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2-Arachidonoyl-glycerol- and arachidonic acid-stimulated neutrophils release antimicrobial effectors against *E. coli*, *S. aureus*, HSV-1, and RSV

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Abstract

The endocannabinoid 2-AG is highly susceptible to its hydrolysis into AA, which activates neutrophils through de novo LTB_4 biosynthesis, independently of CB activation. In this study, we show that 2-AG and AA stimulate neutrophils to release antimicrobial effectors. Supernatants of neutrophils activated with nanomolar concentrations of 2-AG and AA indeed inhibited the infectivity of HSV-1 and RSV. Additionally, the supernatants of 2-AG- and AA-stimulated neutrophils strongly impaired the growth of *Escherichia coli* and *Staphylococcus aureus*. This correlated with the release of a large amount (micrograms) of α-defensins, as well as a limited amount (nanograms) of LL-37. All the effects of AA and 2-AG mentioned above were prevented by inhibiting LTB_4 biosynthesis or by blocking BLT_1. Importantly, neither CB_2 receptor agonists nor antagonists could mimic nor prevent the effects of 2-AG, respectively. In fact, qPCR data show that contaminating eosinophils express ~100-fold more CB_2 receptor mRNA than purified neutrophils, suggesting that CB_2 receptor expression by human neutrophils is limited and that contaminating eosinophils are likely responsible for the previously documented CB_2 expression by freshly isolated human neutrophils. The rapid conversion of 2-AG to AA and their subsequent metabolism into LTB_4 promote 2-AG and AA as multifunctional activators of neutrophils, mainly exerting their effects by activating the BLT_1. Considering that nanomolar concentrations of AA or 2-AG were sufficient to impair viral infectivity, this suggests potential physiological roles for 2-AG and AA as regulators of host defense in vivo.

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AUTHORSHIP

F.C., C.T., M-C.L., X.G., S.P., L.B., and V.P. performed experiments. F.C., C.T., L.F., N.G., and N.F. designed the experiments. F.C. and N.F. wrote the paper. All authors reviewed the paper.

Keywords

leukotriene; cannabinoid receptor; endocannabinoid; eicosanoid; GPR55; 5-lipoxygenase

Introduction

Neutrophils play an important role in host defense by participating in pathogen clearance. They fulfil this important function notably by releasing antimicrobial peptides, by producing ROS, and by ingesting and killing microbes. Neutrophils represent a rich source of antimicrobial peptides, such as α-defensins and the cathelicidin LL-37. α-Defensins are small, cationic peptides accounting for ~5% of neutrophil proteins, and LL-37 is an α-helix peptide containing 37 aa that originates from the C-terminal domain of the cathelicidin human cationic antimicrobial protein-18. In addition to their antimicrobial properties against viruses and bacteria [1–4], α-defensins and LL-37 recruit and activate leukocytes [5–13].

One of the main bioactive ingredients of cannabis, THC was previously shown to increase the susceptibility of mice to bacterial, protozoan, and viral infections [14–17]. This effect of THC is linked to CB_2 receptor activation, decreased phagocytosis by macrophages, down-regulation of proinflammatory cytokine production, and the polarization of T cells toward a Th2 phenotype [18–23]. Although CB_2 receptor activation is usually considered anti-inflammatory, endocannabinoids, such as AEA and 2-AG, can enhance cell functions, such as chemotaxis, phagocytosis, and the release of soluble mediators [21, 24–33].

The pro- and anti-inflammatory effects of endocannabinoids are likely explained by a differential expression of CB receptors among leukocytes and the activation of additional cell surface receptors by endocannabinoid metabolites. 2-AG and AEA are indeed the respective precursors of other bioactive lipids, such as PGs-glycerol and prostamides [34, 35], as well as being a significant source of AA, leading to the biosynthesis of eicosanoids [36, 37]. We indeed documented that 2-AG was rapidly hydrolyzed to AA and further converted into LTB_4 by human neutrophils [36]. Given that LTB_4 promotes the ingestion and killing of microbes and the release of antimicrobial peptides [38–42], we postulated that 2-AG and AA would have similar effects. In this study, we investigated the antimicrobial activities of human neutrophils activated by 2-AG or AA.

MATERIALS AND METHODS

Material

2-AG ether, 2-AG, AA, AEA, SR144528, LTB_4, and MAFP were purchased from Cayman Chemical (Ann Arbor, MI, USA). O-2050, AM-251, and DPI were obtained from Tocris Bioscience (Ellisville, MO, USA). ADA was purchased from Roche Applied Science (Indianapolis, IN, USA). fMLP, cytochalasin B, DMSO, dextran, and methyl cellulose were obtained from Sigma Chemical Co. (St. Louis, MO, USA). HBSS, lymphocyte separation medium, M199, FBS, penicillin, streptomycin, and amphotericin B were obtained from Wisent Laboratories (St-Bruno, Quebec, Canada). The magnetic bead-conjugated anti-CD16 mAb and MACS were purchased from Miltenyi Biotec (Auburn, CA, USA). Purified human

LL-37 and α-defensins, which consist of a 5:4:1 mixture of α-defensin-1, -2, and -3, as well as LL-37 and α-defensin ELISA kits were obtained from Hycult Biotechnologies (Uden, The Netherlands). The FLAP antagonist MK-0591 was provided by Dr. Denis Riendeau from Merck Frosst (Kirkland, Quebec, Canada). *E. coli* (clinical isolate; ATCC #25922*), S. aureus* (clinical isolate; ATCC #25923), Vero cells (ATCC #CCL-81), and HSV-1 (MacIntyre strain; ATCC #VR-539) were obtained from the American Type Culture Collection (Manassas, VA, USA). rRSV encoding GFP was kindly provided by Dr. Peter Collins (U.S. National Institutes of Health, Bethesda, MD, USA). Shaphylokinase and aureolysin were, respectively, obtained from Cedarlane (Burlington, Ontario, Canada) and Axxora (Farmingdale, NY, USA).

Ethics Committee approval

This work required the use of human cells from healthy volunteers and was approved by the Institutional Ethics Committee from the Institut Universitaire de Cardiologie et de Pneumologie de Québec. All of the experiments were conducted with the understanding and the signed consent of each participant.

