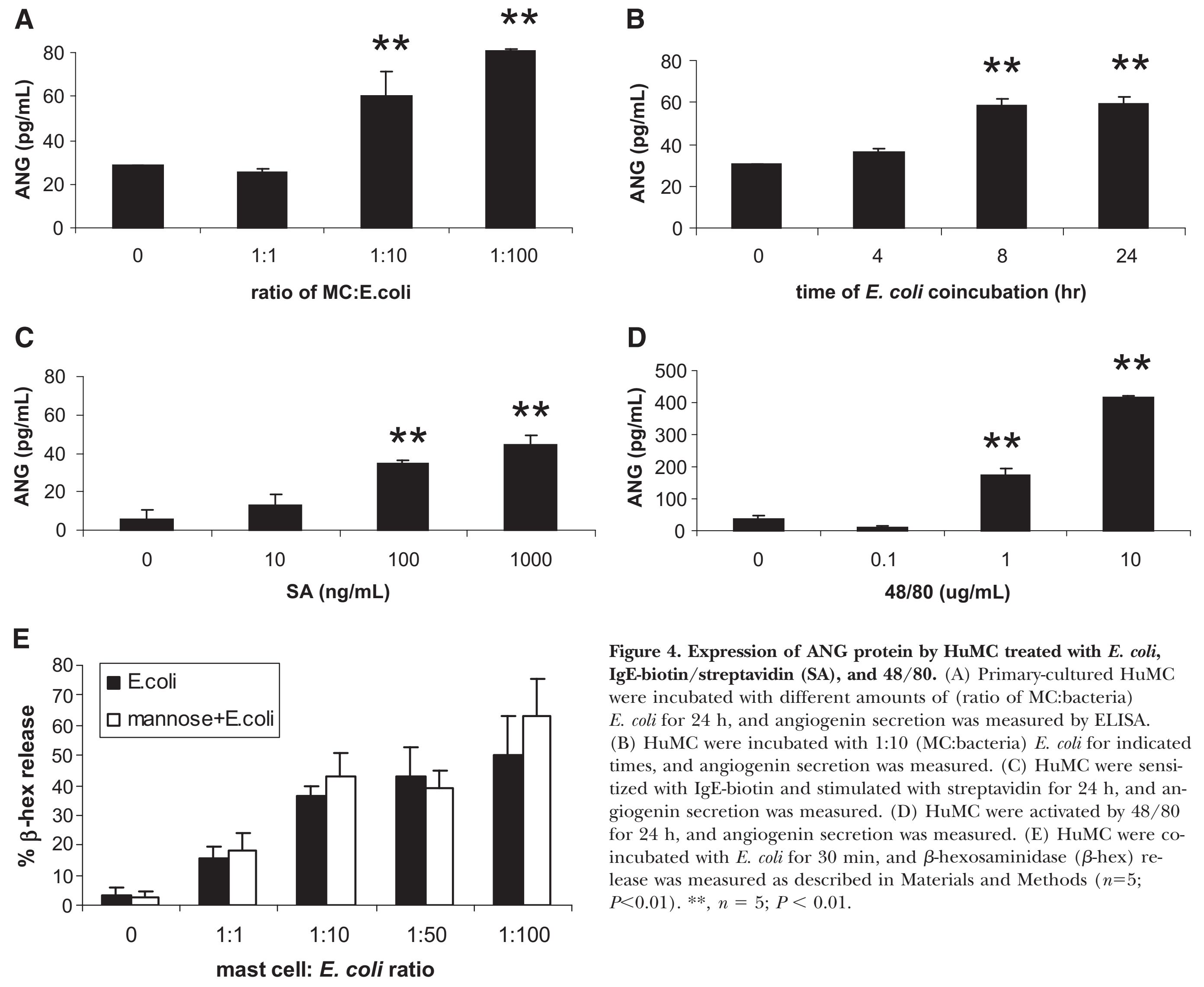

Figure 4. Expression of ANG protein by HuMC treated with *E. coli*, IgE-biotin/streptavidin (SA), and 48/80. (A) Primary-cultured HuMC were incubated with different amounts of (ratio of MC:bacteria) *E. coli* for 24 h, and angiogenin secretion was measured by ELISA. (B) HuMC were incubated with 1:10 (MC:bacteria) *E. coli* for indicated times, and angiogenin secretion was measured. (C) HuMC were sensitized with IgE-biotin and stimulated with streptavidin for 24 h, and angiogenin secretion was measured. (D) HuMC were activated by 48/80 for 24 h, and angiogenin secretion was measured. (E) HuMC were coincubated with *E. coli* for 30 min, and β-hexosaminidase (β-hex) release was measured as described in Materials and Methods (n=5; P<0.01). **, $n = 5$; $P < 0.01$.

bated with *E. coli*. Thus, the amount of ANG produced by HuMC is comparable with TNF, IL-10, and IL-1β, all of which are important MC-derived immunomodulatory cytokines.

