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Synthetic Cationic Peptide IDR-1002 Provides Protection against Bacterial Infections through Chemokine Induction and Enhanced Leukocyte Recruitment

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With the rapid rise in the incidence of multidrug resistant infections, there is substantial interest in host defense peptides as templates for production of new antimicrobial therapeutics. Natural peptides are multifunctional mediators of the innate immune response, with some direct antimicrobial activity and diverse immunomodulatory properties. We have previously developed an innate defense regulator (IDR) 1, with protective activity against bacterial infection mediated entirely through its effects on the immunity of the host, as a novel approach to anti-infective therapy. In this study, an immunomodulatory peptide IDR-1002 was selected from a library of bactenecin derivatives based on its substantially more potent ability to induce chemokines in human PBMCs. The enhanced chemokine induction activity of the peptide in vitro correlated with stronger protective activity in vivo in the *Staphylococcus aureus*-invasive infection model, with a >5-fold reduction in the protective dose in direct comparison with IDR-1. IDR-1002 also afforded protection against the Gram-negative bacterial pathogen *Escherichia coli*. Chemokine induction by IDR-1002 was found to be mediated through a Gi-coupled receptor and the PI3K, NF-κB, and MAPK signaling pathways. The protective activity of the peptide was associated with in vivo augmentation of chemokine production and recruitment of neutrophils and monocytes to the site of infection. These results highlight the importance of the chemokine induction activity of host defense peptides and demonstrate that the optimization of the ex vivo chemokine-induction properties of peptides is a promising method for the rational development of immunomodulatory IDR peptides with enhanced anti-infective activity. *The Journal of Immunology*, 2010, 184: 000–000.

The rise in antibiotic resistance and the dramatic decline in the development of new classes of conventional antibiotics call for new strategies in the treatment of bacterial infections (1). Host defense peptides and their synthetic innate defense regulator (IDR) peptide derivatives are being investigated for their potential value in the therapy and prophylaxis of infections. Natural host defense peptides are cationic amphipathic molecules with diverse immunomodulatory and microbicidal properties (2). Their great diversity in sequence and structure is illustrated by the two main families of mammalian peptides: defensins are characterized by a β-sheet fold with three disulphide bonds (3), whereas cathelicidins are produced by proteolysis from cathelin domain-containing precursor proteins and vary in length and structure, with many linear, α-helical, and β-hairpin examples known (4). The microbicidal activity of peptides is due to their preferential interactions with prokaryotic membranes, leading to bacterial membrane disruption and/or targeting of intracellular bacterial molecules (5, 6). The immunomodulatory activities of such peptides are extremely diverse and include stimulation of chemotaxis and chemokine production (7, 8), promotion of leukocyte antimicrobial functions (9), regulation of neutrophil and epithelial cell apoptosis (10, 11), regulation of dendritic cell differentiation and activation, stimulation of epithelial cell migration and wound healing, promotion of angiogenesis, and many others (reviewed in Refs. 12, 13). This large diversity in the structure and activities of natural host defense peptides provides a wide range of templates for the production of synthetic peptides with activities tailored for clinical applications.

A number of peptides and peptidomimetic compounds are currently undergoing clinical trials, although at this stage most trials are aimed at topical infections and are based on the direct microbicidal properties of peptides (reviewed in Refs. 14, 15). We recently reported the discovery of an IDR-1, which lacked direct antimicrobial activity but still provided broad-spectrum protection against systemic infections with multidrug-resistant bacteria (16). Indeed, an IDR peptide recently completed phase I clinical safety trials, with prospective use for prevention of infections associated with cancer chemotherapy-induced immune suppression (www.inimexpharma.com). IDR-1 offers protection by enhancing innate immune defenses of the host while suppressing potentially harmful excessive inflammatory responses. Such selective enhancement of innate

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Abbreviations used in this paper: FPRL-1, formyl peptide receptor-like 1; IDR, innate defense regulator; MPO, myeloperoxidase; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species.

immunity represents a novel approach to anti-infective therapy with many advantages over directly microbicidal compounds. For example, the microbicidal properties of peptides are strongly inhibited by physiological concentrations of divalent cations, serum, and anionic macromolecules, such as glucosaminoglycans, and in many extracellular environments, peptides exert their immunomodulatory effects at concentrations much lower than those required for direct microbial killing (17–19). As the mode of immunomodulatory action is likely to be independent of interactions with cell membranes, the issues of hemolytic activity and cytotoxicity toward mammalian cells are minimized, as supported by the minimal toxicity of peptide IDR-1 compared with natural cathelicidins like LL-37 (16, 19). Furthermore, the many positive feedback systems that operate in the regulation of immune responses allow an immunomodulatory treatment to have wider effects on the outcome of infection than those of a microbicidal drug (20, 21), and, indeed, IDR-1 has shown broad-spectrum protective activity in many models of infection (16) (R. E. W. Hancock, unpublished observations). Importantly, many host defense peptides combine both anti-infective and anti-inflammatory activities, and thus, IDR-1 therapy is not associated with risks of inflammatory tissue damage, as is often the case with other immunostimulatory treatments. Finally, because the primary target of an immunomodulatory treatment is the host and the effects on the pathogen are exerted indirectly via augmentation of the immune response, the selective pressure for pathogen resistance to the drug is minimized (20).

Despite the high interest in utilizing the immunomodulatory activities of peptides for clinical applications, the research into optimizing and enhancing peptide immunomodulatory properties has been limited to date. Chemotactic activity and chemokine induction are important aspects of the immunomodulatory activities shared by many natural peptides. For example, human cathelicidin peptide LL-37 attracts neutrophils, monocytes, mast cells, and T lymphocytes (7, 8), and also induces the production of neutrophil and monocyte chemoattractants by many cell types (22–25). The reduced production of host defense peptides correlates with impaired influx of leukocytes into the gut in *Shigella flexneri* infections (26), and neutrophil-derived LL-37 is known to be essential for recruitment of inflammatory monocytes in several in vivo models (27). Similarly, IDR-1 also increases macrophage numbers at the site of infection in vivo (16). All these reports highlight the likely importance of the chemokine-induction activity of host defense peptides and indicate that optimization of chemokine induction may be a promising route for the development of peptides with enhanced anti-infective properties.

In the current work, a novel peptide IDR-1002 (VQRWLIVWRIRK-NH_2),selected from a library of bactenecin derivatives for its significantly higher potency compared with IDR-1 in inducing chemokines in vitro, was characterized. Apart from their cationic nature, peptides IDR-1002 and IDR-1 shared no sequence similarity. The enhanced in vitro chemokine-inducing properties of IDR-1002 correlated with stronger in vivo protective activity against *Staphylococcus aureus* infection, with a >5-fold reduction in the therapeutic dose compared with IDR-1. This indicates the value of the screening for chemokine induction as an aid to successful development of IDR peptides with enhanced immunomodulatory properties and improved anti-infective activities.

Materials and Methods

Reagents

Peptides IDR-1002 (VQRWLIVWRIRK-NH_2), IDR-1 (KSRIVPAIPV-SLL-NH_2), and LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNL-VPRTES) were synthesized using F-moc chemistry at the Nucleic Acid/Protein Synthesis Unit (University of British Columbia, Vancouver, British Columbia, Canada) or at GenScript (Piscataway, NJ). Inhibitors used were: pertussis toxin (G_i-protein inhibitor), WRW4 (formyl peptide receptor-like 1 antagonist), Bay11-7082 (NF-κB inhibitor), LY294002 (PI3K inhibitor), PD98059 (MEK-1 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), AG490 (JAK inhibitor), 6-22 amide (protein kinase A inhibitor), and KN-93 (Ca^{2+}/calmodulin-dependent protein kinase II inhibitor) (all from Calbiochem, San Diego, CA), and nocodazole (microtubule assembly inhibitor) and cytochalasin D (actin polymerization inhibitor) (both from Sigma-Aldrich, St. Louis, MO). The inhibitors were resuspended in DMSO (Sigma-Aldrich) and stored at −20°C. The final concentrations of DMSO in cell culture never exceeded 0.02% (v/v), and all experiments included DMSO vehicle controls. The inhibitors were also checked against possible cytotoxic effects, using lactate dehydrogenase release colorimetric cytotoxicity detection kit (Roche, Basel, Switzerland) and WST-1 cell proliferation reagent (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; Roche).

