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The innate defense regulator peptides IDR-HH2, IDR-1002, and IDR-1018 modulate human neutrophil functions

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ABSTRACT

Although HDPs were originally hypothesized to act as antimicrobial agents, they also have been shown to broadly modulate the immune response through the activation of different cell types. We recently developed a series of novel, synthetic peptides, termed IDRs, which are conceptually based on a natural HDP, bovine bactenecin. We showed that IDR-1 and IDR-1002 protect the host against bacterial infections through the induction of chemokines. The objective of this study was to investigate the effects of the IDRs on various functions of human neutrophils. Here, we demonstrated that IDR-HH2, IDR-1002, and IDR-1018 modulated the expression of neutrophil adhesion and activation markers. Moreover, these IDRs enhanced neutrophil adhesion to endothelial cells in a β_2 integrin-dependent manner and induced neutrophil migration and chemokine production. The IDR peptides also increased the release of the neutrophil-generated HDPs (antimicrobial), human α-defensins, and LL-37 and augmented neutrophil-mediated killing of *Escherichia coli*. Notably, the IDRs significantly suppressed LPS-mediated neutrophil degranulation, the release of ROS, and the production of the inflammatory cytokines TNF-α and IL-10, consistent with their ability to dampen inflammation. As evidenced by the inhibitory effects of MAPK-specific inhibitors, IDRs activated the MAPK pathway that was required for chemokine production. In conclusion, our study provides novel evidence regarding the contribution of the IDR peptides to the innate immune response through the modulation of neutrophil functions. The results described here may aid in the development of IDRs as novel, anti-infective and immunomodulatory agents. *J. Leukoc. Biol.* 94: 159–170; 2013.

Introduction

HDPs, also called cationic antimicrobial peptides, are evolutionarily conserved molecules that are involved in the defense mechanisms of a wide range of organisms. They form the first line of defense against invading microbes [1]. With the exception of defensins and other cysteine-rich peptides, HDPs are generally 10–50 aa in length but lack any specific consensus amino acid sequences that are associated with biological activity; however, most of these molecules maintain certain common features, such as a net positive charge as a result of excess arginine and lysine residues, up to 50% hydrophobic amino acids, and an ability to fold into amphiphilic structures [1–3]. In addition to a generally weak direct-killing activity, HDPs exhibit a wide variety of immunomodulatory functions and delicately modulate inflammatory responses without compromising the elements of immunity that are required for the resolution of infections. The immunomodulatory activities of HDPs include the indirect and/or direct promotion of chemotaxis; the stimulation of production of many chemokines and certain cytokines; the modulation of DC and macrophage differentiation; the regulation of neutrophil and epithelial cell apoptosis; the suppression of potentially harmful, proinflammatory responses that are mediated by bacterial products; the induction of angiogenesis and wound healing; and adjuvant activity promoting a vigorous adaptive response [1–8]. Therefore, as a result of the multifunctional properties of HDPs and the increasing bacterial resistance to conventional antibiotics, HDPs and their derivatives are attractive candidates as templates for novel agents that can be used as anti-infective and immunomodulatory therapeutics [9, 10].

The development of natural HDPs has been problematic because of their harmful properties, such as their hemolytic activity and cytotoxicity toward mammalian cells, the stimulation of mast cell degranulation, and the promotion of apopto-

Abbreviations: CD62L=CD62 ligand, DCFDA=2′,7′-dichlorodihydrofluorescein diacetate acetyl ester, hCAP-18=human cationic antimicrobial protein, HDP=host defense peptide (protein), HNP=human neutrophil peptide, IDR=innate defense regulator

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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sis [11–13]. Recently, we screened a series of more than 100 distantly related peptide variants of bovine bactenecin (Bac2A) for their improved immunomodulatory activities, as assessed by chemokine production in human PBMCs [14]. These anti-infective, immunomodulatory peptides, termed IDRs, mediate protection against bacterial infections by selectively enhancing immune-protective mechanisms rather than through direct antimicrobial activity [7]. In contrast, the IDRs decrease proinflammatory responses to microbial products, thereby limiting potentially harmful inflammation [7, 8]. For instance, the prototypical IDR-1 offers prophylactic and therapeutic protection against systemic infections with multidrug-resistant bacteria in mouse models, and this protection is associated with an increase of chemokine production, the suppression of harmful inflammatory cytokines, and an increase of macrophages at the site of infection [7]. IDR-1002 increases protection from bacterial infections via the enhancement of chemokine production and the recruitment of PMN leukocytes/neutrophils and monocytes/macrophages to the site of infection in vivo [8], and IDR-HH2 also shows promise as a component of vaccine adjuvants for single-dose vaccines [15–17]. IDR-1018 is the most potent inducer of chemokines to date [18] and demonstrates anti-infective and anti-inflammatory activity in mouse models, including efficacy in treating *Plasmodium berghei* ANKA cerebral malaria when administered in conjunction with standard first-line antimalarials [19]. The mechanism of IDR-mediated cell recruitment involves local chemokine induction [7, 8, 18, 20] and the promotion of integrin-mediated adhesion [21]. This selective enhancement of innate immunity by IDR peptides represents a novel approach to anti-infective therapy and has many advantages over directly microbicidal compounds [10].

Neutrophils are part of the body's first line of defenses against pathogens and are critical effector cells in innate and adaptive immunity. The principal role of neutrophils in inflammation and the immune responses is primarily driven by phagocytosis and the killing of pathogens through oxidative and nonoxidative mechanisms [22]. Neutrophils respond to a large number of stimulants by enhanced chemotaxis, activation of integrins, and production of inflammatory cytokines and chemokines [22]. In addition to the classical neutrophil stimulants, such as fMLP, PMA, and activated complement fragment 5 [23], a number of HDPs, including human β-defensins [24], cathelicidin LL-37 [25], and S100A7/psoriasin [26], have also been reported to activate various neutrophil functions.

As IDR peptides are known to recruit neutrophils while suppressing inflammation [8], the objective of this study was to characterize further the effects of IDR-HH2, IDR-1002, and IDR-1018 peptides on human neutrophil functions. Here, we demonstrated that these IDR peptides regulated the expression of neutrophil adhesion and activation markers, enhanced neutrophil adhesion to endothelial cells in a β_2 integrin-dependent manner, and induced neutrophil migration and chemokine production. Furthermore, these IDRs also increased the release of the neutrophil-produced HDPs, human α-defensins, and LL-37 and augmented neutrophil killing of *Escherichia coli*. Notably, all of the IDR peptides suppressed LPS-mediated production of the inflammatory cytokines, such as TNF-α and IL-10, the release of ROS, and the degranulation of neutrophils. We also demonstrated that IDR peptides activated the MAPK pathway that was necessary for the production of chemokines. These observations provide novel evidence that IDR-HH2, IDR-1002, and IDR-1018 may contribute to the modulation of the innate immune response by regulating the neutrophil host defense functions at inflammation and infection sites and also by suppressing harmful inflammatory responses.

MATERIALS AND METHODS

Reagents

The peptides, IDR-HH2 (VQLRIRVAVIRA-NH_2), IDR-1002 (VQRWLIVWRIRK-NH_2), and IDR-1018 (VRLIVAVRIWRR-NH_2), were synthesized by solid-phase F-moc chemistry by CPC Scientific (Sunnyvale, CA, USA). A negative control peptide, 1035 (KRWRWIVRNIRR-NH_2), was similarly synthesized by the Biomedical Research Center (University of British Columbia, Vancouver, BC, Canada). The 5- (and 6)-chloromethyl-H_2DCFDA was purchased from Molecular Probes (Eugene, OR, USA). The FITC-labeled anti-human CD11b (CBRM1/5) Ab, anti-human CD62L (DREG-56) Ab, anti-human CD64 (10.1) Ab, anti-human CD66b (G10F5) Ab, and the isotype controls, mouse IgM λ (MOPC-104E) and IgG1 κ (MG1-45) Ab, were purchased from BioLegend (San Diego, CA, USA). The mouse anti-human CD18 (L130) and anti-human CD62L (DREG-56) Ab were obtained from BD Biosciences (San Jose, CA, USA). Rabbit polyclonal antiphosphorylated ERK, JNK, and p38 Ab and ERK, JNK, and p38 Ab were purchased from Cell Signaling Technology (Beverly, MA, USA). The MAPK inhibitors U0126, JNK inhibitor II, and SB203580 were obtained from Calbiochem (La Jolla, CA, USA), and fMLP was from Sigma-Aldrich (St. Louis, MO, USA). LPS was purified from *Pseudomonas aeruginosa*, as described previously [27].

Cell preparation and stimulation

In accordance with the ethics approval and guidelines of the University of British Columbia and the Juntendo University Graduate School of Medicine, informed consent was obtained from healthy volunteers, and blood was drawn from the cubital vein using heparin-containing Vacutainer tubes (BD Biosciences). After sedimentation of the erythrocytes, the upper fraction was layered onto Ficoll-Paque Plus (Amersham Pharmacia Biotech, Piscataway, NJ, USA) prior to density-gradient centrifugation, as reported previously [24]. Neutrophil purity was >95%, and cell viability was determined for each cell preparation by trypan blue exclusion and found to be >98%. The cells were suspended in RPMI-1640 medium, supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate (all from Invitrogen, Carlsbad, CA, USA), and treated with IDR peptides.

The EA.hy926 cells, a hybridoma of HUVEC and the human epithelial cell line A549, which have been shown to retain properties of native endothelial cells [28], were cultured in DMEM (high glucose) containing 10% FBS and hypoxantine, aminopterin, and thymidine media supplement (Invitrogen). These cells were grown in a humidified atmosphere and passaged every 3–4 days using 0.05% trypsin/0.02% EDTA to detach cells.