TLR ligands also induce angiogenin expression and secretion

As *E. coli* could up-regulate ANG expression and induce secretion, we hypothesized that TLR ligands associated with bacterial products, such as LPS, PGN, CpG oligonucleotides, and flagellin, may also up-regulate *ANG* expression. qRT-PCR analysis of HuMC revealed that LPS, PGN, and flagellin treatment up-regulated *ANG* expression (**Fig. 6A**). CpG oligonucleotides, however, had no effect on ANG expression.

To determine if TLR ligands could activate ANG secretion, primary HuMC were activated with LPS, PGN, CpG oligonucleotides, and flagellin for 24 h, and ANG secretion into the supernatant was measured. LPS, PGN, and flagellin induced ANG secretion (Fig. 6, B, C, and E). In confirmation of qPCR results obtained in Figure 6A, CpG oligonucleotides did not induce ANG secretion by HuMC (Fig. 6D). Anti-TLR4 antibodies do not significantly inhibit LPS-mediated activation of ANG production (Fig. 6B). However, anti-TLR2 significantly inhibits PGN-mediated activation of ANG production, suggesting that TLR2 is involved in this process (Fig. 6C).

NGF activates HuMC to produce angiogenin

MCs respond to stimuli that are independent of FcεRI or TLR, such as neuropeptides, hormones, and opiates [23, 24]. Therefore, the ability of HuMC to produce ANG in response to substance P, NGF, gastrin, CGRP, and codeine was determined (**Fig. 7A**). NGF induced the production of significant amounts of ANG, whereas none of the other compounds induced ANG production by HuMC. Further analysis showed that NGF activates NGF production dose-dependently (Fig. 7B).