Isolation of human neutrophils

Venous blood was obtained from healthy volunteers in K_3 EDTA tubes, and neutrophils were purified, as described before with slight modifications [43]. In brief, blood was centrifuged (17 min; 250 *g*) to remove the platelet-rich plasma. Erythrocytes then were sedimented with 3% dextran. PBMCs were discarded from the granulocytes by centrifugation on a discontinuous gradient by using lymphocyte separation medium cushions. A hypotonic lysis with sterile water was next performed on the granulocyte pellet to eliminate the residual erythrocytes. Neutrophils were finally purified from the granulocyte suspension by positive selection with anti-CD16-conjugated magnetic beads, according to the manufacturer's instructions. The purity and viability of the resulting neutrophil and eosinophil suspensions were always ≥98.5 %, as assessed by Diff-Quick staining and trypan blue exclusion, respectively.

Analysis of α-defensins and LL-37

Supernatant of human neutrophils was analyzed for α-defensins and LL-37 content by ELISA (Hycult Biotechnologies), according to the manufacturer's instructions.

Analysis of CB_2 expression by RT-PCR and qPCR

Total RNA of granulocytes, neutrophils, and eosinophils was extracted with TRIzol, according to the manufacturer's instructions. For the analysis of CB_2 expression by RT-PCR, experiments were performed in a single tube using Qiagen OneStep RT-PCR kit. In brief, 50 ng total RNA was reverse-transcribed for 30 min and then amplified on a Peltier Thermal Cycler (PTC-200; MJ Research, Watertown, MA, USA) during 28 cycles with the following parameters: denaturation at 94°C for 1 min (2 min for the first cycle), annealing at 60°C for 30 s, and extension at 72°C for 1 min (5 min for the last cycle). After cycling, the amplicons were resolved by electrophoresis on ethidium bromide-stained 2% agarose gels, and the fluorescent bands were visualized using a UV transilluminator. The chemigenius software

(Syngene, Frederick, MD, USA) was then used to capture the images. Primer sequences were CB_2 (CNR2; NM_001841.2) forward, 5′-CCACAACACAACCCAAAGC-3′; CB_2 reverse, 5′-GCAGAGGTATCGGTCAATGG-3′. For qPCR analyses, total RNA was extracted using TRIzol, followed by a treatment with the RNAse-free DNAse set (Qiagen, Valencia, CA, USA) to eliminate residual genomic DNA. Samples were cleaned and concentrated with the RNeasy MinElute cleanup kit (Qiagen) and then quantified spectrophotometrically at 260 nm using a Synergy H1 microplate reader and the Take3 trio plate (BioTek, Winooski, VT, USA). cDNAs were obtained using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). qPCR reactions were performed with the iQ SYBR Green Supermix (Bio-Rad) on a CFX96 Bio-Rad thermal cycler instrument. cDNA samples, obtained from 8 ng total RNA, were used in a reaction volume of 25 μl with specific primers for human CB_2 (NM_001841) and human 18S rRNA (X03205) from Qiagen.

Dialysis of ADA and removal of endogenous adenosine

ADA was dialyzed using a Slide-A-Lyzer dialysis device (3.5 kDa MW cutoff; Thermo Fisher Scientific, Waltham, MA, USA). Sterile dialysis was performed in a beaker containing 0.9% NaCl and 10 mM Hepes (pH 7.4) with constant stirring. The buffered saline solution was changed after 1.5 and 3 h, and then the content was dialyzed overnight at 4°C. ADA was harvested from the dialysis device and then assessed for its ability to hydrolyze adenosine. To prevent the extracellular buildup of adenosine observed in isolated neutrophil suspensions, which results in the inhibition of neutrophil functions, ADA (0.3 U/ml) was always added to samples 10 min prior to stimulation [44].

Stimulation of human neutrophils

Prewarmed human neutrophil suspensions (37°C; 2×10^7 cells/ml) in HBSS, containing 1.6 mM $CaCl_2$, were treated with 1 μM cytochalasin B for 15 min and then activated with LTB_4, fMLP, 2-AG, AA, AEA, or 2-AG ether for the indicated times and concentrations (see figure legends). Incubations were stopped by placing the samples in an ice-water bath for 5 min. Samples then were centrifuged (4°C; 5 min; 700 *g*), and the cell-free supernatants were collected and frozen until further analyses. Addition of enzyme inhibitors or receptor antagonists was performed 5 min before the addition of the stimuli.

O_2^- release

The release of O_2^- by human neutrophils was assessed by cytochrome *c* reduction, as described previously with minor modifications [45]. In brief, prewarmed human neutrophil suspensions (37°C; 2×10^7 cells/ml) in HBSS, containing 1.6 mM $CaCl_2$, were treated with 10 μM cytochalasin B for 15 min and then activated with 2-AG (3 μM), AA (3 μM), LTB_4 (100 nM), or fMLP (100 nM) for 5 min. Addition of 130 μM cytochrome *c* was done 5 min before the addition of the stimuli. Incubations were stopped by placing the samples in an ice-water bath for 15 min. Samples were next centrifuged (4°C; 5 min; 700 *g*), and cell-free supernatants were analyzed for O_2^- production using the following formula: $(OD_{550}-OD_{540}) \times 47.4 = \text{nmol } O_2^-/10^6$ cells/time units.

Bacterial growth and bactericidal assay

Inocula of *E. coli* or *S. aureus* were grown overnight (18 h, 37°C) in TSB. The obtained cultures were diluted 1/100 in fresh TSB and incubated at 37°C until the OD_{600} reached 0.5. Aliquots of the culture broths (250 μl for *E. coli* and 500 μl for *S. aureus*) were washed twice and then suspended in 1 ml sodium phosphate buffer (10 mM; pH 7.4) containing 1% TSB. Solutions then were diluted with the same buffer (1/3500 for *E. coli* and 1/1750 for *S. aureus*) to obtain suspensions of 10,000 CFU/ml. The resulting bacterial suspensions were then diluted with an equal volume of supernatant from activated neutrophils at 37°C for 4 h. Samples were next diluted 1/300 in sodium phosphate buffer containing 1% TSB, plated on LB agar plates, and incubated overnight to allow the growth of colonies and their enumeration.