ELISA

After incubation of the neutrophils for the indicated periods of time with the IDR peptides, the neutrophils were centrifuged to obtain cell-free samples and stored at −20°C until use for ELISAs, according to the manufacturer's instructions. In some experiments, neutrophils were pretreated with MAPK-specific inhibitors for 2 h before stimulation with the IDR peptides. The ELISA kits for IL-8/CXCL8, IL-10, and MIP-1α/CCL3 were purchased from Invitrogen, and the TNF-α, MCP-1/CCL2, and MCP-3/CCL7 kits were

obtained from eBioscience (San Diego, CA, USA). The ELISA kits for HNP1–3 and LL-37 were purchased from HyCult Biotechnology (Uden, Netherlands).

Flow cytometry analysis for the expression of neutrophil adhesion and activation markers

Neutrophils (5×10^5) were incubated with the IDR peptides for 3 h at 37°C. The cells were washed in Opti-MEM and resuspended in saturating concentrations of the Ab against integrin CD11b, L-selectin CD62L, adhesion molecule CD64, and the GPI-linked glycoprotein CD66b for 30 min on ice. Control samples were stained with nonspecific mouse IgM λ or IgG1 κ Ab at the same concentrations. After washing, the samples were assayed using a FACSCalibur flow cytometer in conjunction with the CellQuest Pro software (BD Biosciences).

Neutrophil degranulation: MPO activity

MPO release was selected as a marker for the degranulation of neutrophil azurophilic granules and was quantified as described previously [8], using assay reagents obtained from Sigma-Aldrich. Briefly, 1×10^6 neutrophils were treated with the IDR peptides in the presence or absence of LPS for 30 min. The cells were then centrifuged to obtain cell-free samples. The MPO released from the neutrophils was quantified in the neutrophil supernatants that were diluted 1:1 in 2× 0.5% (w/v) hexadecyltrimethylammonium in 0.1 M potassium phosphate buffer (pH 6). The supernatants were collected and assayed. The MPO activity was quantified spectrophotometrically at a wavelength of 460 nm in the presence of 0.0005% (v/v) H_2O_2 and 0.5 mM *o*-dianisidine dihydrochloride. One unit of MPO was defined as the amount of enzyme that used 1 μmol/min H_2O_2 at 25°C.

Neutrophil adhesion assay

The neutrophils were resuspended in RPMI-1640 medium with 1% FBS, treated with the various doses of IDR peptides for 1 h at 37°C and washed to remove any excess stimulants. The cells (1×10^5/100 μl) were then added to confluent wells of EA.hy926 cells in a 48-well plate, incubated for 45 min at 37°C, and the wells were washed thrice to remove any nonadherent cells. The neutrophils that were adhered to the EA.hy926 cells were lysed with 0.5% Triton X-100 for 10 min, and the MPO activity was then measured in the cell lysates. For the inhibition experiments, the neutrophils were first pretreated with 10 μg/ml anti-CD18 mAb and/or anti-CD62L mAb for 1 h at 37°C and washed twice prior to the start of the adhesion assay. In some experiments, 48-well plates were coated overnight at 4°C with 25 μg/ml fibronectin (Calbiochem) or 2 μg/ml ICAM-1 (R&D Systems, Minneapolis, MN, USA)/well to evaluate neutrophil adhesion to fibronectin or ICAM-1. The coated wells were washed with PBS, blocked with PBS containing 1% BSA (Roche, Basel, Switzerland) for 1 h at 37°C, and then washed again with PBS prior to use. The neutrophils (1×10^5) were added to each well, followed by stimulation with the IDR peptides at 37°C for 1 h. The nonadherent cells were then removed, and the wells were washed twice with PBS. The adherent cells were lysed with 0.5% Triton X-100, and the MPO activity was measured in these cell lysates.

Measurement of intracellular ROS production

Flow cytometry was used to determine the intracellular levels of ROS, as described previously, using DCFDA [25]. Neutrophils at a density of 2×10^6 cells/ml were resuspended in 150 μl RPMI 1640, supplemented with 10% FBS, and incubated with each IDR peptide in the presence or absence of LPS for 30 min at 37°C. The cells were washed, resuspended in Opti-MEM (Invitrogen) containing 1 μM DCFDA, and then incubated for 30 min at 37°C. Following one wash in ice-cold PBS, the cells were analyzed by flow cytometry.

Chemotaxis assay

The chemotaxis assay was performed using a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD, USA). Neutrophils (1×10^5) were added to the upper wells of the chamber and were separated from the IDR peptide-containing lower wells by a polycarbonate membrane with 3 μm-diameter pores. These membranes were noncoated or were coated with 10 μg/ml fibronectin. Following a 60-min incubation, the number of migrated cells that were adherent to the underside of the filter was counted under a light microscope after the membrane was fixed with methanol and stained with the DiffQuick staining kit (Siemens, Newark, DE, USA). DiffQuick staining was used to confirm that the migrated cells were morphologically neutrophils (>98%).

Bacterial killing assay

The neutrophils were cultured in 1.5 ml microcentrifuge tubes, resuspended in HBSS (Invitrogen) for 30 min at 37°C in a humidified atmosphere with 5% CO_2, and stimulated with the IDR peptides at the indicated concentrations for 1 h. The cells were then washed twice with HBSS by centrifuging the cultures at 500 *g*, followed by resuspension in HBSS, supplemented with 10% autologous serum prior to the assay. Concurrently, luminescent *E. coli* Xen-14 (Caliper Life Sciences, Hopkinton, MA, USA) was harvested by centrifuging the cultures at 11,000 *g*, washing twice with HBSS, and resuspending the cultures in HBSS, supplemented with 10% autologous serum in microcentrifuge tubes. The opsonization of *E. coli* in human serum was performed by gentle inversion of the microcentrifuge tubes for 20 min at 37°C. Antimicrobial killing was assessed by adding 2.5×10^7 CFU of *E. coli* to 5×10^6 neutrophils/treatment condition, resulting in a multiplicity of infection of 5, followed by incubation for 15–60 min. The addition of *E. coli* to the assay medium without neutrophils was performed as a control for bacterial growth. The number of surviving *E. coli* under each treatment condition was determined by removing 50 μl of the coculture at each indicated time-point and adding it to 2.5 ml dilute NaOH solution (pH 11) for 5 min to lyse the neutrophils. This solution was then diluted further in HBSS and plated on LB agar plates. The percentage of *E. coli* killed was calculated according to the following formula: $[1-(CFU_{treatment}/CFU_{bacteria\text{-}only\ controls})] \times 100$.

Western blot analysis

Neutrophils (1×10^6) were incubated with the IDR peptides for 10 min. Following stimulation, cell lysates were obtained by lysing cells in RIPA buffer (Cell Signaling Technology), according to the manufacturer's specifications. Equal amounts of total protein were subjected to 12.5% SDS-PAGE. After nonspecific binding sites were blocked, the blots were incubated with pAb against phosphorylated or unphosphorylated ERK, JNK, and p38 overnight. The membrane was developed with an ECL detection kit (Amersham Pharmacia Biotech).

Statistical analysis

The statistical analysis across multiple treatment groups or time-points was determined with ANOVA, followed by the appropriate post hoc test, whereas the significant differences between paired groups were determined with Student's *t*-test. The statistical analyses were performed with Prism GraphPad for Windows (Prism 5; GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ was considered significant. The results are shown as the mean ± SD.

RESULTS

IDR peptides modulated the expression of adhesion molecules and activation markers on neutrophils

To determine whether the IDRs activate human neutrophils, we first assessed the effects of IDR-HH2, IDR-1002, and IDR-1018 on the expression of the neutrophil adhesion molecules, CD11b and CD62L, and the activation markers, CD64 and CD66b. As observed in **Fig. 1**, all three IDR peptides up-regulated significantly and dose-dependently the expression of

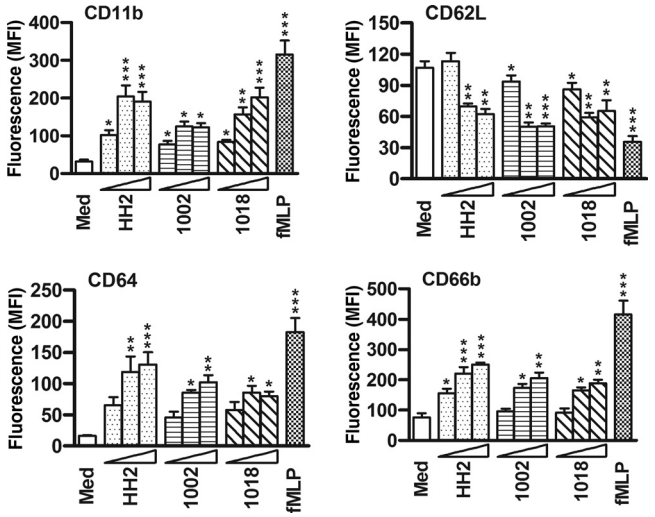

Figure 1. IDR peptides induce up-regulation of CD11b, CD64, and CD66b and shedding of CD62L. Neutrophils (5×10^5) were stimulated with 12.5–50 μg/ml IDR-HH2, IDR-1002, and IDR-1018 or 1 μM fMLP for 3 h at 37°C and incubated with CD11b-, CD62L-, CD64-, and CD66b-specific Ab for 30 min on ice. Samples were assayed using the FACSCalibur flow cytometer. Each bar shows the mean ± SD from four independent experiments using neutrophils from independent donors. The values are expressed as the mean fluorescence intensity (MFI) compared with the nonstimulated cells incubated with the isotype controls. Med, Medium; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

CD11b (four- to sixfold), CD64 (five- to eightfold), and CD66b (two- to threefold) and induced the shedding of CD62L (30–40%) from the surface of the neutrophils, although to a lesser extent than fMLP that was used as a positive control. Similar increases in CD18 expression were also detected in neutrophils treated with the IDR peptides (data not shown). Therefore, increases in the expression of CD11b, CD64, and CD66b and a decrease in the expression of CD62L on neutrophils treated with IDR-HH2, IDR-1002, and IDR-1018 indicate neutrophil activation by these peptides.