Cell isolation and culture

PBMCs were isolated as previously described (28). Venous blood was collected from healthy volunteers into heparin-containing Vacutainer tubes (BD Biosciences, San Jose, CA) in accordance with the ethical approval guidelines of the University of British Columbia Research Ethics Board. The blood was diluted with an equal volume of PBS (pH 7.4) (Invitrogen, Carlsbad, CA) and separated by density gradient centrifugation over Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ). Mononuclear cell layers were collected, washed twice in PBS, and the cells seeded at 1×10^6 cells/ml in RPMI 1640 with 10% heat-inactivated FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate (all from Invitrogen), in a humidified incubator at 37°C and 5% CO_2.

Neutrophils were isolated as previously described (10). Blood was prediluted in 2% (w/v) Dextran T-500 (Amersham Biosciences) in 0.9% saline and sedimented for 30 min at room temperature. The leukocyte-rich upper layer was centrifuged at $250 \times g$ for 7 min, and erythrocytes were hypotonically lysed with water for 30 s, followed by restoration of tonicity with 2.5% (w/v) saline and neutrophil separation by density gradient centrifugation over Ficoll-Paque Plus (Amersham Biosciences) at 4°C. The cells were washed in Krebs-Ringer phosphate buffer (pH 7.3), containing glucose (10 mM) and Mg^{2+} (1.5 mM) at 4°C, and maintained in the same media and conditions as PBMCs.

Mouse bone marrow-derived macrophages were prepared by culturing bone marrow cells of C57BL/6J mice for 7 d in high-glucose DMEM with 20% FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate (all from Invitrogen), and supplemented at 30% with L-conditioned media (supernatant of cell line L-929). Mouse peritoneal lavage was collected in 3 ml sterile saline and cells maintained in RPMI 1640 with 10% FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate, as described above.

The human monocytic cell line THP-1 (29), from the American Type Culture Collection (ATCC TIB-202; Manassas, VA), was maintained in the same media and conditions as PBMCs for a maximum of six passages. THP-1 cells at a density of 1×10^6 cells/ml were treated with 0.3 μg/ml PMA (Sigma-Aldrich) for 24 h to generate adherent macrophage-like cells (30) and rested for further 24 h before stimulation.

Animal experiments and in vivo bioluminescence monitoring of infection

All mouse experiments were conducted in accordance with the Animal Care Ethics Approval and Guidelines of the University of British Columbia. C57BL/6J or CD-1 female mice (from The Jackson Laboratory, Bar Harbor, ME, or Harlan Breeders, Indianapolis, IN, respectively) were maintained under specific pathogen-free conditions. The mice were <12 wk of age and age-matched in all experiments. Peptides IDR-1002 or IDR-1 were administered i.p. in sterile saline and the mice infected i.p. with *S. aureus* (ATCC 25293, 10^8–10^9 CFU/mouse) or *Escherichia coli* Xen-14 (Caliper Life Sciences, Hopkinton, MA; 5×10^7 CFU/mouse). Peritoneal lavage was collected from all the mice in 3 ml sterile saline and the remaining bacterial numbers measured by serial dilution and plating on Mueller-Hinton or Luria-Bertani agar plates. Alternatively, the mice were infected with *S. aureus* Xen-29 engineered for bioluminescence with insertion of a modified complete *lux* operon (Caliper Life Sciences) at $1–2 \times 10^8$ CFU/mouse. The mice were anesthetized via inhalation of aerosolized isoflurane mixed with oxygen and imaged using a Xenogen Imaging System 100 (Xenogen, Hopkinton, MA).

Macrophage and monocyte depletion with liposomal clodronate was performed as previously described (16). Briefly, 10 mg/ml clodronate (Sigma-Aldrich) in PBS was mixed with a phosphatidylcholine:cholesterol (3:1 molar ratio) solution in chloroform and incubated for 2 h at 37°C. Vesicles were formed by vacuum desiccation overnight, suspended in 5 ml saline, and purified by size-exclusion chromatography on Sephadex G-50

beads (Sigma-Aldrich). Mice were injected i.p. with 200 μl purified liposome solution and rested for 4 d before the infection, when the macrophages and monocytes remained depleted whereas normal neutrophil numbers recovered.

Flow cytometry

All flow cytometry data were collected on FACSCalibur and analyzed using CellQuest Pro software (BD Biosciences). For the analysis of cell surface markers, the cells were resuspended in HBSS (pH 7.4), supplemented with 2% FCS (Invitrogen), 0.2% (w/v) sodium azide, and 20 mM HEPES. The staining was at 4°C for 20 min, and the Abs used were: anti-human CD66b FITC (clone G10F5, BioLegend, San Diego, CA), and anti-mouse F4/80-PE (clone C1:A3-1, Cederlane Laboratories, Burlington, Ontario, Canada), Ly-6G-PE (1A8, BD Biosciences), GR1-Alexa Fluor 647 (RB6-8C5), CD40-Alexa Fluor 647 (HM40-3), CD80-Alexa Fluor 647 (16-10A1), CD86-Alexa Fluor 647 (GL1), and I-A/I-E–Alexa Fluor 488 (M5/114.15.2) (all from BioLegend). In some experiments, the cells were pretreated with anti-mouse CD16/CD32 Fc-Blocker (BD Biosciences). After washing, the cells were resuspended in 0.5% (w/v) paraformaldehyde in PBS for analysis.

For the analysis of protein phosphorylation, the stimulated cells were fixed in 2% (w/v) paraformaldehyde in PBS at room temperature for 15 min, permeabilized in 90% (v/v) methanol at 4°C for 20 min, and washed in 0.5% BSA (Roche Applied Science, Indianapolis, IN) in PBS (w/v). The cells were stained for 1 h at room temperature for phospho-p38 (Thr180/Tyr182) using 3D7 rabbit mAb and for phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204) using E10 rabbit mAb (both from Cell Signaling Technology, Beverly, MA). Following washing, the cells were further stained with goat anti-rabbit IgG-Alexa Fluor 647 (Invitrogen) at 2 μg/ml or with goat anti-mouse IgG-Alexa Fluor 488 (Sigma-Aldrich) at 0.8 μg/ml at room temperature for 30 min and counterstained with anti-human CD14-Alexa Fluor 488 or 647 (clone M5E2, BioLegend).

For Ca-flux analysis, the cells were resuspended in HBSS, with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1% FCS (Invitrogen) at 1×10^7/ml, loaded with Fluo-4 calcium-sensitive fluorescent dye (Invitrogen) at 2.5 μM for 20 min at 37°C, washed twice, and analyzed by flow cytometry. For the measurement of reactive oxygen species (ROS) production, neutrophils, resuspended at 10^7 cells/ml in HBSS, were loaded with 1 μM carboxy-H_2DCFDA (Molecular Probes, Eugene, OR) at 37°C for 20 min and washed in fresh HBSS (pH 7.4). Following cell culture and stimulation, the cells were analyzed for fluorescence in the FL-1 channel as the measure of ROS production.

Neutrophils were analyzed for apoptosis and necrosis using the Annexin V-PE Apoptosis Kit (BioVision, Mountain View, CA) and the 7-AAD Cell Viability Solution (BD Biosciences), according to the manufacturers' instructions.