Epithelial cell culture and virucidal assays

For the production of HSV-1, Vero cells were cultured in M199 medium, supplemented with 10% FBS, penicillin, streptomycin, and amphotericin B. Cells were passaged by trypsinization and grown until they reached 80% confluence. Vero cells were then infected with HSV-1. When cellular lysis was almost complete (5 days), the supernatant was collected, filtered (0.45 μm), and pelleted (30,000 *g*; 180 min). The viral pellet was suspended in 5 ml HBSS, aliquoted, and frozen at −80°C. HSV-1 titration was determined by a standard plaque assay on Vero cells. Viral preparations only underwent one freeze-thaw cycle. For the analysis of HSV-1 infectivity, assays were performed, as described before with slight modifications [39]. In brief, the HSV-1 stock solution was diluted in M199 medium to a final working concentration of 1200 PFU/ml and kept on ice until further use. In parallel, 15 μl neutrophil supernatant was added to 85 μl M199 medium and then mixed with 50 μl of the HSV-1 working solution and incubated at 37°C for 10 min. Samples were then mixed with 350 μl M199, and the mixture was added to each well of a 12-well plate containing 90% confluent Vero cells. The plates then were incubated for 60 min at 37°C, after which, the media were replaced with M199 containing 1.5% methylcellulose. After a 3-day incubation at 37°C, wells were washed twice with PBS, fixed with 5% formaldehyde for 10 min, then stained with 0.8% crystal violet in 50% ethanol for 2 min, and finally, washed with sterile water to allow the determination of HSV-1 infectivity by plaque count.

For the production of rRSV encoding GFP, amplification and purification were performed, as described previously [46]. In brief, amplification was done in human epidermoid cancer cell at a multiplicity of infection of 0.1 until a 50% cytopathic effect was observed. Virus was purified by ultra-centrifugation on 30% sucrose cushion centrifugation at 4°C (18 h; 8000 *g*). RSV titration was determined by a standard plaque assay on Vero cells. Viral preparations only underwent one freeze-thaw cycle. For the analysis of RSV infectivity, treatment with neutrophil supernatants was performed, as described for HSV-1, except that DMEM medium was used. Infection of Vero cells was performed for 2 h in DMEM containing 2% FBS. The medium was then replaced with DMEM containing 1% methylcellulose and 2% FBS. Infection was pursued for 7 days, and fluorescent lysis plaques were visualized using a Typhoon apparatus (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analyses

Variables were log-transformed to stabilize variance and analyzed with a multiple comparison using the Tukey method. Results were considered significant if P values were <0.05. The data were analyzed using the statistical package program SAS version 9.1.3 (SAS Institute, Cary, NC, USA).

RESULTS

2-AG and AA induce the release of antimicrobial effectors by cytochalasin B-treated neutrophils

As our initial work on human neutrophils indicated that 2-AG was stimulatory rather than inhibitory [36], we first assessed whether endocannabinoids would also induce the release of antimicrobial effectors. Neutrophil suspensions were incubated with increasing concentrations of 2-AG or AA for 5 min, and then the cell-free supernatants were assayed for their potency to impair HSV-1 or RSV infectivity using Vero cells. The supernatants of 2-AG- and AA-stimulated neutrophils inhibited HSV-1 infectivity with similar efficacies and potencies, with 50% protection at ~30 nM (Fig. 1A). Additionally, 1 μM 2-AG or AA significantly decreased RSV infectivity of Vero cells by ~45%, which was similar to the effect of 100 nM LTB_4 (Fig. 1B).

Given that 2-AG and AA levels are increased significantly during sepsis [47], we investigated whether they would also impair bacterial growth. We consequently performed another series of experiments in which the supernatants of 2-AG- and AA-treated neutrophils were incubated with exponentially growing *E. coli* or *S. aureus* suspensions. 2-AG and AA induced the release of a bactericidal activity from neutrophils, although the EC_{50} were one order of magnitude higher than what we observed with HSV-1 (Fig. 2). The maximal effects of 2-AG and AA were obtained with 3 μM 2-AG or AA, which inhibited the growth of *S. aureus* and *E. coli* by ~95% and ~85%, respectively. Importantly, the low concentration of LTB_4, AA, or 2-AG that we used did not impact virus infectivity or bacterial growth [48, 49].

2-AG and AA induce the release of α-defensins and LL-37

As the antimicrobial effects of AA- and 2-AG-activated neutrophil supernatants were observed during short-term incubations (5 min), we postulated that the mediators involved were rapidly produced or stored in neutrophil granules. 2-AG, AA, and LTB_4 were poor inducers of O_2^- release compared with fMLP (Fig. 3A). Indeed, 100 nM fMLP induced the release of O_2^-, 5× greater than that observed with LTB_4, 2-AG, or AA. Moreover, the NADPH oxidase inhibitor DPI, which blocked O_2^- production (Fig. 3A), did not prevent the effects of 2-AG and AA on bacterial growth (data not shown). In contrast, 2-AG and AA induced an important release of α-defensins (Fig. 3B) and LL-37 (Fig. 3C) in a concentration-dependent manner. This began at a 2-AG or AA concentration of 300 nM, which is also the concentration at which supernatants begin to exert their bactericidal effects (Fig. 2) and was maximal at 3 μM. Interestingly, this mimicked the dose-response curves obtained for the biosynthesis of LTB_4 induced by 2-AG or AA [36]. The release of antimicrobial peptides induced by 2-AG and AA was rapid (Figs. 3D and E), with most of

the peptides released within 2 min. AEA, the GPR55 agonist (and CB_1 antagonist) AM-251, as well as 2-AG ether (a nonhydrolyzable form of 2-AG) did not induce the release of α-defensin or LL-37 (Figs. 3B and C). The amounts of antimicrobial peptides released upon neutrophil activation were different for both classes of peptides. Indeed, whereas α-defensins were detected in the microgram range, LL-37 was found in the nanogram range (~250:1 ratio). In an attempt to dissect the contribution of α-defensins and LL-37 in the antimicrobial effects that we observed, additional experiments were performed by treating neutrophil supernatants with commercially available staphylokinase and aureolysin to impair the efficacy of α-defensins and LL-37. This strategy was, however, unsuccessful, as both protein preparations directly inhibited the growth of our bacterial suspensions (data not shown). We, nonetheless, investigated the potency of purified α-defensins and LL-37 on bacterial growth in our experimental model. Purified peptides effectively inhibited the growth of *E. coli* (Fig. 3F), with LL-37 being more potent by one order of magnitude. Essentially similar results were obtained for *S. aureus* (data not shown). Interestingly, when 10 μg/ml α-defensins were combined with 40 ng/ml LL-37 (250:1 ratio), the antimicrobial peptides prevented the growth of *E. coli* suspension in a synergistic manner (Fig. 3G).