Effects of IDR peptides on neutrophil adhesion to endothelial cells

The increased expression of CD11b and the decreased expression of CD62L on the neutrophils caused by treatment with the IDR peptides suggested that these peptides modify neutrophil adhesion. Therefore, we examined the effects of the IDRs on neutrophil adherence to endothelial cells. The number of adherent neutrophils to endothelial cells was assessed by extracellular MPO release and was increased significantly (up to eightfold) following treatment of neutrophils with IDR-HH2, IDR-1002, and IDR-1018. This IDR peptide-induced increase in adherence of the neutrophils to the endothelial cells was dose-dependent (**Fig. 2A**). Similarly, in separate experiments, we confirmed that all three IDR peptides caused increased neutrophil adhesion to fibronectin and ICAM-1 (up to four- and 10-fold, respectively) compared with control, untreated neutrophils (Supplemental Fig. 1A and B). The combination of each IDR peptide with fMLP seemed only to have an additive effect on neutrophil adherence to the endothelial cells. This might indicate that the IDR peptides activate neutrophils through similar or completely independent mechanism(s) as fMLP. An interaction between the neutrophils and endothelial cells, which is important for neutrophil adhesion, occurs through the β_2 integrin, CD11b/CD18, and CD62L that are expressed on neutrophils [29]. Therefore, the contribution of this β_2 integrin and CD62L to the binding interaction was assessed. Ab directed against CD18 and CD62L significantly blocked the attachment of neutrophils to the endothelial cells, and the combination of both Ab further inhibited neutrophil adhesion to the endothelial cells (Fig. 2B). These results demonstrate that IDR-stimulated neutrophil adhesion to the endothelial

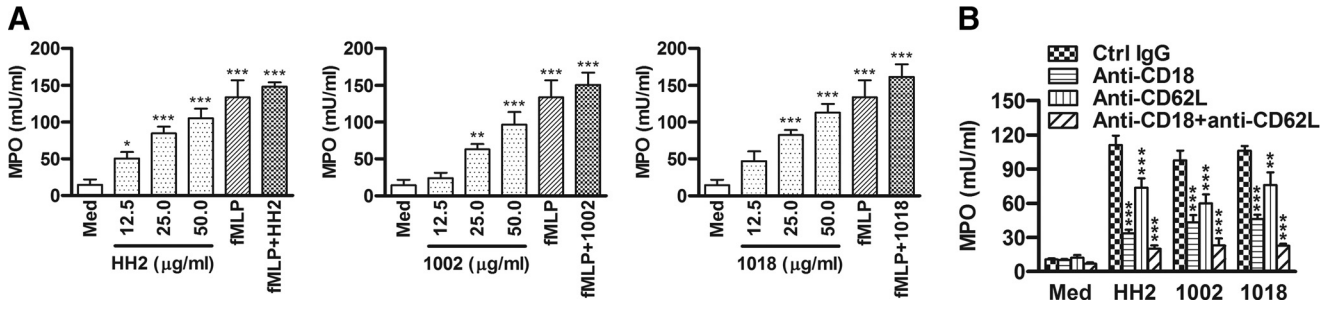

Figure 2. IDR peptides increase neutrophil adherence to endothelial cells. (A) Neutrophils (1×10^5) were treated with 12.5–50 μg/ml IDR-HH2, IDR-1002, and IDR-1018, 1 μM fMLP, or with a combination of fMLP and 25 μg/ml each IDR peptide for 1 h at 37°C and were then added to confluent wells of EA.hy926 cells and incubated for 45 min at 37°C. After washing, the neutrophils that were adherent to the EA.hy926 cells were lysed. The MPO enzyme activity was measured spectrophotometrically in the cell lysates at a wavelength of 460 nm in the presence of 0.0005% H_2O_2 and 0.5 mM *o*-dianisidine dihydrochloride. One unit of MPO is defined as the amount of enzyme that uses 1 μmol/min H_2O_2. The values are the mean ± SD of three to five separate experiments using cells from an independent donor, and the results are compared with the untreated group (Med); $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (B) Neutrophils were pretreated with 10 μg/ml anti-CD18 Ab, anti-CD62L Ab, a combination of 10 μg/ml anti-CD18 and anti-CD62L Ab, or 10 μg/ml isotype control Ab (Ctrl IgG) for 1 h. Neutrophils were then challenged for 1 h with 25 μg/ml IDR-HH2, IDR-1002, or IDR-1018. The IDR-stimulated neutrophils were added to EA.hy926 cells and incubated for 45 min at 37°C, and the adhesion assay was performed as described. The values are the mean ± SD of three separate experiments using cells from independent donors; $^{**}P < 0.01$; $^{***}P < 0.001$ compared in the presence and absence of each Ab.

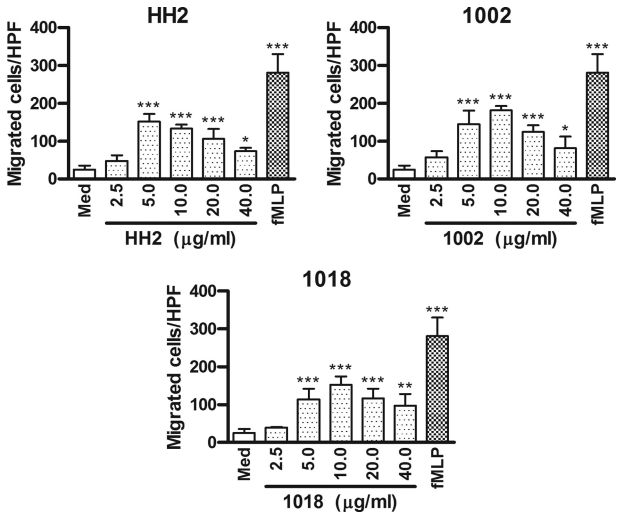

Figure 3. IDR peptides induce neutrophil migration. Neutrophils (1×10^5) were placed in the upper wells of a chemotaxis microchamber and allowed to migrate toward the lower well containing 2.5–40 μg/ml IDR-HH2, IDR-1002, and IDR-1018, 10 nM fMLP, or diluent (Med) for 1 h at 37°C. Chemotaxis was assessed by counting the number of cells that migrated through the polycarbonate membrane with 3 μm-diameter pores in five randomly chosen high power fields (HPF) under a light microscope. The values were compared between the stimulated and nonstimulated cells (Med); $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Each bar represents the mean ± SD of four separate experiments using neutrophils from independent donors.

cells is mediated through the β_2 integrin, CD11b/CD18, and CD62L.

IDR peptides induced neutrophil migration on fibronectin-coated surfaces

The effects of the IDR peptides on neutrophil migration were tested in an in vitro chemotaxis assay using modified Boyden chambers with a fibronectin-coated membrane. As shown in **Fig. 3**, IDR-HH2, IDR-1002, and IDR-1018 promoted chemotaxis of the neutrophils at concentrations varying from 5 to 40 μg/ml. This IDR-mediated neutrophil migration was dose-dependent and bell-shaped, and 5 μg/ml was the optimal chemotactic dose for IDR-HH2, whereas 10 μg/ml was optimal for IDR-1002 and IDR-1018. The IDR peptide-induced chemotactic activity was ~50% of the cell activity seen with fMLP. A significant increase in chemotaxis was also observed using a non-coated membrane (data not shown). The presence of IDR in only the upper compartment did not induce any substantial increase of cell migration, implying that IDR-mediated neutrophil migration is based predominantly on chemotaxis rather than chemokinesis (data not shown).

IDR peptides attenuated LPS-induced neutrophil activation

As the IDR peptides increased the expression of CD11b and CD66b, which are stored in specific and gelatinase-containing granules, respectively, these data indicated the possibility that the IDRs induced neutrophil degranulation. Therefore, we investigated the effects of the IDR peptides on neutrophil degranulation by assessing the extracellular release of MPO, an azurophilic granule component known as a neutrophil degranulation marker. The results depicted in **Fig. 4** demonstrated that IDR-HH2, IDR-1002, and IDR-1018 modestly (up to twofold) and dose-dependently induced MPO release from the neutrophils compared with nonstimulated cells. fMLP was used as a positive control, and costimulation of the neutrophils with the IDRs and fMLP increased MPO release further, although this effect was additive but not synergistic. In our experimental conditions, 427.29 ± 31.64 mU/ml MPO was present in 1×10^6 neutrophils when these cells were lysed.

LPS is an inflammatory stimulus and is known to stimulate various neutrophil functions, including degranulation, ROS production, and cytokine/chemokine production [30]. As we have reported recently—that IDRs inhibit LPS-induced proinflammatory responses in human PBMCs [7, 8, 18]—we assessed the impact of IDR-HH2, IDR-1002, or IDR-1018 on LPS-induced neutrophil degranulation. LPS alone increased neutrophil degranulation significantly (four- to fivefold compared with the individual IDR peptides), and this effect was reduced significantly by treatment with IDR-HH2, IDR-1002, or IDR-1018 (Fig. 4).