ELISA

Tissue culture or peritoneal lavage samples were centrifuged at $1000 \times g$ for 10 min to obtain cell-free samples and stored at −20°C. Cytokine levels were measured by ELISA using anti-human CCL2 Ab clones 5D3-F7 and 2H5 (eBioscience, San Diego, CA), anti-human CXCL8 clones 893A6G8 and 790A28G2 (Biosource, Carlsbad, CA), anti-mouse TNF-α clones 1F3F3D4 and XT3/XT22, anti-mouse IL-6 clones MP5-20F3 and MP5-32C11, anti-mouse IL-10 clones JES5-16E3 and JES5-2A5, anti-mouse CCL2 clones 4E2 and 2H5, anti-mouse GM-CSF MP1-31G6 and MP1-22E9 (all from eBioscience), and an anti-human CXCL1 Ab pair (clone 20326 and goat polyclonal, from R&D Systems, Minneapolis, MN), all followed by avidin HRP (eBioscience) as per manufacturers' protocols. Human CCL7 and mouse CXCL1 and CXCL5 Duoset ELISA kits (R&D Systems) were also used. The ELISAs were developed using TMB Liquid Substrate System (Sigma-Aldrich) and imaged with a PowerWave ×340 plate-reader (Bio-Tek Instruments, Winooski, VT). Cytokine quantification was done against serial dilutions of recombinant cytokines (from R&D Systems and eBioscience). Mouse Inflammation Cytometric Bead Array kit (BD Biosciences) was also used for cytokine quantification according to the manufacturer's protocol.

Other colorimetric assays

The colorimetric NO Assay kit (Calbiochem) was used according to manufacturer's instructions to measure NO levels in the peritoneal lavage of mice treated with saline or IDR-1002, with or without *S. aureus* infection. Myeloperoxidase (MPO) was quantified using the enzymatic method described by Ormrod et al. (31), with modifications to optimize the assay for cells collected by peritoneal lavage (32). Briefly, cell pellets from 1 ml peritoneal lavage were suspended in 200 μl HTAB buffer [0.5% (w/v) hexadecyltrimethylammonium (Sigma-Aldrich) in 0.1 M potassium phosphate buffer (pH 6.0)] and frozen at −80°C overnight. The following day, thawed cell suspensions were lysed by sonication and then incubated at 60°C for 2 h to solubilize the MPO. Supernatants were assayed in the presence of 0.0005% (v/v) hydrogen peroxide (Sigma-Aldrich) and 0.5 mM *o*-dianisidine dihydrochloride, with color change read spectrophotometrically at 460 nm every 1 min. One unit of MPO was defined as the amount of enzyme that used 1 μmol/min peroxide at 25°C.

Quantitative real-time PCR

RNA was isolated using the RNEasy Mini Kit (Qiagen, Valencia, CA), treated with RNase-Free DNase (Qiagen), and cDNA prepared with the SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen). cDNA levels were assessed by quantitative real-time PCR (qRT-PCR) with the SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen) and the Applied Biosystems PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA). The data were analyzed using the comparative Ct method (33) and normalized against GAPDH or β-actin expression. The primers used are listed in Supplemental Table I.

Phagocytosis assay

The protocol was adopted from (34). *S. aureus* bacteria were preloaded with CFSE fluorescent dye (Molecular Probes) at 10 μM in PBS for 30 min at 37°C, killed by incubation with 1% paraformaldehyde in PBS for 1 h at 37°C, and washed five times in fresh PBS. THP-1 human monocytic cells were cultured with the CFSE-loaded bacterial particles at multiplicity of infection 100 for 4 h. At different time points, the cells were thoroughly washed, extracellular fluorescence quenched with 15 μg/ml trypan blue in PBS, and the cells analyzed by flow cytometry for CFSE fluorescence as a measure of the phagocytic uptake of the bacterial particles.

Statistical analyses

Prism 4.0 Software (GraphPad, San Diego, CA) was used for statistical data analyses, with a two-tailed Student *t* test used for comparisons of two datasets and ANOVA for multiple comparisons, with post hoc comparisons using Tukey's or Bonferroni tests.

Results

Peptide IDR-1002 potently induced chemokines in human PBMCs

Peptide IDR-1002 (VQRWLIVWRIRK-NH_2) was selected from an iterative library of artificial cationic peptides (H. Jenssen, J. Kindrachuk, M. Elliot, M. Waldbrook, K. Hilpert, E. Yuen, K. Wee, and R. E. W. Hancock, submitted for publication) as one of the strongest chemokine inducers in an ex vivo screen with human PBMCs. The peptide showed low cytotoxicity to mammalian cells, being nontoxic to human neutrophils and PBMCs up to concentrations of 100 and 200 μg/ml, respectively, in contrast to natural human cathelicidin LL-37 that showed some cytotoxicity at levels above 25 and 50 μg/ml under the same conditions (data not shown).

Peptide IDR-1002 was a potent inducer of chemokine production in human PBMCs ex vivo, with stronger activity than the human natural cathelicidin LL-37 and the linear derivative of bactenecin Bac2a, which is the parental peptide of the library (Fig. 1*A*). Furthermore, IDR-1002 also had significantly stronger activity than IDR-1 and within the 20–100 μg/ml concentration range induced over 10-fold more CCL2 and CXCL8 than IDR-1 (Fig. 1*B*). The chemokines induced by IDR-1002 included both neutrophil chemoattractants CXCL1 and CXCL8 and monocyte chemoattractants CCL2 and CCL7 (Fig. 1) (35, 36).

The chemokine induction activity of the peptide was also confirmed in human monocytic cell line THP-1 at the transcriptional level, with IDR-1002 inducing the expression of chemokines CCL2 and CCL4 and an anti-inflammatory cytokine IL-10 (data not shown). We did not detect any induction of proinflammatory cytokines, such as TNF-α, at either the protein or mRNA level (data not shown). This confirmed the strong chemokine-inducing properties of peptide IDR-1002 and suggested that monocytic cells may be one of the targets of the peptide activity.