CBs are not involved in the 2-AG-induced release of antimicrobial peptides

The results obtained using AEA and 2-AG ether (Fig. 3B and C), which activate CBs and GPR55 [50–55], suggested that the effects of 2-AG were likely independent of GPR55 or CB activation. The expression of CBs by neutrophils is controversial. Neutrophils were reported to express the CB_2 receptor [56–59], whereas others [27] and we [36] reported otherwise. Interestingly, the absence of CB_2 receptor mRNA by human neutrophils coincided with the removal of contaminating eosinophils. This prompted us to determine the expression of the CB_2 receptor in isolated granulocytes (which many research groups use as purified neutrophils), eosinophil-depleted neutrophils, and eosinophils. As shown in Fig. 4, CB_2 receptor mRNA expression was detected by RT-PCR in isolated granulocytes and eosinophils, in sharp contrast to purified (eosinophil-depleted) neutrophils. Additional experiments using qPCR (Fig. 4E) also yielded similar results: the removal of contaminating eosinophils significantly decreased the CB_2 mRNA signal found in granulocytes. Importantly, the expression of CB_2 receptor mRNA by human eosinophils was ~100-fold higher than that observed with highly purified neutrophils. We also undertook a series of experiments, in which human neutrophils were pretreated with the CB_1 receptor antagonist O-2050 or the CB_2 antagonist SR144528. Both antagonists did not prevent the bactericidal effects of 2-AG (Fig. 5A) nor did they prevent the release of antimicrobial peptides (Fig. 5B and C). Additionally, AEA and the GPR55 agonist (and CB_1 antagonist) AM-251 did not inhibit nor enhance the 2-AG-induced release of α-defensins or LL-37. Altogether, the lack of effect of AEA and 2-AG ether combined with the lack of efficacy of CB antagonists to block the 2-AG-induced antimicrobial peptide release and bactericidal effect indicate that these phenomena are likely independent of CB_1, CB_2, or GPR55 activation.

2-AG hydrolysis, AA, and LTB_4 biosynthesis are involved in the antimicrobial effects of 2-AG

The similarity between the effects induced by 2-AG and AA and the absence of response of AEA and 2-AG ether suggested that the effects of 2-AG and AA were mediated through

their metabolism. Importantly, 2-AG and AA induce a robust biosynthesis of LTB_4 by human neutrophils [36, 60], and LTB_4 is an important regulator of host defense [38–42, 61] Blockade of 2-AG hydrolysis to AA (MAFP), LTB_4 biosynthesis (MK-0591), or BLT_1 activation (CP 105,696) inhibited the effects of 2-AG on the release of antimicrobial activity (Fig. 6A) and antimicrobial peptides (Figs. 6A–C). LT modifiers, but not MAFP, also prevented the effect of AA on antimicrobial peptide release and the antimicrobial effects observed. Importantly, the release of α-defensins and LL-37, induced by fMLP, was not affected by any of the tested compounds. These results demonstrate that the release antimicrobial effects of 2-AG and AA relies on de novo LTB_4 biosynthesis, as opposed to the effects of fMLP. They clearly underscore the essential role of LTB_4 in the release of the antimicrobial activity by 2-AG and AA. Moreover, they underline the potential of 2-AG as a source of AA for neutrophils in vivo.

DISCUSSION

In the present study, we evaluated the effects of AEA, 2-AG, and AA on the activation of neutrophil functions related to host defense. We found that in contrast to AEA, very low (mid-nanometer) concentrations of 2-AG and AA induce the release of antimicrobial effectors against *S. aureus*, *E. coli*, HSV-1, and RSV. The activation of neutrophils by 2-AG and AA was associated with: 1) the release of α-defensins and LL-37; 2) a lack of CB involvement; and 3) an essential role of endogenous LTB_4 and BLT_1 activation. This suggests that low 2-AG concentration possibly plays an important role in host defense by promoting human neutrophil functions.

Plasma and tissue endocannabinoid levels are usually in the low nanomolar range [62, 63] but increase following cell activation. Many inflammatory mediators, present at infectious sites, such as LPS, platelet-activating factor, and M-CSF, induce the biosynthesis of endocannabinoids by leukocytes [64–67]. In this respect, endocannabinoid levels in sepsis and inflamed tissues are in the micromolar range [47, 68], well above the EC_{50} that we observed in this study (Figs. 1 and 2). Importantly, experiments performed with HSV-1 and RSV were done in the presence of only 10% (v/v) of neutrophil supernatants, possibly underestimating the efficacy of 2-AG and AA by up to one order of magnitude.

Studies investigating the involvement of CBs at regulating neutrophil functions are limited. Moreover, inconsistencies are found regarding the expression of the CB receptors [27, 36, 56, 57, 69–77]. Importantly, studies that reported a lack of CB_2 were performed using cell preparations in which contaminating eosinophils were removed [27, 36]. The comparative experiments among granulocytes (neutrophils and eosinophils), neutrophils, and eosinophils, which we provided here, demonstrate that >95% of CB_2 mRNA for CB_2 originates from eosinophils rather than neutrophils (Fig. 4). The 100-fold higher CB_2 receptor expression (mRNA) that we observed in human eosinophils indicates that even a 1% contamination of neutrophil suspensions with eosinophils is enough to yield a false-positive signal when performing RT-PCR or qPCR analyses. Furthermore, the inefficacy of other endocannabinoids (AEA, 2-AG ether) or CB antagonists to, respectively, mimic or block the effects of 2-AG supports an activation mechanism independent of CB receptors.

Following its uptake, 2-AG can be hydrolyzed to AA by serine hydrolases, such as monoacylglycerol lipase, or metabolized into other bioactive lipids by eicosanoid biosynthetic enzymes [34, 35, 78–81]. The data presented here (Fig. 5) indicate that 2-AG and AA induce the release of antimicrobial activities through their metabolism into LTB_4 and the activation of BLT_1, in agreement with our previous study [36]. This again supports the physiological role of LTB_4 in host defense. Indeed, LTB_4 enhances host defense in vivo, promoting antimicrobial peptide release and enhancing phagocyte functions through BLT_1 receptor activation [39, 40].

AA is usually esterified at the *sn-2* position of glycerophospholipid. One of the dogmata regarding AA release is that upon cell activation, the group IVA PLA_2 translocates to membranes, where it releases AA. Interestingly, other PLA_2, notably the secreted PLA_2 groups V, X, and XII, are also participating in AA release [82]. The important impact of exogenously added AA on the release of antimicrobial peptides and antimicrobial effects that we observed indicates that secreted PLA_2 might also play an important role in the regulation of host defense. This, however, fosters additional studies to delineate the putative role of the secreted PLA_2 in these processes.