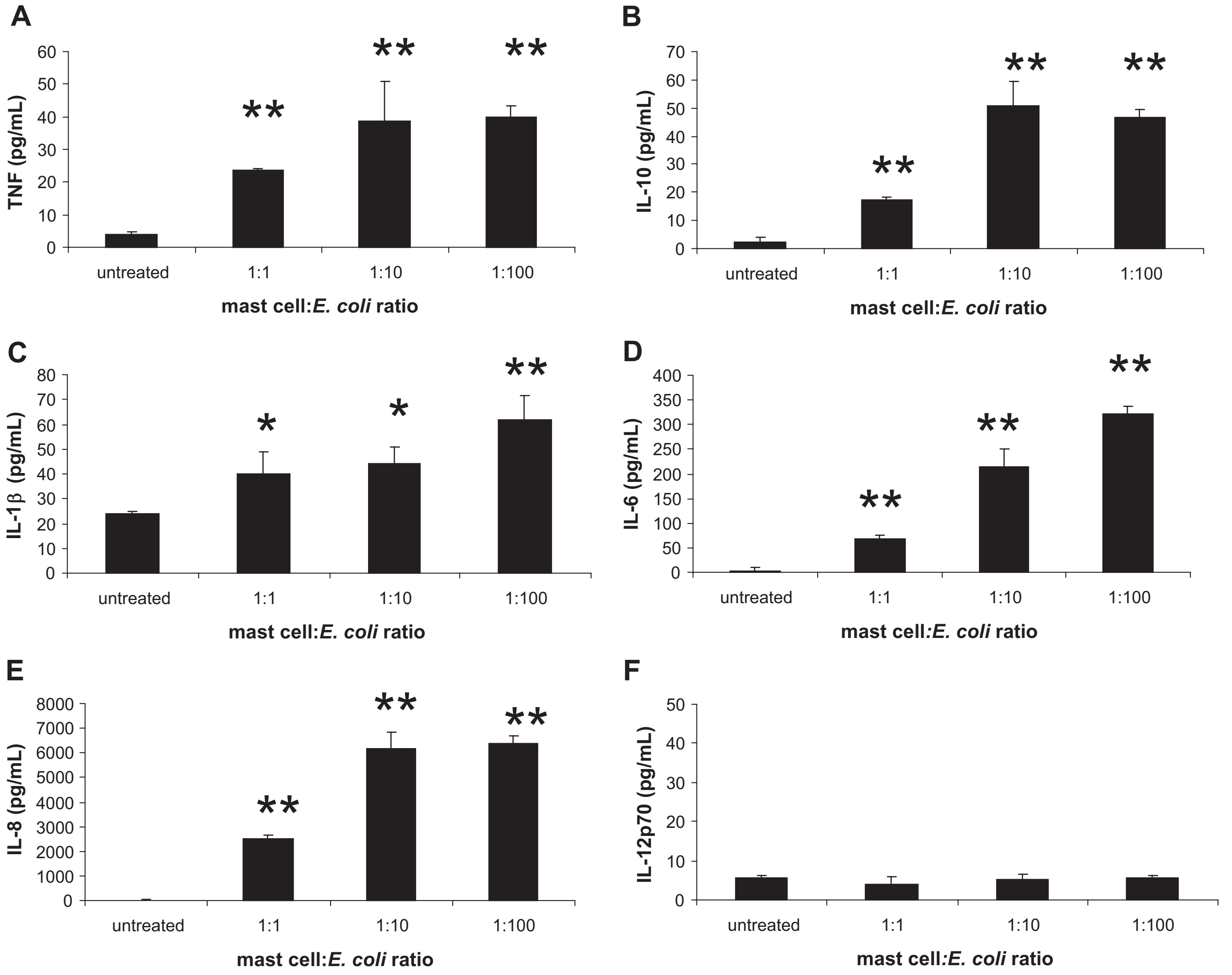

Figure 5. Cytokine production by HuMC following exposure to *E. coli*. HuMC were exposed to *E. coli* for 24 h, and cell-free supernatants were analyzed for TNF (A), IL-10 (B), IL-1β (C), IL-6 (D), IL-8 (E), and IL12p70 (F) production by ELISA. All values represent the mean of $n = 5$ ($P<0.01$). *, $n = 5$; $P < 0.01$; **, $n = 5$; $P < 0.05$.

DISCUSSION

This study shows that HuMC (two HMC as well as CD34+-derived MCs) express ANG. Furthermore, HuMC store ANG in their granules and release ANG to a variety of stimuli, including FcεRI-mediated signals, TLR ligands, and NGF, implying that MC-derived ANG is released in a variety of physiological conditions.

ANG was isolated as a tumor angiogenic factor based solely on its angiogenic activity, and subsequent studies have focused mainly on its angiogenic capacity. However, several reports have suggested that ANG is important in regulating rRNA transcription in cancer cells [4], involved in chronic heart failure [5], important in wound healing [6], and elevated in patients with asthma [7–10]. Although the reports are conflicting, ANG may also have antimicrobial activity and may be important in clearing infections [11, 12].

ANG is one of several unusual members of the pancreatic RNase superfamily. It was first isolated as a 14-kDa-soluble protein from culture medium conditioned by human colon carcinoma (HT-29) cells and was identified as an angiogenic substance based on its capacity to induce blood-vessel formation on the chorioallantoic membrane of the chicken embryo. Although ANG is secreted by many tumor cells and has been shown to be essential for tumor growth, it is not a tumor-specific protein. It is present at a concentration of 250–360 ng/mL in normal human plasma [3, 5]. Higher or lower concentrations have been seen in a variety of conditions, including endometrial cancer, pregnancy, and renal dialysis, but thus far, its plasma level has not been shown to have diagnostic relevance. Recently, a protein that inhibits the degranulation of polymorphonuclear leukocytes was isolated from plasma ultrafiltrates of patients with uremia and shown to be identical to ANG [25].