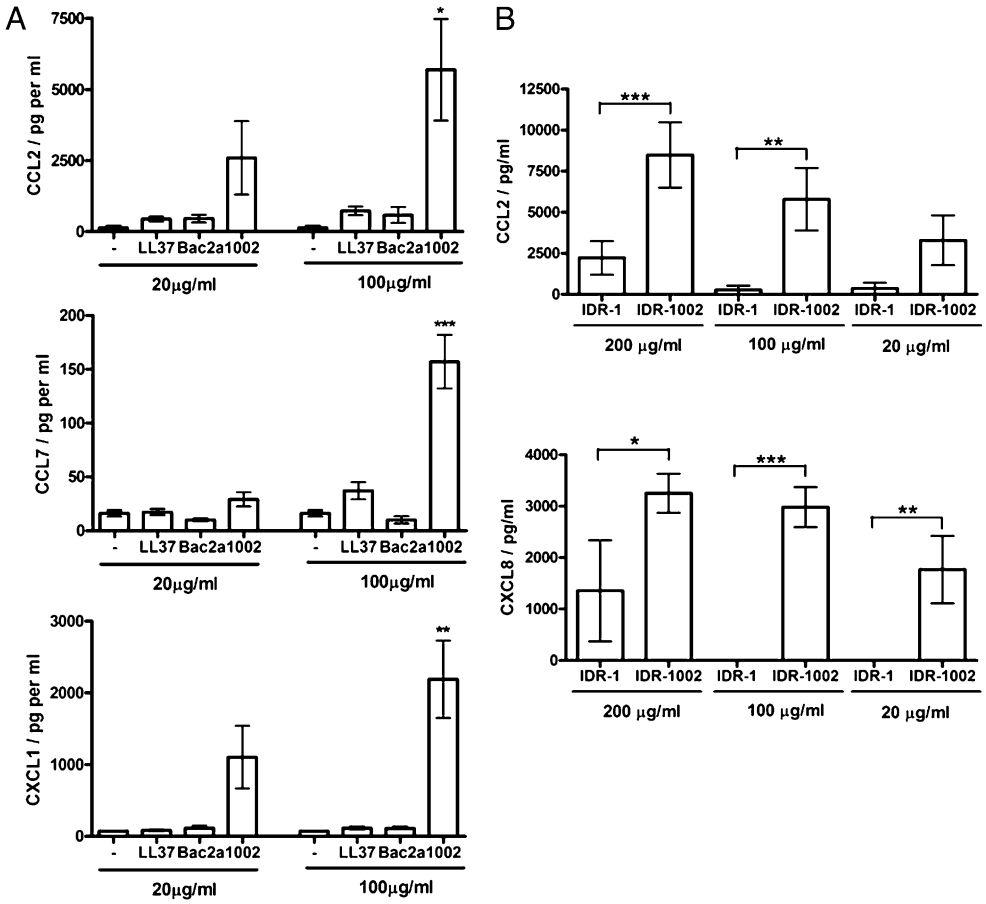

FIGURE 1. Chemokine-inducing activities of synthetic peptide IDR-1002. IDR-1002 induced stronger production of monocyte and neutrophil chemoattractants in human PBMCs compared with peptides LL-37, Bac2a (*A*), and IDR-1 (*B*). Chemokine levels measured by ELISA after 24 h of stimulation. Bars represent means and SEs from three to six independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; comparison by ANOVA.

Peptide IDR-1002 induced a wide range of chemokines in mouse cells

To assess the biological activity of peptide IDR-1002 in vivo, it was necessary to move from a human to a mouse system. Thus, we tested the chemokine-inducing properties of IDR-1002 in mouse cells. Mouse bone marrow-derived macrophages were stimulated with IDR-1002 at 100 μg/ml for 4 h and analyzed for chemokine and cytokine induction using qRT-PCR. The induction of a range of chemokines and cytokines was detected, including CCL4, CCL7, CCL20, and CXCL1 (Fig. 2*A*). Similar results were observed with mouse peritoneal lavage cells stimulated ex vivo with IDR-1002 (100 μg/ml) for 24 h, with secretion of CCL2, CXCL1, and IL-10 detected by ELISA (Fig. 2*B*). The activity of IDR-1002 on bone marrow-derived macrophages further confirmed that monocytic cells were one of the targets of the peptide activity and suggested that the peritoneal macrophages were the responding cell type within the peritoneal lavage.

Protective activity of IDR-1002 against Gram-positive and Gram-negative bacterial pathogens

We previously showed that synthetic immunomodulatory peptide IDR-1 can offer protection in murine models of bacterial infection (16). Thus, the anti-infective properties of IDR-1002 were similarly tested using an invasive *S. aureus* infection model. *S. aureus* was

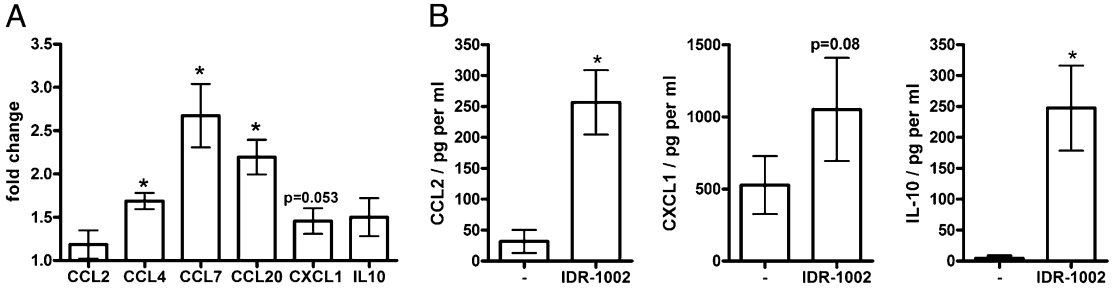

FIGURE 2. Peptide IDR-1002 induced chemokine production in mouse cells. *A*, Mouse bone marrow-derived macrophages were stimulated with peptide IDR-1002 (100 μg/ml) for 4 h and analyzed for chemokine production by qRT-PCR. The data were analyzed using the comparative Ct method (33) and normalized against GAPDH; significant changes relative to unstimulated control as determined by one-sample two-tailed *t* test in GraphPad Prism are indicated. $*p < 0.05$. *B*, Peritoneal lavage cells were stimulated with peptide IDR-1002 (100 μg/ml) for 24 h and analyzed for chemokine production by ELISA. Bars represent means and SEs from three to five C57BL/6 mice. $*p < 0.05$ using a paired *t* test.

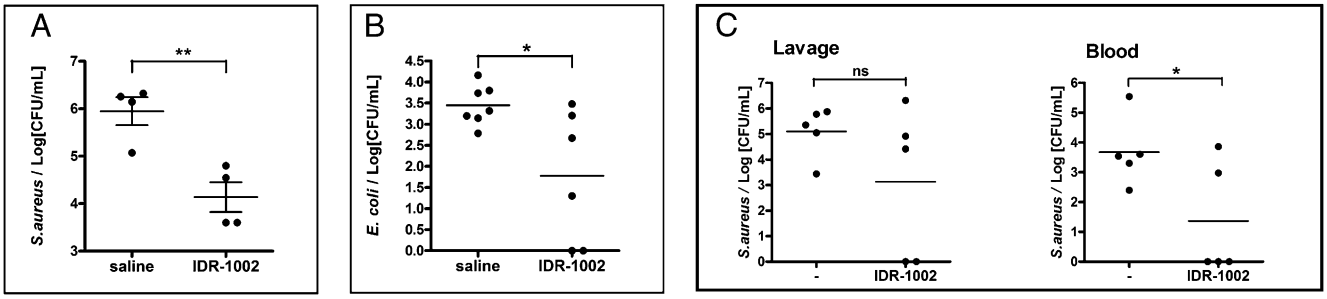

FIGURE 3. Protective activity of IDR-1002 against Gram-positive and Gram-negative bacterial pathogens. Prophylactic protective activity of IDR-1002 in murine models of invasive *S. aureus* (*A*) and *E.coli* (*B*) infections: the mice were pretreated i.p. with a single dose of IDR-1002 (200 μg/mouse) for 4 h and infected i.p. with 2×10^8 CFU/mouse *S. aureus* or 5×10^7 CFU/mouse *E.coli*. *C*, Therapeutic administration of IDR-1002 protects against systemic spread of infection: mice infected i.p. with 5×10^8 CFU/mouse *S. aureus* and treated with two doses of IDR-1002 (200 μg/mouse) at 1 and 22 h postinfection. All the mice were analyzed for bacterial counts in the peritoneal lavage (*A–C*) and blood (*C*) at 24 h of infection. Bars represent means and SEs. Comparisons by *t* test. $*p < 0.05$; $**p < 0.01$.

chosen as the test pathogen because historically it shows rapid evolution of antibiotic resistance with a wide spread of methicillin-resistant *Staphylococcus aureus*, increasing resistance to vancomycin, and recent emergence of community-associated methicillin-resistant *Staphylococcus aureus* infections (37). Pretreatment with peptide was routinely used in this model because this best reflects the immunomodulatory capabilities given the rapid progression of infections. Mice were treated i.p. with 200 μg IDR-1002 in sterile saline and 4 h later infected with 2×10^8 CFU *S. aureus*. Peritoneal lavage was collected 24 h after the onset of infection and analyzed for bacterial counts. A significant decrease in the bacterial counts in the peptide-treated animals was observed (Fig. 3*A*), showing the strong protective activity of peptide IDR-1002.