We also attempted to delineate the exact contribution of some antimicrobial products. Our data indicate that 2-AG and AA induced a modest production of ROS compared with fMLP and that the inhibition of NADPH oxidase with DPI did not blunt the effect of 2-AG and AA (Fig. 3, and data not shown). Interestingly, LL-37 and α-defensins were documented to mediate the LTB_4-induced antimicrobial effects of neutrophils [8, 39]. Our data show that there is an ~250:1 ratio between α-defensins and LL-37 release following the activation of human neutrophils by 2-AG or AA. Furthermore, our data indicate that LL-37 is more potent than α-defensins against *E. coli* and *S. aureus* by one order of magnitude (ED_{50} of 1 vs. 10 μg/ml). Importantly, we provided evidence that α-defensins and LL-37 acted synergistically rather than redundantly (Fig. 3G) in agreement with a previous study [83]. This suggests that the bactericidal effect of 2-AG and AA is likely the consequence of LL-37 and α-defensins. Interestingly, the inhibitory effect of 2-AG- and AA-stimulated neutrophil supernatants on HSV-1 infectivity does not correlate with the release of LL-37 or α-defensins (Figs. 1 and 3). This suggests that other soluble mediators might also participate in the virucidal effect of 2-AG- and AA-stimulated neutrophil supernatants and fosters additional studies.

Cytochalasin B acts by disrupting the actin cytoskeleton, which enhances many neutrophil functions, including degranulation. Cytoskeleton-disrupting agents are not necessary in vivo, as adherence to entothelium, which is linked to cytoskeleton rearrangement, is sufficient to prime neutrophils and promote the release of granule components (reviewed in ref. [39]). To better mimic the fate of in vivo neutrophils, we treated our neutrophil suspensions with 1 μM cytochalasin B to enhance their cellular responses. In the absence of cytochalasin B, only a minimal release of LL-37 and α-defensins was observed (data not shown).

Finally, our experiments were performed under serum-free conditions, given that serum can trap fatty acids. For example, the use of serum or BSA has long been used to analyze AA release by stimulated cells ex vivo. Moreover, the incubation of isolated neutrophils in plasma results in the trapping of de novo-synthesized LTA_4 [84]. Nonetheless, LTB_4 can be

found in activated whole blood under many circumstances. In our own hands, we still observed a stimulatory effect of 2-AG and AA, with up to 2% BSA (data not shown), which represents the protein content of an aqueous solution containing 5% serum [85]. We favored using BSA over plasma or serum to avoid the stimulatory effect that TGF-β has on the LT pathway [86]. That being said, AA-specific secreted PLA_2 enzymes have many biological functions involving eicosanoids, albeit they release AA in the extracellular space.

In conclusion, we provide evidence that 2-AG and AA are potential regulators of human neutrophil functions associated to host defense. Importantly, this occurred at physiological (nanomolar) concentrations of 2-AG and AA and was associated with the release of LL-37 and α-defensins from cytochalasin B-treated neutrophils. Numerous events associated with the innate and adaptive immune responses are linked to antimicrobial peptides: they recruit and activate leukocytes [5–13], the complement cascade [87], and the phagocytosis by macrophages. Consequently, our data support that 2-AG and AA may be important physiological regulators of the innate and adaptive immune responses in humans. Additional studies will be needed to establish the roles of 2-AG and AA as regulators of neutrophil-mediated innate immunity in vivo.

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Abbreviations

2-AG	2-arachidonoyl-glycerol
AA	arachidonic acid
ADA	adenosine deaminase
AEA	N-arachidonylethanolamine
AM-251	N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole 3-carboxamide
BLT_1	leukotriene B_4 receptor 1
CB	cannabinoid receptor
CIHR	Canadian Institutes of Health Research
DPI	diphenyleneiodonium
FLAP	5-lipoxygenase-activating protein
GPR55	GPCR 55
HSV-1	herpes simplex virus 1
LT	leukotriene

MAFP	methyl-arachidonyl-fluorophosphonate
O-2050	(6aR,10aR)-3-(1-methane-sulfonyl-amino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzopyran
O_2^-	superoxide anion
qPCR	quantitative PCR
RSV	respiratory syncytial virus
SR144528	5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3-carboxamide
THC	(−)-Δ^9-tetrahydrocannabinol
TSB	tryptic soy broth

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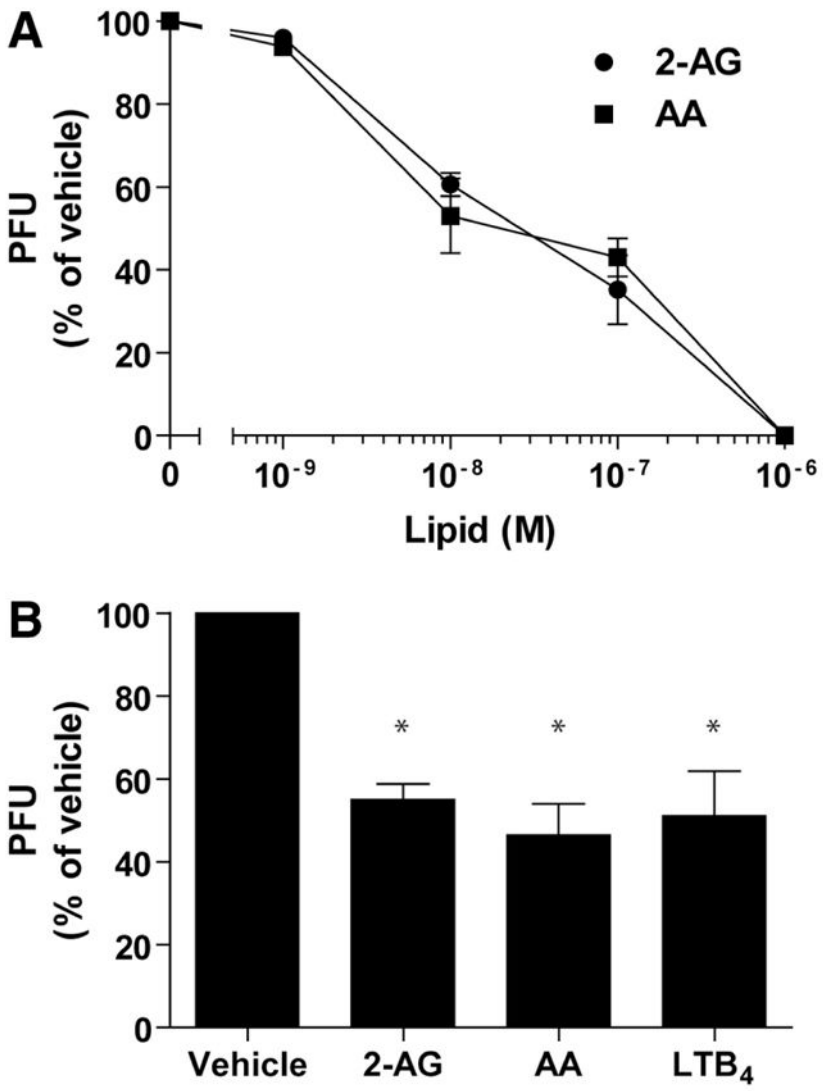