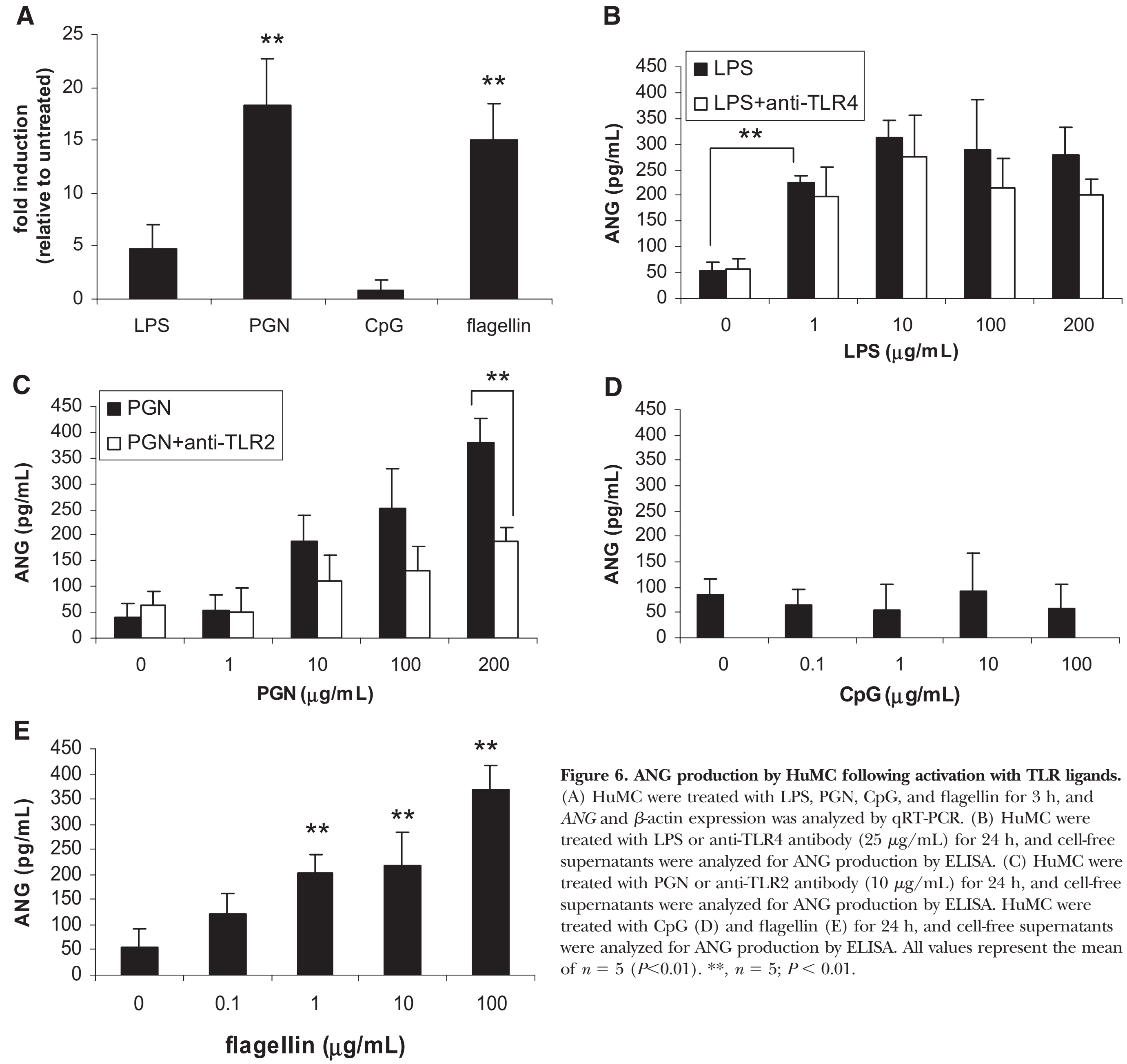

Figure 6. ANG production by HuMC following activation with TLR ligands. (A) HuMC were treated with LPS, PGN, CpG, and flagellin for 3 h, and *ANG* and β-actin expression was analyzed by qRT-PCR. (B) HuMC were treated with LPS or anti-TLR4 antibody (25 μg/mL) for 24 h, and cell-free supernatants were analyzed for ANG production by ELISA. (C) HuMC were treated with PGN or anti-TLR2 antibody (10 μg/mL) for 24 h, and cell-free supernatants were analyzed for ANG production by ELISA. HuMC were treated with CpG (D) and flagellin (E) for 24 h, and cell-free supernatants were analyzed for ANG production by ELISA. All values represent the mean of $n = 5$ ($P<0.01$). **, $n = 5$; $P < 0.01$.

One finding of this study is that HuMC produce ANG in response to activation of TLR4, TLR2, and TLR5 via LPS, PGN, and flagellin, respectively. These findings suggest that ANG may be important in MC responses to bacterial pathogens and the associated inflammatory response leading to bacterial clearance. MCs have been implicated in innate immune responses, in that they produce cytokines, leukotrienes, and chemokines in response to bacteria and viruses, mediated in part through the cell-surface pattern recognition receptors, such as TLR1, -2, -4, -6, and -9 [26, 27], and the FimH receptor CD48 for bacterial fimbriae [20]. MCs also recognize and attach to a variety of opsonized bacteria and release antimicrobial peptides such as cathelicidin or cytokines [28], which promote the inflammatory response to bacterial insults. Other and recent evidence shows that bacterial products such as LPS and PGN modulate HuMC differentiation, cytokine production, and expression of chymase and tryptase [29]. The location and immune functions associated with MCs and observations of antimicrobial peptides in MCs of lower vertebrates led us to hypothesize that HuMC may also express and release ANG in response to bacterial or TLR ligand activation.