To test the activity of peptide IDR-1002 in a Gram-negative infection model, mice were pretreated as previously with 200 μg IDR-1002 or sterile saline and 4 h later infected with *E. coli* at 5×10^7 CFU/mouse. The lavage was analyzed for bacterial counts at 24 h of infection, and a significant reduction in bacterial counts was observed in the peptide-pretreated animals (Fig. 3*B*), indicating that the peptide offered protection against both Gram-positive and Gram-negative bacterial pathogens.

The activity of IDR-1002 was also tested by therapeutic administration, with the peptide given at +1 and +22 h following infection with 5×10^8 CFU *S. aureus*. A significant decrease in the bacterial load in the blood of the peptide-treated animals was observed (Fig. 3*C*), indicating that the peptide provided partial protection against the systemic spread of *S. aureus* infection. Thus, the peptide may be used in both therapeutic and prophylactic applications, with prophylactic uses in patients with very high risk of infection (e.g., in intensive care units) and in cancer chemotherapy-induced immune suppression.

Enhanced in vivo protective activity of IDR-1002 in direct comparison with IDR-1

The protective activity of IDR-1002 was directly compared with the previously developed innate defense regulator peptide IDR-1, which has been previously shown to be protective in vivo at doses of 24 mg/kg

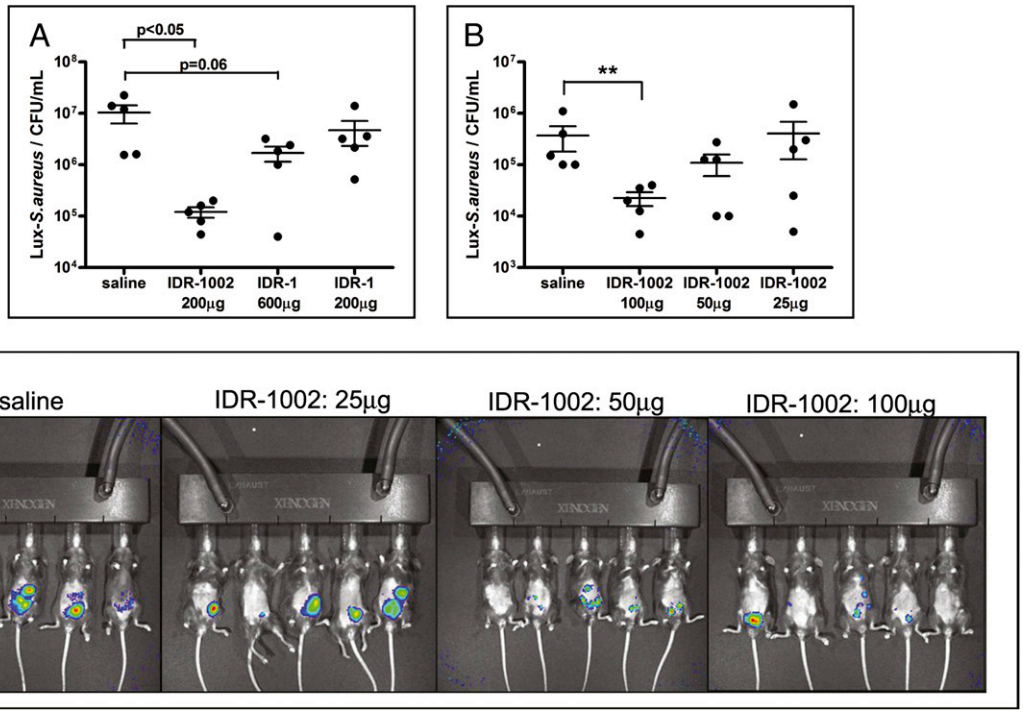

FIGURE 4. Enhanced protective activity of peptide IDR-1002 in a mouse model of *S. aureus* infection. C57BL/6 female mice pretreated i.p. with the indicated doses of the peptides for 4 h and infected i.p. with 2×10^8 CFU *S. aureus*-Xen29 were imaged with the Xenogen 100 system and analyzed for bacterial counts in the lavage at 24 h of infection. *A*, Direct comparison of the protective activities of IDR-1002 and IDR-1. *B* and *C*, Dose titration of peptide IDR-1002. Bars represent means and SEs. $*p < 0.05$; $**p < 0.01$.