Figure 1. 2-AG and AA induce the release of antiviral activities

Human neutrophil suspensions (2×10^7 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were incubated with 1 μM cytochalasin B for 15 min and then stimulated with 2-AG or AA for 5 min. Incubations were stopped by placing the samples in an ice-water bath. Cell-free supernatants were diluted in M199 medium (10:90) and incubated with (A) HSV-1 or (B) RSV for 10 min before infection, as described in Materials and Methods. Data are the mean (±SEM) of four experiments, each performed in duplicate. Statistical analyses were done using mixed-effects ANOVA, followed by the Tukey a posteriori test and were considered significant when *P* values were <0.05. *Statistically significant difference compared with vehicle (DMSO).

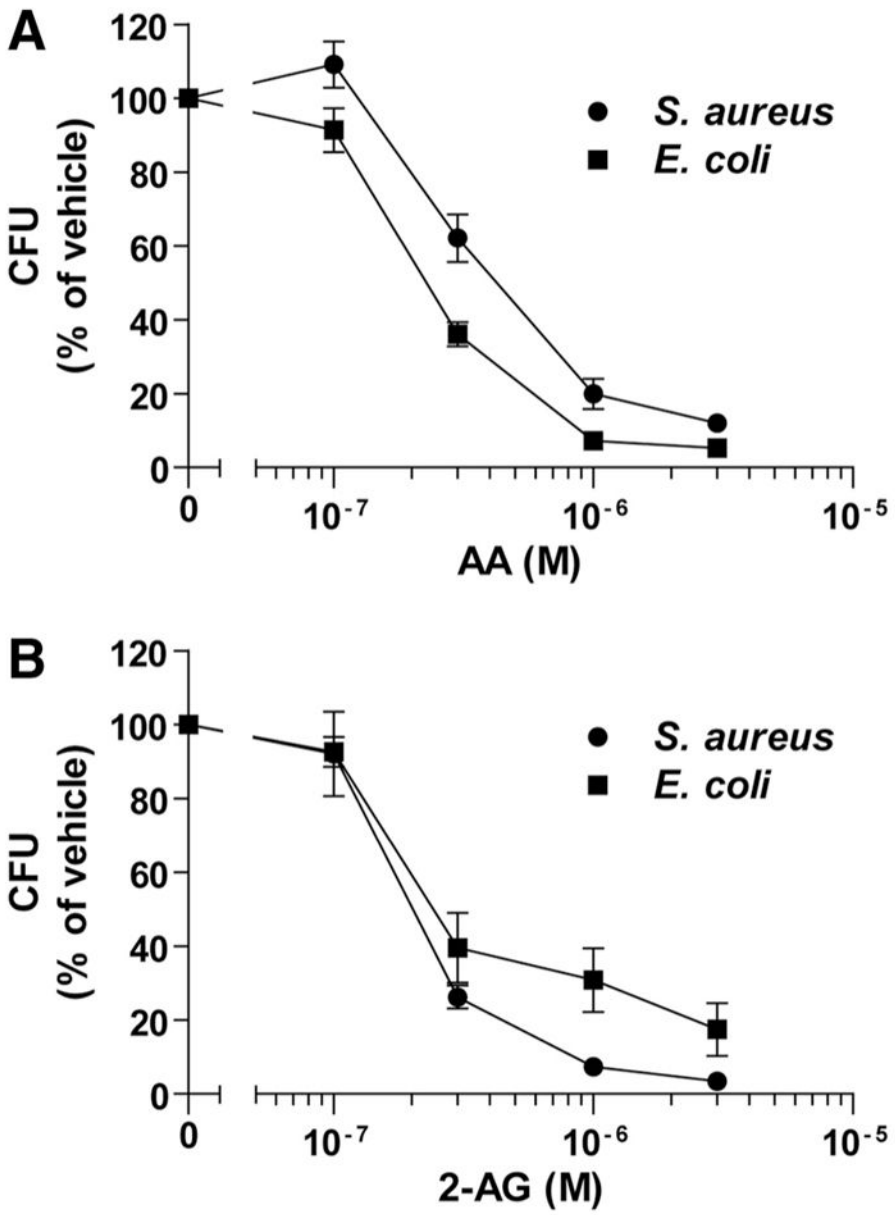

Figure 2. 2-AG and AA induce the release of antibacterial activities

Human neutrophils (2×10^7 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were incubated with 1 μM cytochalasin B for 15 min and then stimulated with (A) AA or (B) 2-AG for 5 min. Incubations were stopped by placing the samples in an ice-water bath and then centrifuged. Cell-free supernatants were incubated (1:1) with exponentially growing *E. coli* or *S. aureus* suspensions (10,000 CFU/ml) in sodium phosphate buffer containing 1% TSB for 4 h at 37°C. Samples then were spread on LB agar plates and incubated overnight at 37°C, followed by CFU enumeration. The results represent the mean (±SEM) of four independent experiments, each performed in triplicate. Vehicle represents an amount of DMSO that equals the volume used in each experiment involving 2-AG or AA.