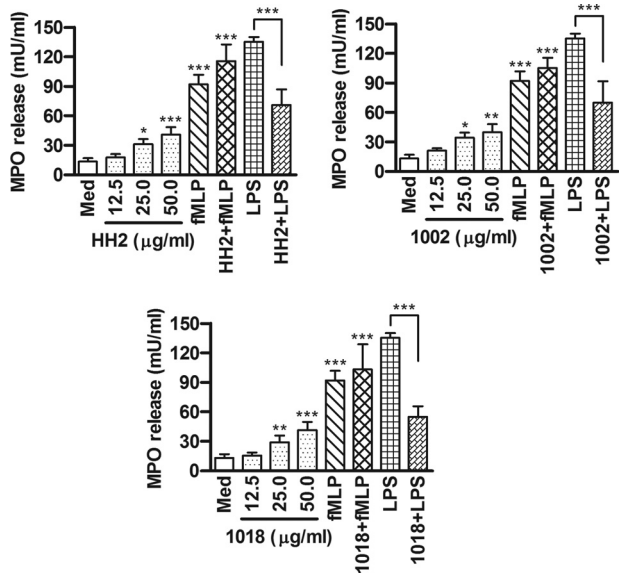

Figure 4. Effects of IDR peptides on LPS-mediated neutrophil degranulation. Neutrophils (1×10^6) were treated with 12.5–50 μg/ml IDR-HH2, IDR-1002, and IDR-1018, 1 μM fMLP alone or in combination with 50 μg/ml of each peptide, or 50 ng/ml LPS alone or in combination with 50 μg/ml of each IDR peptide for 30 min at 37°C. After washing, MPO enzyme activity was measured spectrophotometrically in the cell-free supernatants at 460 nm in the presence of 0.0005% H_2O_2 and 0.5 mM *o*-dianisidine dihydrochloride. One unit of MPO is defined as the amount of enzyme that used 1 μmol/min H_2O_2. The values are the mean ± SD of four independent experiments using neutrophils from independent donors. The values were compared between the stimulated and nonstimulated cells (Med) or between the cells treated with LPS alone and LPS in combination with each IDR peptide; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

We next evaluated the actions of the IDR peptides on LPS-mediated ROS production. First, as shown in **Fig. 5A**, IDR peptides alone caused a slight increase of ROS generation, and costimulation of the neutrophils with the IDR peptides and fMLP only had an additive effect, if any at all. In preliminary, dose-dependent experiments, we observed that 50 μg/ml of each IDR peptide induced the highest levels of ROS (Supplemental Fig. 2). Analogous to neutrophil degranulation, LPS greatly induced the ROS production, and the combination of each IDR peptide with LPS suppressed LPS-mediated ROS production significantly compared with LPS alone. IDR-1002 and IDR-1018 were more effective than IDR-HH2 in inhibiting LPS-caused ROS generation (Fig. 5B).

As IDR-1 and IDR-1002 are known to induce the production of specific cytokines/chemokines in human PBMCs [7, 8], we examined whether IDR-HH2, IDR-1002, and IDR-1018 affected cytokine/chemokine production in neutrophils. Following treatment of the neutrophils with the individual IDR peptides, the production of TNF-α and IL-10 was increased modestly. As expected, LPS strongly induced the TNF-α and IL-10 production, and the combination of each IDR peptide with LPS inhibited this cytokine production significantly (**Fig. 6A**). We also observed that IDR-HH2, IDR-1002, and IDR-1018 considerably increased the production of the chemokines IL-8/CXCL8, MCP-1/CCL2, MCP-3/CCL7, and MIP-1α/CCL3 in a dose-dependent manner in the absence of LPS; however, in contrast to the situation with TNF-α and IL-10 production, costimulation of the neutrophils with the IDR peptides and LPS did not lead to suppression of the production of these chemokines, but rather, to some extent, the IDRs cooperated with LPS to enhance this production (Fig. 6B). Consistent with previous reports [7], we confirmed that all three peptides did not affect the binding of LPS to the LPS-binding protein, confirming that these peptides did not modulate neutrophil activation by blocking LPS binding directly (data not shown).

IDR peptides enhanced neutrophil killing of *E. coli*

To determine whether the IDR peptides could also modulate neutrophil killing, in addition to neutrophil migration, degranulation, and cytokine/chemokine production, we investigated the effects of IDR-HH2, IDR-1002, and IDR-1018 on the neutrophil-mediated killing of *E. coli*. As shown in **Fig. 7A–C**, the neutrophils treated with various doses of the IDR peptides displayed increased killing of *E. coli* compared with the untreated neutrophils. Overall, the neutrophil killing effect peaked 30 min after the addition of *E. coli* to the IDR-treated neutrophils, and 25 μg/ml of each peptide was determined to be the optimal killing dose. Longer treatments of up to 60 min resulted in elevated, spontaneous killing of the *E. coli*. Costimulation of the neutrophils with IDR peptides and fMLP increased the neutrophil-mediated killing of the *E. coli* only slightly further (Fig. 7D).

IDR peptides induced extracellular release of HDPs from neutrophils

It has been shown previously that stimulation of neutrophils by HDPs resulted in an extracellular release of neutrophil-produced HDPs, including α-defensins and cathelicidin cationic antibacterial polypeptide of 11 kDa [25, 26, 31]. As we reported recently that some IDR peptides lack direct antimicrobial activities [7, 8] (and unpublished data), it was postulated that the IDR peptides might partly increase the killing activity of the neutrophils through their ability to induce the release of HDPs contained in the neutrophil granules. We evaluated the release of HNP1, -2, and -3, which are members of human α-defensins that are located in the neutrophil azurophilic granules [32], and cathelicidin LL-37 that is stored in specific granules [33]. Incubation of the neutrophils with IDR-HH2, IDR-1002, or IDR-1018 resulted in dose-dependent increases in the release of HNP1–3 and LL-37. This effect was observed first, as early as 30 min after treatment with the IDR peptides

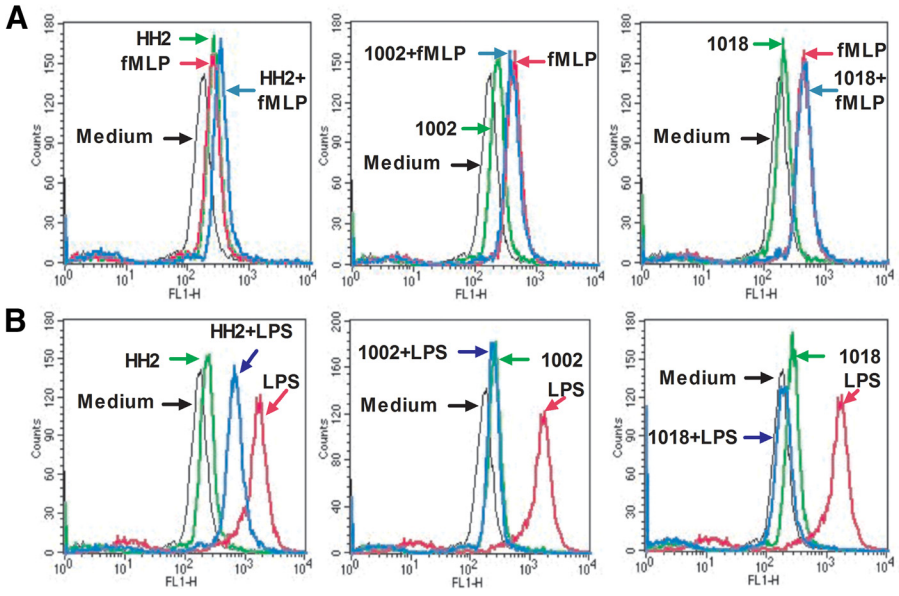

Figure 5. Effects of IDR peptides on LPS-induced ROS generation. (A) Neutrophils (3×10^5) were treated with 50 μg/ml IDR-HH2, IDR-1002, and IDR-1018 or with 1 μM fMLP alone or in combination with each IDR peptide for 30 min at 37°C. Representative FACS profiles of DCFDA oxidation at 30 min with nonstimulated cells (black lines) are shown. IDR-induced ROS generation is represented by green lines, fMLP-induced ROS generation is represented by red lines, and fMLP in combination with each IDR is represented by blue lines. (B) Neutrophils were also treated with 50 μg/ml IDR-HH2, IDR-1002, and IDR-1018 in the presence or absence of 50 ng/ml LPS for 30 min at 37°C, and ROS generation was analyzed as above. Spontaneous ROS generation from nonstimulated cells is shown in black lines. IDR-induced ROS generation is represented by green lines, LPS-induced ROS generation is represented by red lines, and LPS in combination with each IDR is represented by blue lines. FL1-H, Fluorescence 1-height.