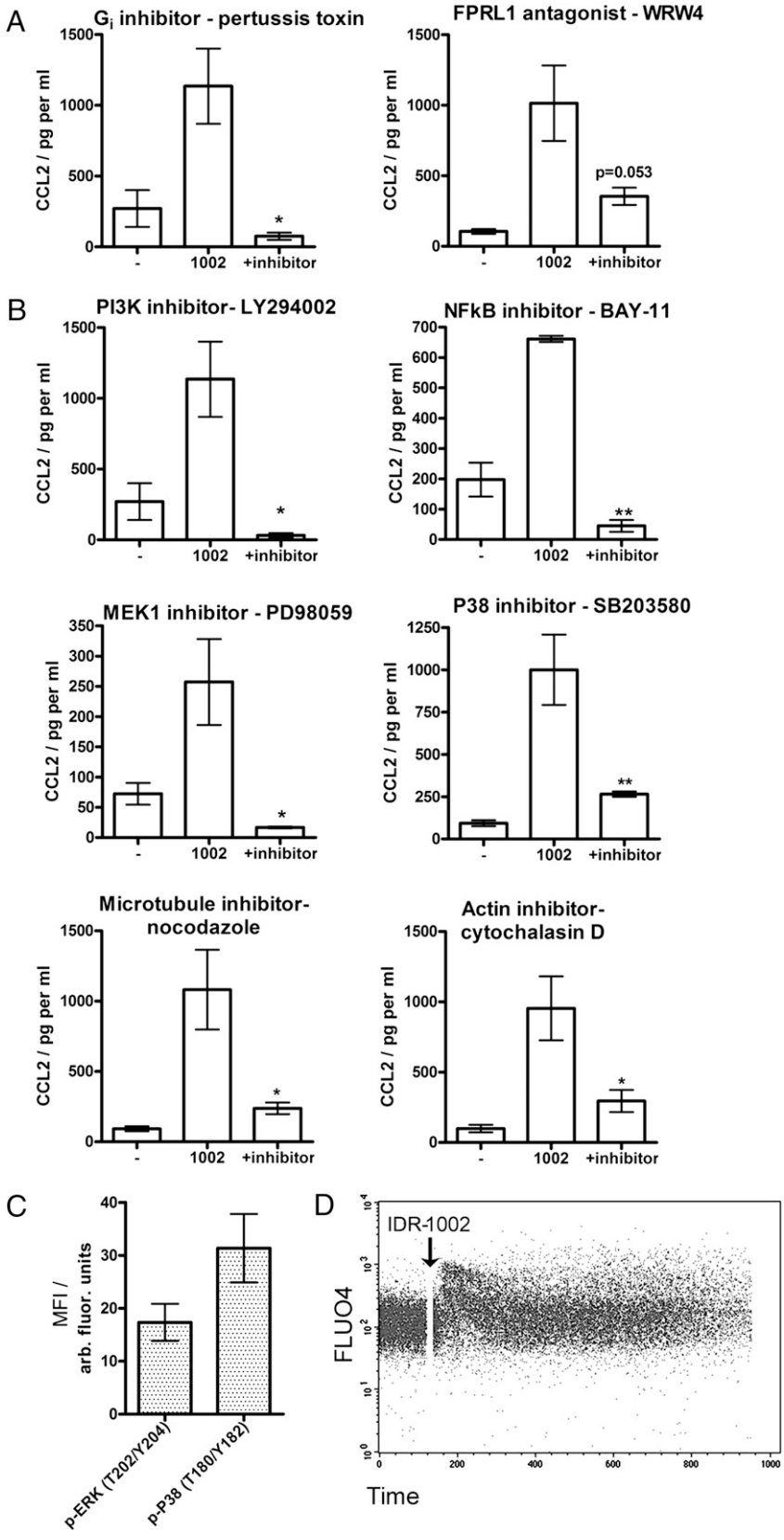

FIGURE 5. Signaling pathways mediating IDR-1002 chemokine induction in human PBMCs. *A* and *B*, The effects of specific chemical inhibitors on IDR-1002–induced chemokine responses: cells were pretreated for 1 h with inhibitors pertussis toxin (G_i-protein inhibitor, 100 ng/ml), WRW4 (FPRL-1 antagonist, 10 μM), LY294002 (PI3K inhibitor, 10 μM), Bay11-7082 (NF-κB inhibitor, 1 μM), PD98059 (MEK-1 inhibitor, 10 μM), SB203580 (p38 inhibitor, 10 μM), nocodazole (microtubule assembly inhibitor, 500 ng/ml), and cytochalasin D (actin polymerization inhibitor, 500 ng/ml) before stimulation with IDR-1002 at 100 μg/ml for 18 h; CCL2 assessed by ELISA. All bars represent means and SEs from three to five independent biological replicates. $*p < 0.05$; $**p < 0.01$ using *t* test. *C*, IDR-1002 induced phosphorylation of ERK and p38 MAPKs in primary monocytes: cells stimulated with IDR-1002 at 100 μg/ml for 30 min were stained with the phospho-specific Abs and analyzed by flow cytometry gating on $CD14^+$ monocytes. Mean fluorescence intensity of the stimulated cells following background subtraction of the mean fluorescence intensity of unstimulated controls is shown. Results were significantly different ($p < 0.05$) as determined using the one-sample two-tailed *t* test in GraphPad Prism. *D*, IDR-1002 induced Ca^{2+} flux in primary monocytes: FL-1 fluorescence of Fluo4-loaded $CD14^+$ monocytes, with IDR-1002 added at 100 μg/ml 1 min after the beginning of data acquisition.

(~600 μg/mouse) (16). We hypothesized that the enhanced in vitro chemokine-induction properties of IDR-1002 would correlate with a stronger protective action in vivo. C57BL/6 female mice were treated i.p. with IDR-1002 (200 μg) or IDR-1 (200 μg and 600 μg) and infected with 2×10^8 CFU *S. aureus*. A significant reduction in bacterial load was again seen in the IDR-1002 (200 μg/mouse) treated animals, whereas in this study IDR-1 had no significant activity at the lower 200 μg/mouse dose and had only moderate activity at the 600 μg/mouse dose (Fig. 4*A*), demonstrating the stronger protective properties of the new peptide IDR-1002.

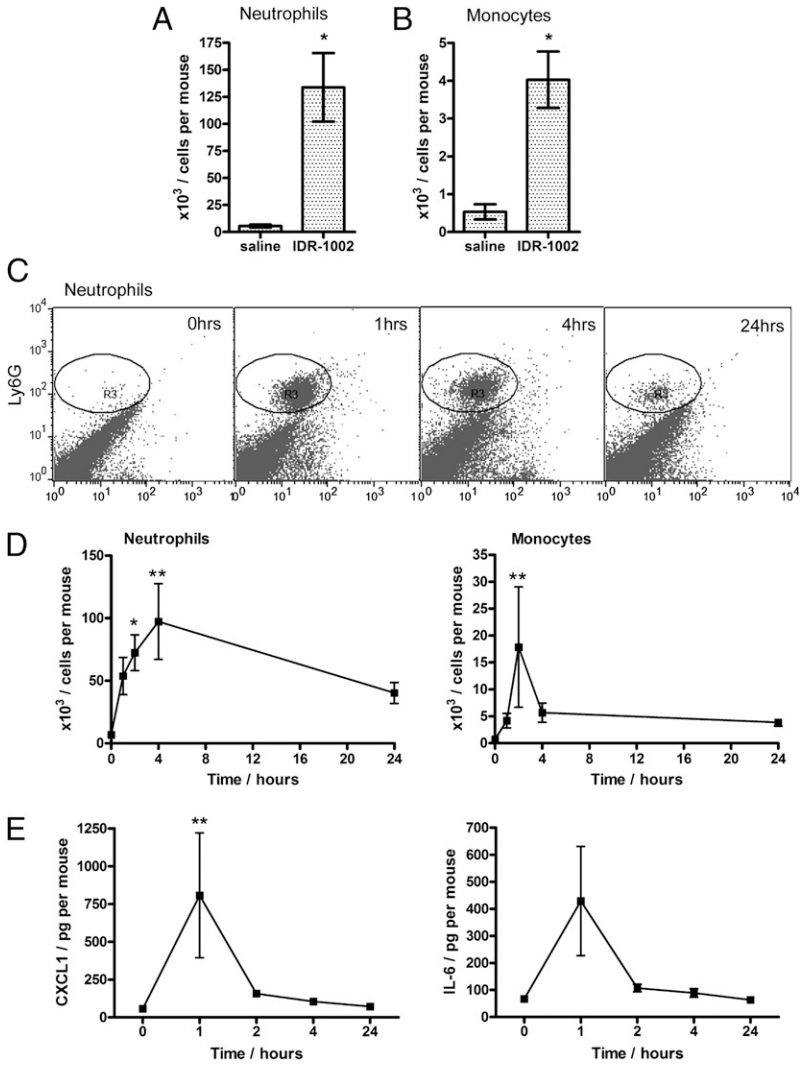

FIGURE 6. Peptide IDR-1002 induced neutrophil and monocyte recruitment in vivo. C57BL/6 mice were injected i.p. with 200 μg peptide IDR-1002 in sterile saline, sacrificed, and lavaged at 0–24 h postinjection. The numbers of neutrophils ($Ly6G^+$) and monocytes ($Gr1^+F4/80^+$) were calculated using the total peritoneal cell counts and the percentages for each cell population estimated by flow cytometry. *A* and *B*, Peptide IDR-1002 induced significant recruitment of neutrophils and monocytes at 1 h postinjection. *C* and *D*, Time course of neutrophil and monocyte recruitment into the peritoneum by peptide IDR-1002. *E*, Induction of a neutrophil chemoattractant CXCL1 and cytokine IL-6 in the peritoneal lavage of the mice by peptide IDR-1002, calculated as the concentration of the cytokine multiplied by the total volume of lavage recovered. All bars represent means and SEs from four mice. $*p < 0.05$; $**p < 0.01$ using *t* test (*A*, *B*) or ANOVA (*D*, *E*).

The protective activity of IDR-1002 was further tested at lower doses, using *S. aureus*-Xen29 carrying a constitutively expressed modified *lux*-operon permitting in vivo bioluminescence imaging of infection with the Xenogen 100 system. Strong and significant protection was observed in the mice treated with 100 μg/mouse IDR-1002, and a trend toward protection was still observed at the 50 μg/mouse dose (Fig. 4*B*, 4*C*). This further confirmed the stronger protective properties of the novel peptide IDR-1002 as compared with the previously developed candidate peptide IDR-1, with IDR-1002 being protective at doses at least 5–10-fold lower than IDR-1.

Receptors and signaling pathways mediating the chemokine-induction activity of IDR-1002

The involvement of various signaling pathways in the induction of chemokines by peptide IDR-1002 in human mononuclear cells was analyzed. Human PBMCs were pretreated with specific chemical inhibitors for 1 h, stimulated with peptide IDR-1002 at 100 μg/ml for further 18 h, and the peptide activity was assessed by measuring CCL2 release by ELISA. All the inhibitors were tested for cytotoxicity using both WST-1 and lactate dehydrogenase release assays and showed no significant toxicity at the concentrations used (data not shown). All the experiments also included DMSO vehicle controls at levels identical to those used as solvents for inhibitor treatments. The levels of DMSO in the cell cultures never exceeded 0.02%, and no significant DMSO toxicity or effects on chemokine production were detected (data not shown).