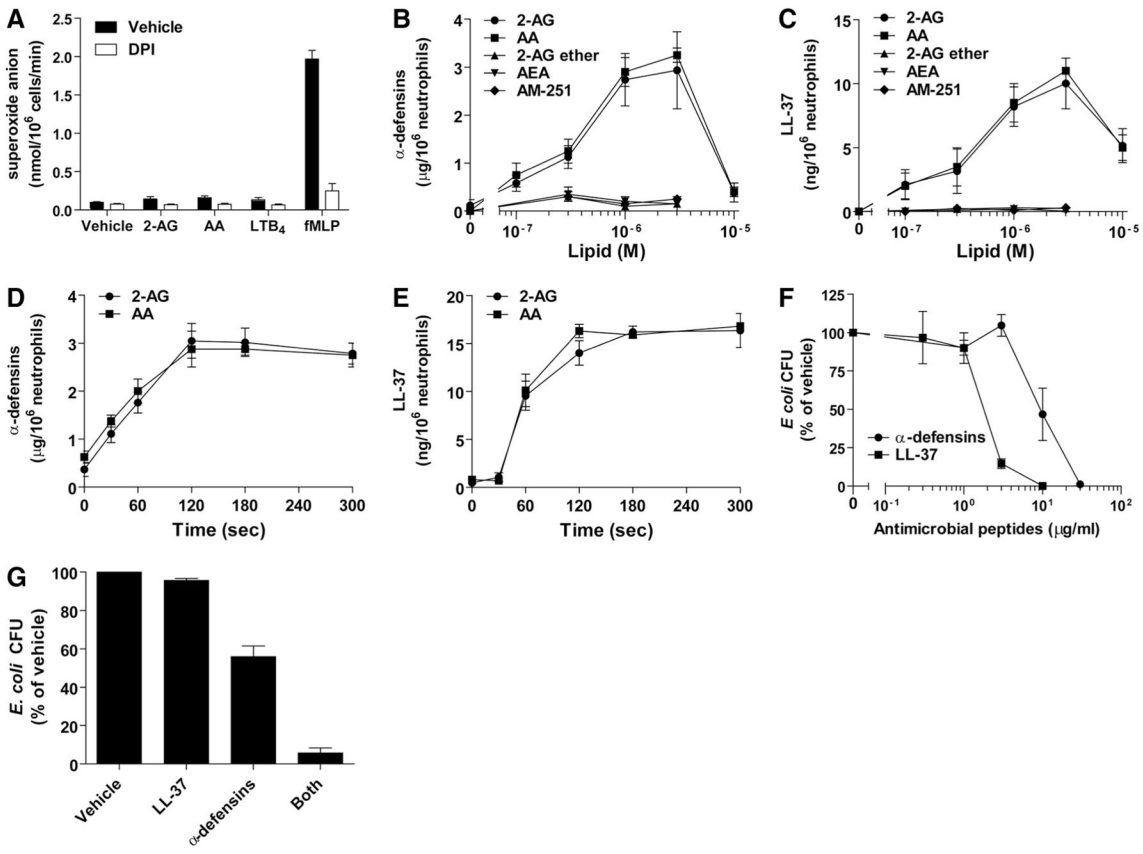

Figure 3. 2-AG and AA induce the release of antimicrobial factors

(A) Human neutrophils (2×10^7 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were incubated with 1 μM cytochalasin B for 15 min and then stimulated with 3 μM 2-AG, 3 μM AA, 100 nM LTB_4, or 100 nM fMLP for 5 min in the presence or absence of 10 μM DPI. Incubation was stopped by placing the samples in an ice-water bath and analyzed for O_2^- production, as described in Materials and Methods. (B and C) Human neutrophils (2×10^7 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were stimulated with 2-AG, AA, 2-AG ether, AEA, or AM-251 at the indicated concentration for 5 min. Incubations were stopped by placing the samples in an ice-water bath, and then cell-free supernatants were analyzed for their content in α-defensins or LL-37, as described in Materials and Methods. (D and E) Human neutrophils (2×10^7 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were stimulated with 3 μM 2-AG or AA for the indicated times. Incubations were stopped by placing the samples in an ice-water bath, and then the cell-free supernatants were analyzed for their content in α-defensins or LL-37, as described in Materials and Methods. (F) Purified LL-37 or α-defensins were incubated with exponentially growing *E. coli* suspensions (10,000 CFU/ml) in sodium phosphate buffer containing 1% TSB for 4 h at 37°C. (G) Purified LL-37 (40 ng/ml), α-defensins (10 μg/ml), or both were incubated with exponentially growing *E. coli* suspensions (10,000 CFU/ml) in sodium phosphate buffer containing 1% TSB for 4 h at 37°C. (F and G) Samples were diluted 1/300 in incubation medium, plated on LB agar plates, and incubated overnight at 37°C to allow CFU enumeration. (A–G) The data

represent the mean (±SEM) of at least three independent experiments, each performed in duplicate.

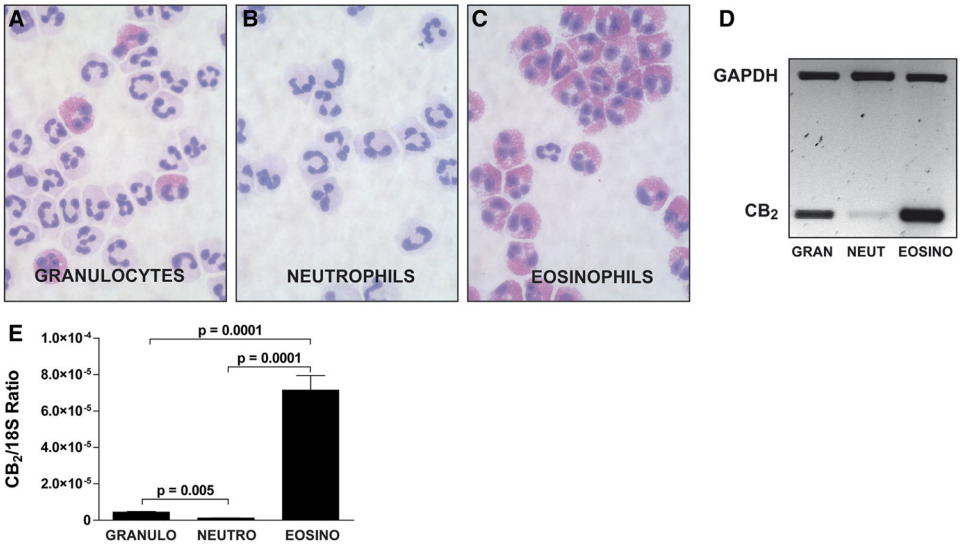

Figure 4. Expression of CB_2 by granulocytes, eosinophil-depleted neutrophils, and eosinophils

Granulocytes (A) were purified further into neutrophils ($CD16^+$; B) and eosinophils ($CD16^-$; C) and then stained with Diff-Quik. Total RNA was extracted from the cell pellets, and amplification of the CB_2 receptor mRNA by (D) RT-PCR or by (E) RT-qPCR was performed, as described in Materials and Methods. (D) The data represent a typical RT-PCR result obtained using the mRNA from cells of the same donor. (E) Data are the mean (±SEM) of six independent experiments, each performed in triplicate. Statistical analyses were done using mixed-effects ANOVA, followed by the Tukey a posteriori test. 18S refers to 18S rRNA and is used as an expression control.