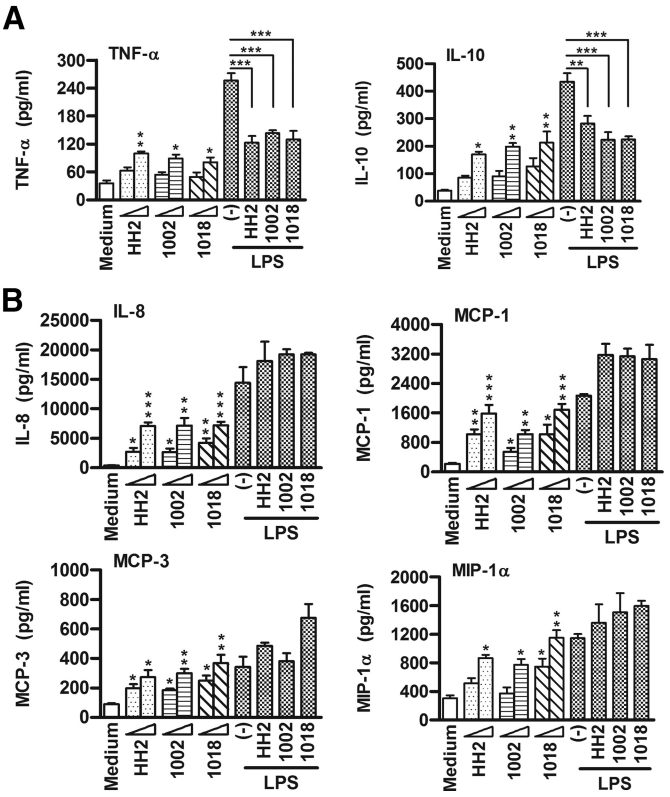

Figure 6. IDR peptides modulated LPS-mediated cytokine and chemokine production. (A and B) Neutrophils (1×10^6) were treated with 25–50 μg/ml IDR-HH2, IDR-1002, and IDR-1018 or with 50 ng/ml LPS alone or in combination with 50 μg/ml of each IDR peptide for 6 h at 37°C, and the concentrations of (A) TNF-α and IL-10 and (B) IL-8, MCP-1, MCP-3, and MIP-1α released into the culture supernatants were determined by ELISA. The values were compared between the stimulated and untreated cells (Medium) or between the cells treated with LPS alone (−) and LPS in combination with each IDR peptide. The bars show the mean ± SD of three independent experiments using cells from independent donors; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

and was sustained even up to 3 h later (**Fig. 8A** and **B**). The combination of the IDR peptides with fMLP increased HNP and LL-37 release further, but this effect was only additive (Fig. 8C). These data are consistent with the suggestion that IDR-mediated killing by neutrophils might be, in part, a result of IDR-induced extracellular release of neutrophil-generated HDPs and/or the immunomodulatory activities of HDPs.

Activation of the MAPK pathway by IDR peptides is necessary for the production of chemokines

As the MAPK pathway occupies a central role in innate immunity and as IDR-1 and IDR-1002 have been reported to stimulate chemokine production by PBMCs through the MAPK pathway [7, 8], we reasoned that IDR-HH2, IDR-1002, and IDR-1018 might also activate MAPKs in human neutrophils. As pictured in **Fig. 9A**, all IDR peptides markedly induced phosphorylation of ERK, JNK, and p38. In preliminary experiments, the optimal activation of MAPKs induced by IDRs was observed after 10 min. Likewise, fMLP, used as a positive control, also enhanced MAPK phosphorylation.

The activation of MAPKs was required for the production of chemokines IL-8, MCP-1, MCP-3, and MIP-1α by IDR peptides. This was shown by the noteworthy suppression of chemokine production by specific inhibitors of ERK (U0126), JNK (JNK inhibitor II), and p38 (SB203580; Fig. 9B). These inhibitors also suppressed fMLP-induced chemokine production, suggesting a possible overlap among the signaling pathways used by IDR peptides and fMLP. The doses of inhibitors used in this study were not toxic to neutrophils, as tested by lactate dehydrogenase activity (data not shown). All of the experiments included DMSO vehicle controls, and the levels of DMSO in the cell cultures never exceeded 0.1% (data not shown).

DISCUSSION

We showed recently that IDRs recruit neutrophils to the site of infection in vivo while suppressing inflammation [8]. To understand this further, we investigated the effects of IDRs on various functions of human neutrophils. This study demonstrated that the three tested IDR peptides—IDR-HH2, IDR-1002, and IDR-1018—enhanced neutrophil adhesion, migration, and cytokine/chemokine production. These peptides also stimulated the release of neutrophil-generated HDPs and augmented neutrophil-mediated killing of *E. coli*. Moreover, the IDRs suppressed LPS-induced proinflammatory cytokine secretion, as well as the LPS-induced release of ROS and neutrophil degranulation. IDR peptides also activated the MAPK pathway, which was required for chemokine production. These findings indicated that IDR-HH2, IDR-1002, and IDR-1018 regulate a range of neutrophil functions and provided novel insights regarding the contribution of the IDR peptides to the innate immune response through the modulation of neutrophil activation. We confirmed that a negative control peptide, 1035, isolated as part of the same design series as IDR-HH2, IDR-1002, and IDR-1018 but with no immunomodulatory properties, as measured by its lack of enhancement of chemokine production in human PBMCs (unpublished data), did not elicit neutrophil activation above the baseline levels (data not shown). This observation confirms that the modulation of neutrophil functions that is mediated by the IDR peptides is specific.

The migration of neutrophils from the circulation into an area of inflammation or infection involves the regulated expression of neutrophil surface adhesion molecules. L-selectin (CD62L) is important in the initial attachment of the neutrophils to the endothelium and is shed rapidly after neutrophil activation [34]. This is followed by tight adhesion and transendothelial migration of the neutrophils, which are mediated by β_2 integrins. CD11b/CD18 represents the predominant β_2 integrin that is expressed on the neutrophils, and this protein is known to interact with fibronectin and ICAM-1 [35]. CD11b/CD18 also regulates several neutrophil functions, including adhesion, migration, degranulation, and phagocytosis [36–38]. CD66b is another glycoprotein hypothesized to be involved in neutrophil activation and migration through the regulation of

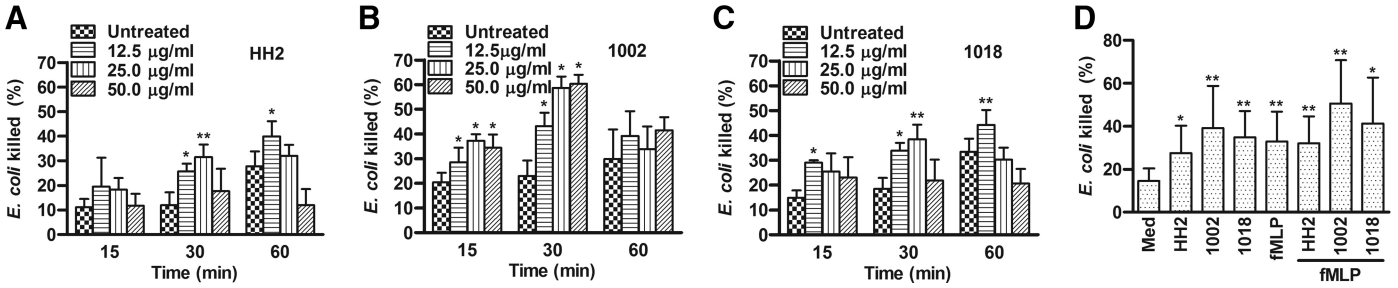

Figure 7. IDR peptides potentiate neutrophil killing of E. coli. (A–C) Neutrophils (5×10^6) were incubated with 12.5–50 μg/ml IDR-HH2, IDR-1002, or IDR-1018 or diluent for 1 h at 37°C and infected with luminescent *E. coli* at a multiplicity of infection of five for 15–60 min. Neutrophils were lysed, the mixture was plated on LB agar plates, and the percentage of *E. coli* killed was calculated as described in Materials and Methods. (D) Cells were also incubated with 25 μg/ml IDR-HH2, IDR-1002, and IDR-1018 or with 1 μM fMLP alone or in combination with each IDR peptide for 1 h and infected with *E. coli* for 30 min. The percentage of *E. coli* killed was calculated as above. The values are compared between the stimulated and nonstimulated cells (Untreated or Med). The bars show the mean ± SD of the percent killing of three independent experiments using neutrophils from independent donors; $*P < 0.05$; $**P < 0.01$.

CD11b/CD18 adhesive activity [39]. Another neutrophil activation marker is CD64, which initiates ROS generation, degranulation, and phagocytosis [40]. Here, we demonstrated that the IDR peptides increased the expression levels of CD11b/CD18, CD64, and CD66b and lowered the expression of CD62L, demonstrating that the IDR peptides induced neutrophil activation.

The ability of the IDR peptides to regulate the expression of the neutrophil adhesion molecules and activation markers was supported first by the observation that these peptides enhanced the adherence of neutrophils to the endothelial cells, fibronectin, and ICAM-1, and this effect was controlled almost completely by CD11b/CD18 and CD62L. Furthermore, all three IDR peptides induced neutrophil migration significantly.