An inhibitor of G_i-proteins, pertussis toxin, abolished chemokine induction by peptide IDR-1002, suggesting the involvement of a G_i-coupled receptor in peptide signaling (Fig. 5*A*). Previously, G_i-coupled receptors have been shown to mediate some of the effects of human cathelicidin LL-37 on leukocytes, with formyl peptide receptor-like 1 (FPRL-1) being one of the receptors involved (7, 8, 10, 11). Therefore, we further analyzed the effects of an FPRL-1 antagonist WRW4 on the activity of peptide IDR-1002. Although there was a strong trend toward inhibition of IDR-1002 activity by WRW4, it did not reach statistical significance (Fig. 5*A*). Based on this data, the involvement of FPRL-1 cannot be ruled out, although other G_i-coupled receptors are also likely to be involved.

The use of further chemical inhibitors addressed the downstream signaling pathways involved in the chemokine-induction activity of peptide IDR-1002. As shown in Fig. 5*B*, the MAPKs MEK-1 (upstream of ERK1/2) and p38, PI3K, and the NF-κB signaling pathway were all essential for IDR-1002 activity, mirroring previous findings for peptides IDR-1 and LL-37 (16, 38–40). In contrast, inhibition of the JAK-STAT pathway with inhibitor AG490, JNK with SP600125, protein kinase A with amide 6-22, or Ca^{2+}/calmodulin-dependent protein kinase II with KN-93 did not have significant effects on IDR-1002 activity (Supplemental Fig. 1). Of these inhibitors, AG490 and amide 6-22 were previously shown to have no inhibitory effects on LL-37 activity (40), and AG490 and SP600125 had no inhibitory effects on IDR-1 (16). These findings further suggest a strong overlap among the signaling pathways used

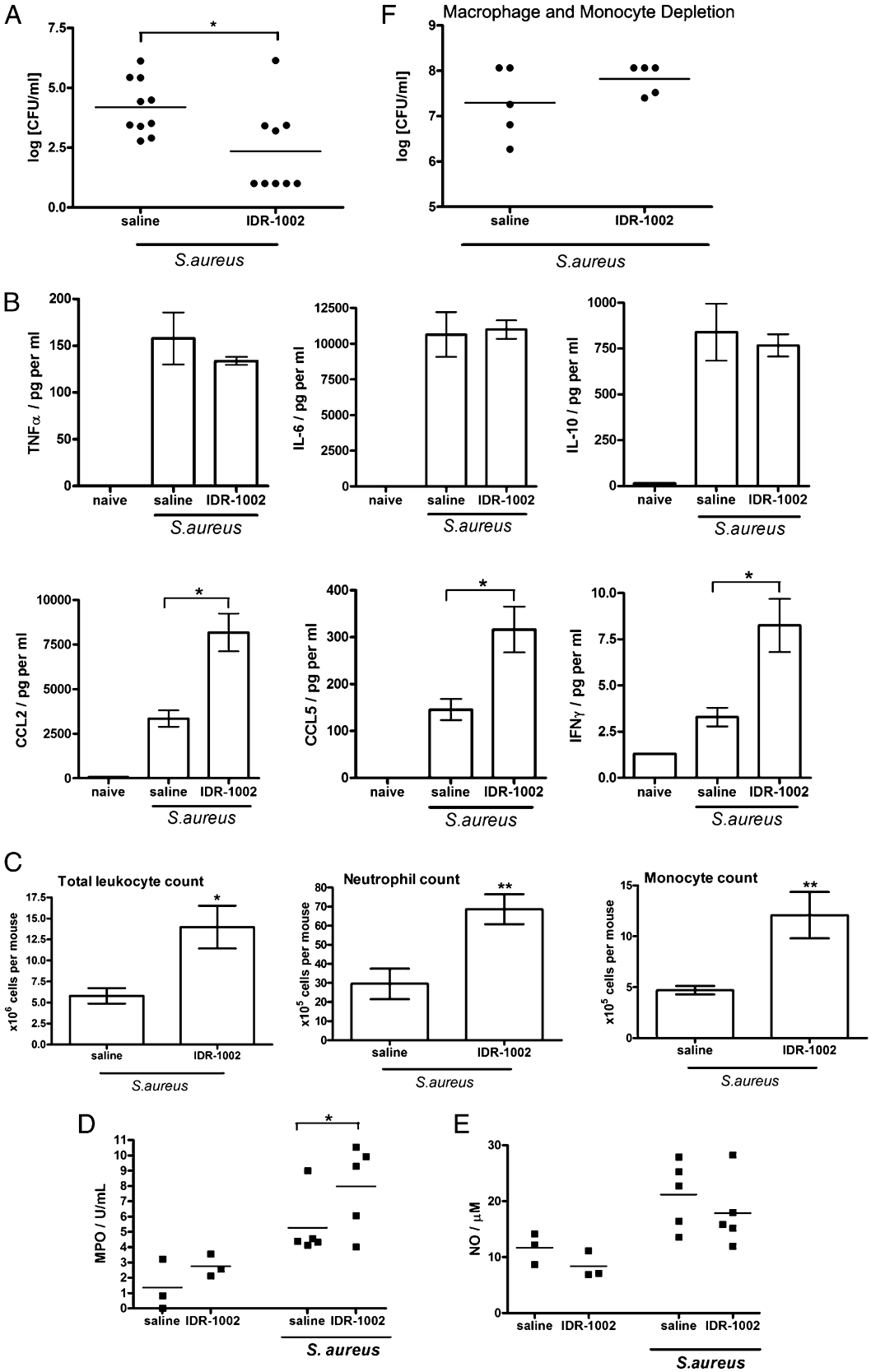

FIGURE 7. The protective activity of peptide IDR-1002 in a murine model of *S. aureus* infection was associated with increased chemokine induction and leukocyte recruitment and was macrophage- and/or monocyte-dependent. Mice were treated i.p. with 200 μg peptide IDR-1002 or sterile saline for 4 h, infected i.p. with $\sim 1 \times 10^9$ CFU *S. aureus*, and lavaged after a further 4 or 24 h. *A*, IDR-1002 protection in i.p. *S. aureus* infection: CFU of *S. aureus* per ml lavage in the IDR-1002–treated and control groups at 24 h of infection. *B*, Peptide IDR-1002 affected cytokine and chemokine production at 4 h at the site of infection assessed by CBA assay or ELISA. *C*, Peptide IDR-1002 increased leukocyte recruitment to the site of infection: total cell numbers and neutrophil ($Gr1^+F4/80^-$) and monocyte ($Gr1^+F4/80^+$) numbers in the lavage of IDR-1002–treated and control animals at 4 h of infection. *D*, Enhanced MPO activity in the cells present in the peritoneal lavage of IDR-1002 pretreated versus control mice at 4 h of *S. aureus* infection expressed per ml lavage. *E*, NO levels in the

by IDR-1002, IDR-1 (16), and natural immunomodulatory peptides like human cathelicidin LL-37 (38, 39) despite the lack of any obvious sequence similarities. Interestingly, the microtubule assembly inhibitor nocodazole and actin polymerization inhibitor cytochalasin D also abolished chemokine induction by peptide IDR-1002 (Fig. 5*B*), suggesting that cytoskeletal reorganization and endocytic uptake may be required for peptide activity. This raised the possibility that, like LL-37 and IDR-1 (41–43), peptide IDR-1002 may be actively internalized by cells and may act on intracellular, as well as cell-surface, targets.