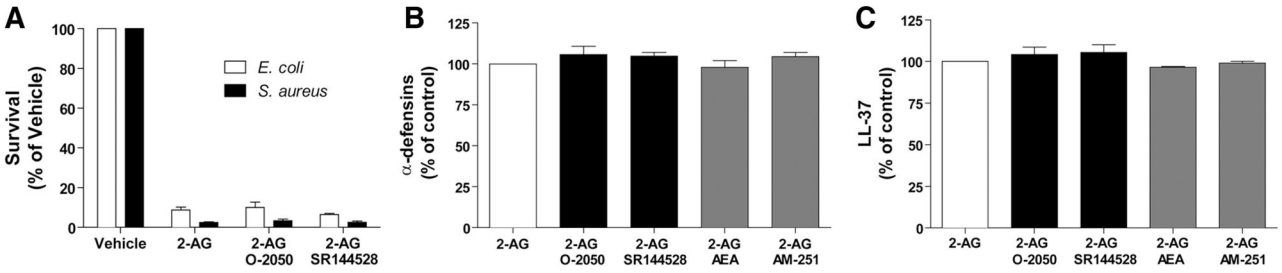

Figure 5. The 2-AG-mediated bacterial killing and antimicrobial peptide release are independent of CB activation

(A) Neutrophil suspensions (2×10^7 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were incubated with 1 μM cytochalasin B for 15 min and then stimulated with 1 μM 2-AG for 5 min. Incubations were stopped by adding 1 vol ice-cold buffer, immediately placing the samples in an ice-water bath for 5 min, and then centrifuged. Cell-free supernatants then were incubated with exponentially growing *E. coli* or *S. aureus* for 4 h and then plated on LB agar plates overnight to allow CFU quantification, as described in Materials and Methods. (B and C) Neutrophil suspensions (2×10^7 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were incubated with 1 μM cytochalasin B for 15 min and then stimulated with 1 μM 2-AG for 5 min. Incubations were stopped by adding 1 vol cold incubation buffer and then rapidly centrifuged. Supernatants then were assessed for their content in (B) α-defensins 1–3 and (C) LL-37 by ELISA, according to the manufacturer's instructions. Addition of 1 μM AEA, the GPR55 agonist (and CB_1 antagonist) AM-251, the CB_1 antagonist O-2050, or the CB_2 antagonist SR144528 was done 5 min before stimulating neutrophils with 2-AG in all experimental settings. The data represent the mean (±SEM) of three independent experiments performed in duplicate.

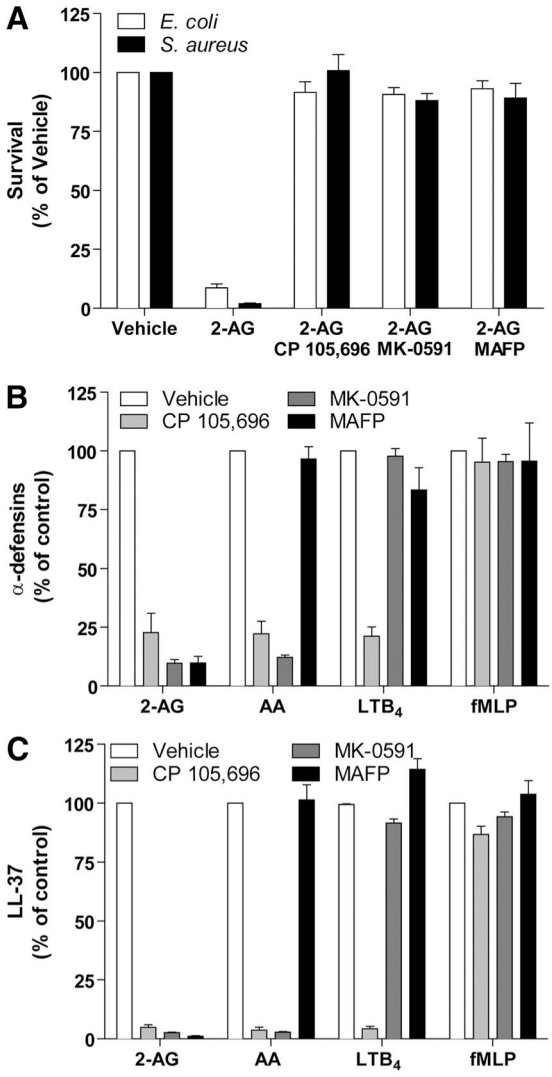

Figure 6. Involvement of LTB_4 in the effects of 2-AG and AA on bacterial growth and antimicrobial peptide release by neutrophils

(A) Neutrophil suspensions (2×10^7 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were incubated with 1 μM cytochalasin B for 15 min and then stimulated with 3 μM 2-AG for 5 min. Incubations were stopped by placing the samples in an ice-water bath and then centrifuged. Cell-free supernatants were incubated with exponentially growing *E. coli* or *S. aureus* for 4 h and then plated on LB agar plates overnight to allow CFU quantification, as described in Materials and Methods. (B and C) Neutrophil suspensions (2×10^7 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were incubated with 1 μM cytochalasin B for 15 min and then stimulated with 3 μM 2-AG, 3 μM AA, 100 nM LTB_4, or 100 nM fMLP for 5 min. Incubations were stopped by adding 1 vol cold incubation buffer and then rapidly centrifuged. Supernatants were next assessed for their content in (B) α-defensins 1–3 and (C) LL-37 by ELISA, according to the manufacturer's instructions. The addition of the BLT_1 antagonist CP 105,696 (100 nM), the FLAP antagonist MK-0591 (100 nM), and the 2-AG hydrolysis inhibitor MAFP (30 nM) was done 5 min prior to the stimulation with 2-AG,

AA, LTB_4, or fMLP in all experimental settings. The results represent the mean (±SEM) of at least three independent experiments, each performed in duplicate.