However, the precise role of ANG in bacterial infections is controversial. Hooper et al. [11] have shown that mouse and human ANG is able to reduce growth of *Streptococcus pneumoniae* and *Candida albicans* by at least 100-fold, yet Avdeeva et al. [12] reported that the antimicrobial activities of commer-

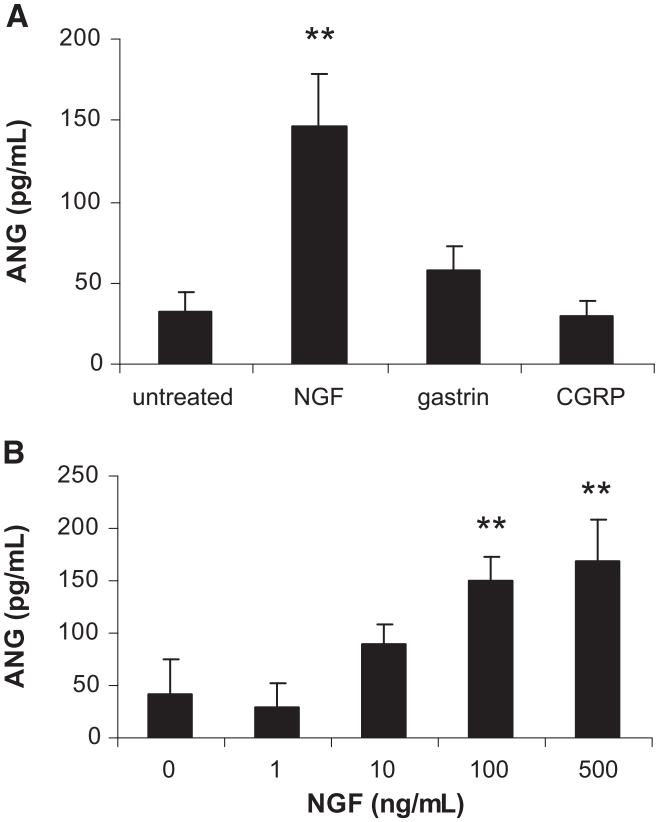

Figure 7. ANG production by HuMC following activation with neuropeptides, gastrin, and codeine. (A) HuMC were treated with NGF (1 μg/mL), gastrin (10 μg/mL), and CGRP (1 μg/mL) for 24 h, and cell-free supernatants were analyzed for angiogenin production by ELISA. (B) HuMC were treated with NGF for 24 h, and ANG production was measured by ELISA. All values represent the mean of $n = 5$ ($P<0.01$). **, $P < 0.01$.

cially prepared human ANG were comparable with that of BSA. A study by Cognasse et al. [30] showed that *E. coli*-derived LPS inhibited constitutive ANG expression (~5%) by human blood-derived platelets. Yet, angiogenesis is extremely important during tissue regeneration throughout a bacterial or viral infection, and expression of angiogenic factors such as ANG increases during hepatitis C infection of the liver [31]. Therefore, it is possible that the role of ANG in infection is not antimicrobial but serves as a modulator of tissue regeneration and remodeling necessary for pathogen clearance and re-establishment of tissue homeostasis.

Our study has also shown that NGF stimulates HuMC to release greater amounts of ANG (Fig. 7) compared with MCs stimulated by FcεRI cross-linking (Fig. 4C). This observation has some interesting implications in light of some recent studies in which ANG gene mutations have been associated with amyotrophic lateral sclerosis [32, 33]. Human ANG has been shown to be neuroprotective and promotes the survival and neurite extension formation of motor neurons [34]. MCs are intimately associated with nerves and are therefore ideally suited to communicate with neurons. As such, it is possible that NGF stimulation of MCs in neuroinflammatory conditions may ultimately lead to ANG production and promotion of neuron survival.

In this study, we have shown that HuMC secrete ANG in response to a variety of stimuli. Some of the stimuli tested, namely the TLR ligands LPS, PGN, and flagellin and the neuropeptide NGF, induced significant levels of ANG (160 pg/mL or more), and FcεRI cross-linking induced smaller amounts of ANG (<100 pg/mL). These findings suggest that ANG may be important in certain physiological settings, particularly those mediated by TLR ligands and NGF. MC-derived ANG may be an important immune modulator in nonallergic conditions.

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