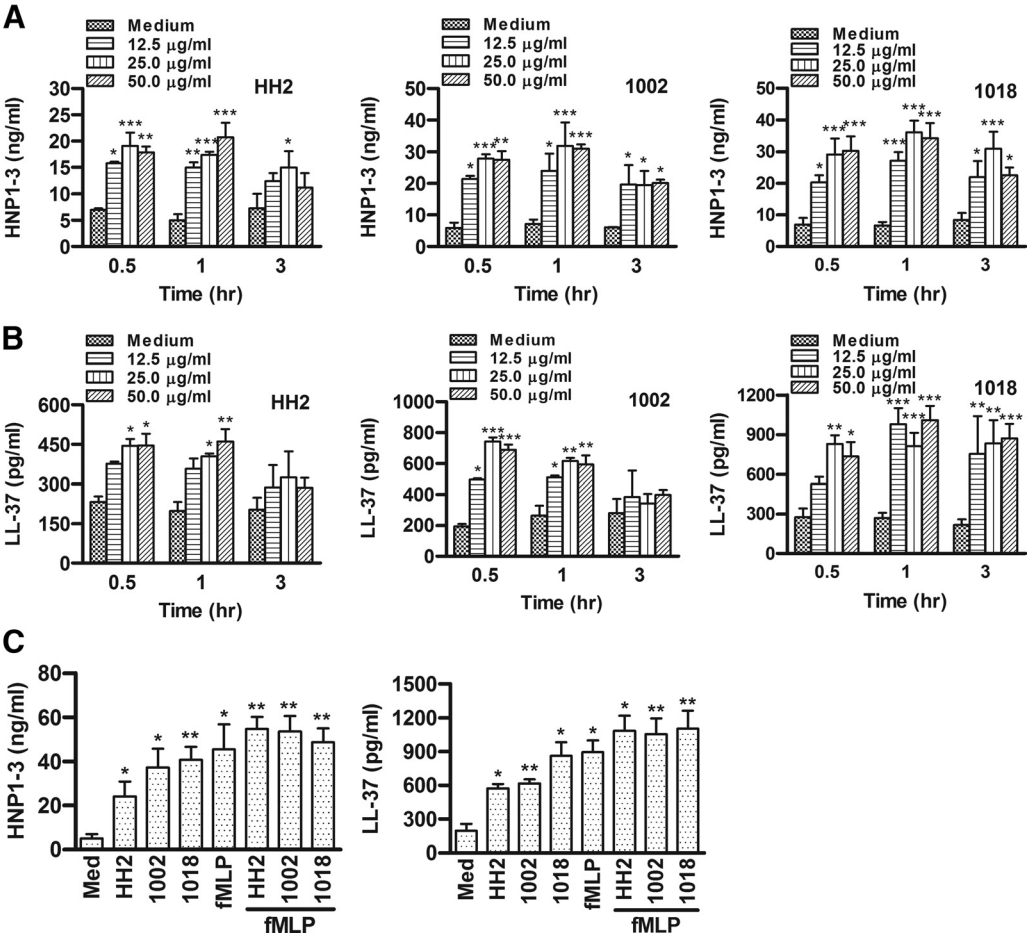

Figure 8. IDR peptides increase the extracellular release of HNP1–3 and LL-37. Neutrophils (1×10^6) were incubated with 12.5–50 μg/ml IDR-HH2, IDR-1002, or IDR-1018 or diluent (Medium) for 0.5–3 h at 37°C. Following the incubation, the cell-free supernatants were used in ELISAs for the detection of released (A) HNP1–3 and (B) LL-37 proteins. (C) Cells were also incubated with 25 μg/ml IDR-HH2, IDR-1002, and IDR-1018 or with 1 μM fMLP alone or in combination with each IDR peptide for 30 min, and the amounts of HNP1–3 and LL-37 released were detected as above. The values are compared between the stimulated and nonstimulated cells (Medium or Med); $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Each bar represents the mean ± SD of four separate experiments using neutrophils from independent donors.

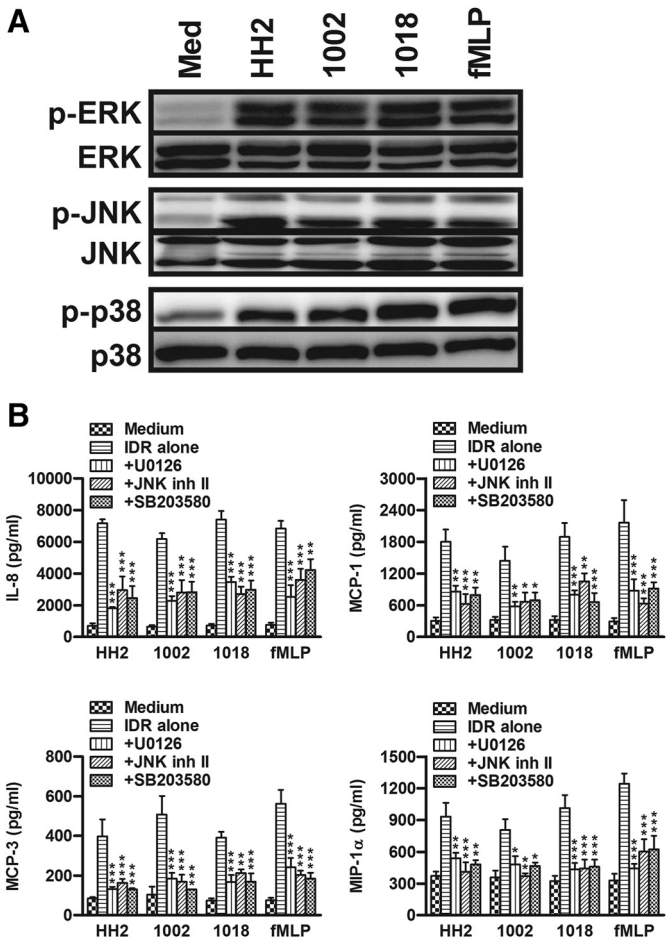

Figure 9. IDR peptides induce the activation of MAPKs, which are responsible for chemokine production by neutrophils. (A) Neutrophils (1×10^6) were stimulated with 25 μg/ml IDR-HH2, IDR-1002, and IDR-1018, 1 μM fMLP, or the diluent (Med) for 10 min. The levels of phosphorylated (p) and unphosphorylated ERK (p-ERK and ERK), JNK (p-JNK and JNK), and p38 (p-p38 and p38) in cell lysates were then determined by Western blot analysis. One representative experiment of three separate experiments with similar results is shown. (B) The effects of ERK, JNK, and p38 inhibitors on chemokine production. Neutrophils (1×10^6) were pretreated with 10 μM U0126 (ERK inhibitor), JNK inhibitor II (JNK inh II), and SB203580 (p38 inhibitor) or 0.1% DMSO for 2 h, and the cells were then exposed to 25 μg/ml IDR-HH2, IDR-1002, and IDR-1018, 1 μM fMLP, or the diluent (Medium) for 6 h. The concentrations of IL-8, MCP-1, MCP-3, and MIP-1α released into the culture supernatants were determined by ELISA. The values are the mean ± SD of three separate experiments and were compared between the presence and absence of each inhibitor; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Upon arrival at the site of infection, the neutrophils recognize and migrate toward invading pathogens and potentially degranulate. This action deploys a potent antimicrobial arsenal that includes various antimicrobial peptides/proteins, cytotoxic and proteolytic enzymes, and ROS [41, 42]. This process is executed rapidly by the neutrophils, as the granule proteins are not synthesized de novo at the sites of infection but rather, are synthesized during neutrophil differentiation and stored in azurophilic, specific, and gelatinase granules [42]. Here, we demonstrated that at an early, 30-min time-point, the IDR peptides induced the exocytosis of MPO and the antimicrobial granule components, human α-defensins (HNP1–3) and cathelicidin LL-37. Although HNP and LL-37 secretion was increased fourfold, there was only limited secretion of MPO (up to twofold), equivalent to 10% of total MPO release. The most plausible explanation for this result is the previous finding that although HNPs and MPO are stored in azurophilic granules, HNPs are the dominating protein in a major subset of azurophilic granules [43]. As for LL-37, a component of specific granules, the relatively higher exocytosis of this protein upon stimulation with IDR peptides may be a result of the fact that specific granules are exocytosed easily compared with azurophilic granules [44]. Among α-defensins, HNP1–3 account for 5–7% of the total neutrophil proteins [45], whereas HNP4 comprises <2% of the total defensins present in neutrophils [46]. LL-37 is the unique human cathelicidin, which is normally generated by proteolytic cleavage after the extracellular release of preproprotein hCAP-18. In addition to neutrophils, hCAP18/LL-37 is present in lymphocytes, PBMCs, squamous epithelia, and keratinocytes [2, 3]. Besides their (relatively weak) microbicidal activities, HNPs and LL-37 also have a range of immunomodulatory roles in various immune and inflammatory cells, including neutrophils [2, 3]. Although the concentrations of HNPs and LL-37 released from neutrophils by IDRs are lower than the doses required for killing *E. coli*, especially under physiological salt conditions [47], it is possible that this could be mitigated by the high concentrations of peptides in granules during phagosome-lysosome fusion, the higher local concentrations at sites of degranulation, elevated concentrations in patients with bacterial infections, or local inflammation [48, 49], and the synergistic action of HNPs and LL-37 [47] might enable direct killing. Alternatively, these natural HDPs may increase some of the immunomodulatory effects observed here. Our finding that the IDR peptides enhance neutrophil-mediated killing of *E. coli* may be partly a result of the release of HNPs and LL-37, although neutrophils possess other antimicrobial defenses, including ROS, cationic proteins, and granule proteases, which are delivered to phagosomes and the extracellular environment [41, 42].

Although neutrophils are somewhat specialized in handling immediate host defenses during tissue infection, they can have a deleterious effect in magnifying the inflammatory response. Especially in the presence of an uncontrolled infection—sustained, proinflammatory signals from other immune cells and resident tissue cells or during inappropriate activation—neutrophils can exacerbate inflammation and create local tissue damage[50]. In these circumstances, the uncontrolled release of proteases, ROS, and other cytotoxic products leads to additional tissue destruction, further neutrophil and immune cell infiltration, and the perpetuation of inflammation [50]. Therefore, to limit collateral host tissue damage, the release of these cytotoxic mediators needs to be tightly regulated [50, 51]. Intriguingly, although treatment with the IDR peptides alone induced a modest increase in MPO release and a slight enhancement of ROS generation, these peptides markedly sup-

pressed LPS-induced neutrophil degranulation and ROS production. In addition to its microbicidal activities, ROS has other roles in physiological and pathophysiological processes that are relevant to infection, including tissue injury and mediation of inflammation [52]. Therefore, the observation that the IDR peptides inhibited LPS-mediated neutrophil degranulation and ROS generation indicates that these peptides act as protective agents that regulate the excessive release of proinflammatory mediators and is consistent with the selective modulation (control) of inflammatory responses observed with monocytes and in animal models [7, 8]. During inflammation, activated neutrophils initiate an apoptotic program, which facilitates the resolution of inflammation and prevents tissue damage caused by necrotic cell lysis and the spilling of cytotoxic proteins and ROS into the extracellular environment [53, 54]. Although a number of HDPs, including LL-37, HNPs, and human β-defensins, have been reported to inhibit neutrophil apoptosis [55–57], in this study, the IDR peptides were not found to have any significant effect on neutrophil apoptosis (data not shown). This suggests that the IDR peptides display selective effects in the regulation of neutrophil functions.

Whereas treatment with the IDR peptides alone modestly increased the production/secretion of the inflammatory cytokines—TNF-α and IL-10—they actually reduced, by as much as 50%, the secretion of these inflammatory cytokines in response to LPS, again consistent with a modulatory role. In contrast, the IDRs had no inhibitory effects on LPS-induced chemokine production and tended to cooperate with LPS to enhance the production of chemokines. In contrast to these IDR peptides, we found previously in PBMCs and macrophages that IDR-1 not only reduced LPS-induced TNF-α production but also reduced the production of IL-8 and other cytokines while increasing IL-10 production further in the presence of LPS [7]. This indicates that different IDR peptides act differently depending on the cell type. Our finding that the IDR peptides used in this study induced production of the chemokines IL-8/CXCL8, MCP-1/CCL2, MCP-3/CCL7, and MIP-1α/CCL3 in neutrophils was consistent with previous reports, demonstrating that IDR-1 and IDR-1002 enhanced chemokine production in PBMCs and neutrophils [7, 8]. This result is consistent with the suggestion that the protective properties of the IDR peptides are, in part, a result of their abilities to induce chemokine production, which leads to the recruitment of neutrophils to the site of infection [7, 8]. No study to date has performed a detailed characterization of the interactions of IDR peptides with neutrophils. However, all of the animal model studies have demonstrated that these peptides, in the context of an infection, enhance the recruitment of neutrophils to the site of infection while suppressing proinflammatory cytokines but resolving infections [7, 8], indicating that these data are relevant to the in vivo mechanism of IDR peptide action.

The relative potency of IDR peptides was similar to or less than that of fMLP, a well-known neutrophil agonist, and the combination of each IDR peptide with fMLP seemed to have only an additive effect on cell activity, if any at all. This indicates that IDR peptides might activate neutrophils through similar or completely independent mechanism(s) as fMLP. HDPs have been shown to have multiple surface and intracellular receptors, and we suspect that this is also true of these IDR peptides. Whereas the receptors for IDR peptides are not well-defined, there is some evidence for the involvement of FPR-1 and other GPCRs [8], sequestosome 1 [58], and GAPDH [59]. As one of the candidate receptors is FPR-1 [8], because fMLP also activates neutrophils through MAPK activation [60], and as IDR-1 and IDR-1002 stimulate PBMCs via the MAPK pathway, which is downstream of several of these receptors [7, 8], we investigated the involvement of MAPKs in neutrophil activation by IDR-HH2, IDR-1002, and IDR-1018. The MAPK family mainly consists of ERK, JNK, and p38, which are activated by different stimuli and target different downstream molecules, therefore performing diverse functions, including regulation of inflammation and production of cytokines and chemokines [61]. We demonstrated that IDR peptides induced the activation of ERK, JNK, and p38 and that this activation was required further for neutrophil stimulation, as MAPK-specific inhibitors significantly suppressed chemokine secretion caused by IDR peptides. Similar effects were observed for fMLP-induced cell activation. Further studies would be necessary to determine which of the above receptors were used by IDRs to activate neutrophils.

Collectively, the selective modulation of neutrophil functions by the IDR peptides emphasizes the observation that these peptides balance inflammation rather than merely suppressing it. This selective enhancement of the innate immune response represents a novel approach to anti-infective therapy by IDR peptides and has many advantages over directly microbicidal compounds. As neutrophils are known to participate in the innate immune response, our finding that IDR peptides regulate various neutrophil functions provides novel insight into the mechanism by which the IDR peptides may contribute to the modulation of the host defense, particularly at inflammation/infection sites. To the best of our knowledge, this control of neutrophil-mediated immunity by the IDR peptides was previously unknown.

AUTHORSHIP

F.N., L.M., and N.A. performed the experiments. K.O. and H.O. contributed to the study's conception. F.N. and R.E.W.H. designed and supervised the work and wrote the manuscript.

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DISCLOSURES

The University of British Columbia has filed the IDR peptides described here for patent protection, and they have been licensed out as adjuvant components and as therapeutics.

REFERENCES

1. Hancock, R. E., Chapple, D. S. (1999) Peptide antibiotics. *Antimicrob. Agents Chemother.* **43,** 1317–1323.
2. Niyonsaba, F., Nagaoka, I., Ogawa, H. (2006) Human defensins and cathelicidins in the skin: beyond direct antimicrobial properties. *Crit. Rev. Immunol.* **26,** 545–576.
3. Niyonsaba, F., Nagaoka, I., Ogawa, H., Okumura, K. (2009) Multifunctional antimicrobial proteins and peptides: natural activators of immune systems. *Curr. Pharm. Des.* **15,** 2393–2413.
4. Oppenheim, J. J., Yang, D. (2005) Alarmins: chemotactic activators of immune responses. *Curr. Opin. Immunol.* **17,** 359–365.
5. Kruse, T., Kristensen, H. H. (2008) Using antimicrobial host defense peptides as anti-infective and immunomodulatory agents. *Expert Rev. Anti Infect. Ther.* **6,** 887–895.
6. Diamond, G., Beckloff, N., Weinberg, A., Kisich, K. O. (2009) The roles of antimicrobial peptides in innate host defense. *Curr. Pharm. Des.* **15,** 2377–2392.
7. Scott, M. G., Dullaghan, E., Mookherjee, N., Glavas, N., Waldbrook, M., Thompson, A., Wang, A., Lee, K., Doria, S., Hamill, P., Yu, J. J., Li, Y., Donini, O., Guarna, M. M., Finlay, B. B., North, J. R., Hancock, R. E. W. (2007) An anti-infective peptide that selectively modulates the innate immune response. *Nat. Biotechnol.* **25,** 465–472.
8. Nijnik, A., Madera, L., Ma, S., Waldbrook, M., Elliott, M. R., Easton, D. M., Mayer, M. L., Mullaly, S. C., Kindrachuk, J., Jenssen, H., Hancock, R. E. W. (2010) Synthetic cationic peptide IDR-1002 provides protection against bacterial infections through chemokine induction and enhanced leukocyte recruitment. *J. Immunol.* **184,** 2539–2550.
9. Yeung, A. T., Gellatly, S. L., Hancock, R. E. W. (2011) Multifunctional cationic host defence peptides and their clinical applications. *Cell. Mol. Life Sci.* **68,** 2161–2176.
10. Hancock, R. E. W., Nijnik, A., Philpott, D. J. (2012) Modulating immunity as a therapy for bacterial infections. *Nat. Rev. Microbiol.* **10,** 243–254.
11. Niyonsaba, F., Someya, A., Hirata, M., Ogawa, H., Nagaoka, I. (2001) Evaluation of the effects of peptide antibiotics human β-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells. *Eur. J. Immunol.* **31,** 1066–1075.
12. Chen, X., Niyonsaba, F., Ushio, H., Hara, M., Yokoi, H., Matsumoto, K., Saito, H., Nagaoka, I., Ikeda, S., Okumura, K., Ogawa, H. (2007) Antimicrobial peptides human β-defensin (hBD)-3 and hBD-4 activate mast cells and increase skin vascular permeability. *Eur. J. Immunol.* **37,** 434–444.
13. Lau, Y. E., Bowdish, D. M., Cosseau, C., Hancock, R. E. W., Davidson, D. J. (2006) Apoptosis of airway epithelial cells: human serum sensitive induction by the cathelicidin LL-37. *Am. J. Respir. Cell Mol. Biol.* **34,** 399–409.
14. Easton, D. M., Nijnik, A., Mayer, M. L., Hancock, R. E. W. (2009) Potential of immunomodulatory host defense peptides as novel anti-infectives. *Trends Biotechnol.* **27,** 582–590.
15. Kindrachuk, J., Jenssen, H., Elliott, M., Townsend, R., Nijnik, A., Lee, S. F., Gerdts, V., Babiuk, L. A., Halperin, S. A., Hancock, R. E. W. (2009) A novel vaccine adjuvant comprised of a synthetic innate defence regulator peptide and CpG oligonucleotide links innate and adaptive immunity. *Vaccine* **27,** 4662–4671.
16. Gracia, A., Polewicz, M., Halperin, S. A., Hancock, R. E. W., Potter, A. A., Babiuk, L. A., Gerdts, V. (2011) Antibody responses in adult and neonatal BALB/c mice to immunization with novel *Bordetella pertussis* vaccine formulations. *Vaccine* **29,** 1595–1604.
17. Brown, T. H., David, J., Acosta-Ramirez, E., Moore, J. M., Lee, S., Zhong, G., Hancock, R. E. W., Xing, Z., Halperin, S. A., Wang, J. (2012) Comparison of immune responses and protective efficacy of intranasal prime-boost immunization regimens using adenovirus-based and CpG/HH2 adjuvanted-subunit vaccines against genital *Chlamydia muridarum* infection. *Vaccine* **30,** 350–360.
18. Wieczorek, M., Jenssen, H., Kindrachuk, J., Scott, W. R., Elliott, M., Hilpert, K., Cheng, J. T., Hancock, R. E. W., Straus, S. K. (2010) Structural studies of a peptide with immune modulating and direct antimicrobial activity. *Chem. Biol.* **17,** 970–980.
19. Achtman, A. H., Pilat, S., Law, C. W., Lynn, D. J., Janot, L., Mayer, M. L., Ma, S., Kindrachuk, J., Finlay, B. B., Brinkman, F. S., Smyth, G. K., Hancock, R. E. W., Schofield, L. (2012) Effective adjunctive therapy by an innate defense regulatory peptide in a preclinical model of severe malaria. *Sci. Transl. Med.* **4,** 135ra64.
20. Lee, H. Y., Bae, Y. S. (2008) The anti-infective peptide, innate defense-regulator peptide, stimulates neutrophil chemotaxis via a formyl peptide receptor. *Biochem. Biophys. Res. Commun.* **369,** 573–578.
21. Madera, L., Hancock, R. E. W. (2012) Synthetic immunomodulatory peptide IDR-1002 enhances monocyte migration and adhesion on fibronectin. *J. Innate Immun.* **4,** 553–568.
22. Theilgaard-Monch, K., Porse, B. T., Borregaard, N. (2006) Systems biology of neutrophil differentiation and immune response. *Curr. Opin. Immunol.* **18,** 54–60.
23. Rollins, B. J. (1997) Chemokines. *Blood* **90,** 909–928.
24. Niyonsaba, F., Ogawa, H., Nagaoka, I. (2004) Human β-defensin-2 functions as a chemotactic agent for tumour necrosis factor-α-treated human neutrophils. *Immunology* **111,** 273–281.
25. Zheng, Y., Niyonsaba, F., Ushio, H., Nagaoka, I., Ikeda, S., Okumura, K., Ogawa, H. (2007) Cathelicidin LL-37 induces the generation of reactive oxygen species and release of human α-defensins from neutrophils. *Br. J. Dermatol.* **157,** 1124–1131.
26. Zheng, Y., Niyonsaba, F., Ushio, H., Ikeda, S., Nagaoka, I., Okumura, K., Ogawa, H. (2008) Microbicidal protein psoriasin is a multifunctional modulator of neutrophil activation. *Immunology* **124,** 357–367.
27. Mookherjee, N., Brown, K. L., Bowdish, D. M., Doria, S., Falsafi, R., Hokamp, K., Roche, F. M., Mu, R., Doho, G. H., Pistolic, J., Powers, J. P., Bryan, J., Brinkman, F. S., Hancock, R. E. W. (2006) Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. *J. Immunol.* **176,** 2455–2464.
28. Edgell, C. J., McDonald, C. C., Graham, J. B. (1983) Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc. Natl. Acad. Sci. USA* **80,** 3734–3737.
29. Simon, S. I., Green, C. E. (2005) Molecular mechanics and dynamics of leukocyte recruitment during inflammation. *Annu. Rev. Biomed. Eng.* **7,** 151–185.
30. Malcolm, K. C., Arndt, P. G., Manos, E. J., Jones, D. A., Worthen, G. S. (2003) Microarray analysis of lipopolysaccharide-treated human neutrophils. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **284,** L663–L670.
31. Yomogida, S., Nagaoka, I., Yamashita, T. (1997) Comparative studies on the extracellular release and biological activity of guinea pig neutrophil cationic antibacterial polypeptide of 11 kDa (CAP11) and defensins. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **116,** 99–107.
32. Ganz, T. (2003) Defensins: antimicrobial peptides of innate immunity. *Nat. Rev. Immunol.* **3,** 710–720.
33. Zanetti, M., Gennaro, R., Romeo, D. (1995) Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Lett.* **374,** 1–5.
34. Ley, K., Gaehtgens, P., Fennie, C., Singer, M. S., Lasky, L. A., Rosen, S. D. (1991) Lectin-like cell adhesion molecule 1 mediates leukocyte rolling in mesenteric venules in vivo. *Blood* **77,** 2553–2555.
35. Berton, G., Lowell, C. A. (1999) Integrin signalling in neutrophils and macrophages. *Cell. Signal.* **11,** 621–635.
36. Arnaout, M. A. (1990) Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood* **75,** 1037–1050.
37. Nakayama, H., Yoshizaki, F., Prinetti, A., Sonnino, S., Mauri, L., Takamori, K., Ogawa, H., Iwabuchi, K. (2008) Lyn-coupled LacCer-enriched lipid rafts are required for CD11b/CD18-mediated neutrophil phagocytosis of nonopsonized microorganisms. *J. Leukoc. Biol.* **83,** 728–741.
38. Schymeinsky, J., Mocsai, A., Walzog, B. (2007) Neutrophil activation via β2 integrins (CD11/CD18): molecular mechanisms and clinical implications. *Thromb. Haemost.* **98,** 262–273.
39. Skubitz, K. M., Campbell, K. D., Skubitz, A. P. (1996) CD66a, CD66b, CD66c, and CD66d each independently stimulate neutrophils. *J. Leukoc. Biol.* **60,** 106–117.
40. Ureten, K., Ertenli, I., Ozturk, M. A., Kiraz, S., Onat, A. M., Tuncer, M., Okur, H., Akdogan, A., Apras, S., Calguneri, M. (2005) Neutrophil CD64 expression in Behcet's disease. *J. Rheumatol.* **32,** 849–852.
41. Segal, A. W. (2005) How neutrophils kill microbes. *Annu. Rev. Immunol.* **23,** 197–223.
42. Faurschou, M., Borregaard, N. (2003) Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect.* **5,** 1317–1327.
43. Rice, W. G., Ganz, T., Kinkade, J. M. Jr., Selsted, M. E., Lehrer, R. I., Parmley, R. T. (1987) Defensin-rich dense granules of human neutrophils. *Blood* **70,** 757–765.
44. Sengeløv, H., Kjeldsen, L., Borregaard, N. (1993) Control of exocytosis in early neutrophil activation. *J. Immunol.* **150,** 1535–1543.
45. Selsted, M. E., Harwig, S. S., Ganz, T., Schilling, J. W., Lehrer, R. I. (1985) Primary structures of three human neutrophil defensins. *J. Clin. Invest.* **76,** 1436–1439.

46. Wilde, C. G., Griffith, J. E., Marra, M. N., Snable, J. L., Scott, R. W. (1989) Purification and characterization of human neutrophil peptide 4, a novel member of the defensin family. *J. Biol. Chem.* **264,** 11200–11203.
47. Nagaoka, I., Hirota, S., Yomogida, S., Ohwada, A., Hirata, M. (2000) Synergistic actions of antibacterial neutrophil defensins and cathelicidins. *Inflamm. Res.* **49,** 73–79.
48. Panyutich, A. V., Panyutich, E. A., Krapivin, V. A., Baturevich, E. A., Ganz, T. (1993) Plasma defensin concentrations are elevated in patients with septicemia or bacterial meningitis. *J. Lab. Clin. Med.* **122,** 202–207.
49. Parameswaran, G. I., Sethi, S., Murphy, T. F. (2011) Effects of bacterial infection on airway antimicrobial peptides and proteins in COPD. *Chest* **140,** 611–617.
50. Eyles, J. L., Roberts, A. W., Metcalf, D., Wicks, I. P. (2006) Granulocyte colony-stimulating factor and neutrophils—forgotten mediators of inflammatory disease. *Nat. Clin. Pract. Rheumatol.* **2,** 500–510.
51. Nathan, C. (2006) Neutrophils and immunity: challenges and opportunities. *Nat. Rev. Immunol.* **6,** 173–182.
52. Fang, F. C. (2004) Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat. Rev. Microbiol.* **2,** 820–832.
53. Kobayashi, S. D., Braughton, K. R., Whitney, A. R., Voyich, J. M., Schwan, T. G., Musser, J. M., DeLeo, F. R. (2003) Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils. *Proc. Natl. Acad. Sci. USA* **100,** 10948–10953.
54. Kobayashi, S. D., Voyich, J. M., Somerville, G. A., Braughton, K. R., Malech, H. L., Musser, J. M., DeLeo, F. R. (2003) An apoptosis-differentiation program in human polymorphonuclear leukocytes facilitates resolution of inflammation. *J. Leukoc. Biol.* **73,** 315–322.
55. Nagaoka, I., Tamura, H., Hirata, M. (2006) An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2X7. *J. Immunol.* **176,** 3044–3052.
56. Nagaoka, I., Suzuki, K., Murakami, T., Niyonsaba, F., Tamura, H., Hirata, M. (2010) Evaluation of the effect of α-defensin human neutrophil peptides on neutrophil apoptosis. *Int. J. Mol. Med.* **26,** 925–934.
57. Nagaoka, I., Niyonsaba, F., Tsutsumi-Ishii, Y., Tamura, H., Hirata, M. (2008) Evaluation of the effect of human β-defensins on neutrophil apoptosis. *Int. Immunol.* **20,** 543–553.
58. Yu, H. B., Kielczewska, A., Rozek, A., Takenaka, S., Li, Y., Thorson, L., Hancock, R. E. W., Guarna, M. M., North, J. R., Foster, L. J., Donini, O., Finlay, B. B. (2009) Sequestosome-1/p62 is the key intracellular target of innate defense regulator peptide. *J. Biol. Chem.* **284,** 36007–36011.
59. Mookherjee, N., Lippert, D. N., Hamill, P., Falsafi, R., Nijnik, A., Kindrachuk, J., Pistolic, J., Gardy, J., Miri, P., Naseer, M., Foster, L. J., Hancock, R. E. W. (2009) Intracellular receptor for human host defense peptide LL-37 in monocytes. *J. Immunol.* **183,** 2688–2696.
60. Kuroki, M., O'Flaherty, J. T. (1997) Differential effects of a mitogen-activated protein kinase kinase inhibitor on human neutrophil responses to chemotactic factors. *Biochem. Biophys. Res. Commun.* **232,** 474–477.
61. Ballif, B. A., Blenis, J. (2001) Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. *Cell Growth Differ.* **12,** 397–408.

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