Activation of MAPK signaling pathways by peptide IDR-1002 was further confirmed by measuring ERK1/2 and p38 phosphorylation in response to peptide stimulation in primary human monocytes using flow cytometry (Fig. 5*C*). PBMCs stimulated for 30 min with peptide IDR-1002 at 100 μg/ml were stained for phospho-ERK1/2 (T202/Y204) and phospho-p38 (T180/Y182) following fixation and permeabilization according to standard protocols. Monocytes were gated as $CD14^+$ cells, and a significant increase in phospho-ERK and phospho-p38 levels was observed in the IDR-1002–stimulated samples (Fig. 5*C*; $p < 0.05$, $n = 4$). This confirmed the results of the inhibitor studies, showing the role of MAPKs ERK1/2 and p38 in IDR-1002 signaling.

IDR-1002 also induced a transient increase in intracellular Ca^{2+} levels in monocytes, as measured by the increased fluorescence of a Ca^{2+}-sensitive dye Fluo-4 following IDR-1002 stimulation (Fig. 5*D*). Natural cathelicidin LL-37 was also previously shown to induce Ca^{2+} flux in monocytes (7), suggesting that Ca^{2+} is one of the second messengers mediating signaling responses of synthetic as well as natural host defense peptides.

IDR-1002 induced neutrophil and monocyte recruitment and chemokine production in vivo

Previous findings and those described in this study have indicated that chemokines are induced by IDR peptides but have not demonstrated that the chemokines are functional in vivo. In this paper, we examined whether the chemokine induction activity of peptide IDR-1002, as observed ex vivo, could result in a significant increase in leukocyte recruitment in vivo following peptide administration. Cohorts of C57BL/6 mice were injected i.p. with 200 μg peptide IDR-1002 or the vehicle sterile saline, and the peritoneal lavage was collected at 1 h postinjection and analyzed by flow cytometry. There was a substantial and significant increase in the numbers of monocytes (×8) and neutrophils (×30) in the peritoneal lavage of the peptide-treated animals (Fig. 6*A*, 6*B*), with neutrophils and inflammatory monocytes gated as $Ly6G^+$ (or $Gr1^+F4/80^-$) and $F4/80^+Gr1^+$ cells, respectively.

The time course of IDR-1002–induced leukocyte recruitment in the animals was further analyzed. Mice were similarly treated with 200 μg peptide IDR-1002 and lavaged at different time points over the following 24 h. Leukocyte recruitment was observed as early as 1 h after peptide administration and peaked at 2–4 h (Fig. 6*C*, 6*D*). A small increase in leukocyte numbers continued to be seen as late as 24 h after the peptide administration (Fig. 6*C*, 6*D*).

Leukocyte recruitment was associated with increased levels of the neutrophil chemoattractant CXCL1 and the cytokine IL-6 in the peritoneal lavage (Fig. 6*E*). No significant increase in the monocyte chemoattractant CCL2 was detected (data not shown), suggesting that other monocyte chemoattractants may mediate the monocyte recruitment. There was also no induction of TNF-α or activation of resident macrophages, as measured by CD80, CD86, and MHC class II expression levels (data not shown), suggesting that the effects of peptide IDR-1002, by itself, were due to selective chemokine/cytokine induction rather than a classical inflammatory response.

Protective activity of IDR-1002 was associated with enhanced leukocyte recruitment

To analyze the mechanisms of IDR-1002 protective activity in murine infection models, peptide-treated and control animals were compared for cytokine levels and leukocyte recruitment during the course of *S. aureus* infection. IDR-1002–treated *S. aureus*-infected mice had elevated levels of the monocyte chemoattractant CCL2 compared with the control group and unexpectedly also increased levels of T cell/eosinophil/basophil chemoattractant CCL5 (RANTES) and the macrophage-activating factor IFN-γ (Fig. 7*A*, 7*B*). Importantly, no increases in the levels of the proinflammatory cytokines TNF-α and IL-6 were detected between peptide-treated and control groups (Fig. 7*B*), showing that the protective activity of IDR-1002 is not associated with overall enhanced inflammatory response.

There was a significant increase in the total cell counts in the peritoneal lavage of the peptide-treated animals at 4 h of infection, with increased recruitment of inflammatory monocytes ($F4/80^+Gr1^+$) and neutrophils ($Gr1^+F4/80^-$) to the site of infection (Fig. 7*C*). The enhanced recruitment of neutrophils in the peptide-treated mice was further confirmed by the increased MPO levels in the cell pellets from the peritoneal lavage at 4 h of infection, with the mean MPO levels of 8.0 U/ml in the IDR-1002–treated group as compared with 5.3 U/ml in the control group ($p < 0.05$; Fig. 7*D*). There were no changes in the percentage of neutrophils in the blood or the bone marrow of the IDR-1002–treated mice at 4 h of infection, and there was no effect of the peptide on the GM-CSF levels in the serum of the infected animals (Supplemental Fig. 2), suggesting that the effects of IDR-1002 were largely mediated through augmentation of leukocyte recruitment to the site of infection rather than systemic changes in leukocyte numbers. Further studies also showed that peptide IDR-1002 similarly augmented recruitment of leukocytes (in particular inflammatory monocytes) in mice treated with heat-inactivated *S. aureus* bacteria (Supplemental Fig. 3), indicating that this peptide activity is independent of any interactions of the peptide with live pathogens.

Effects of IDR-1002 on microbicidal activities of leukocytes

In order to determine whether, in addition to its effects on the numbers of leukocytes at the site of infection, peptide IDR-1002 could also modulate microbicidal activities of the recruited cells, we investigated the effects of IDR-1002 on the activation and antimicrobial activities of neutrophils and macrophages. IDR-1002 did not affect the activation of peritoneal macrophages at the site of infection, as measured by the expression of CD80 and CD86 activation markers on $F4/80^+$ peritoneal cells at 4 and 24 h of infection (data not shown). Furthermore, there was no increase in the levels of NO in the peritoneal lavage of the IDR-1002–treated compared with the control mice at 4 h of infection (Fig. 7*E*), suggesting no increase in

peritoneal lavage of IDR-1002 pretreated and control mice at 4 h of *S. aureus* infection. *F*, Peptide IDR-1002 did not protect macrophage- and monocyte-depleted mice from *S. aureus* infection: mice were depleted of macrophages/monocytes by an i.p. injection of liposomal clodronate and 4 d later treated with 200 μg of IDR-1002, infected with $\sim 1 \times 10^9$ CFU *S. aureus* 4 h later, and euthanized at 24 h postinfection. Animals that died of the infection were assigned the highest observed colony counts in the experiment. Bars represent mean and SEs from four to five animals. $*p < 0.05$; $**p < 0.01$ using *t* test (*A–C*) or ANOVA with Tukey's post hoc test (*D*).

inducible NO synthase expression and the antimicrobial activities of classically activated macrophages (44) at least at this early time point. Peptide IDR-1002 also did not promote in vitro phagocytosis of fluorescently labeled bacterial particles by human monocytic cell line THP-1 (Supplemental Fig. 4), suggesting no direct stimulation of the phagocytic activity of macrophages by the peptide.

The effects of IDR-1002 on the activation and survival of neutrophils were also investigated, using cells freshly isolated from the human blood. Peptide IDR-1002 (5–125 μg/ml) did not promote ROS production by the neutrophils over 30 min of stimulation (Supplemental Fig. 5*A*) and also did not promote neutrophil activation as measured by upregulation of cell surface levels of the adhesion molecule CD66b (Supplemental Fig. 5*B*) (45). IDR-1002 also did not affect the in vitro viability of human neutrophils at concentrations up to 125 μg/ml (Supplemental Fig. 6). This suggests that the protective activity of IDR-1002 was likely mediated through enhanced leukocyte recruitment to the site of infection rather than through direct effects of the peptide on the microbicidal activities of the recruited leukocytes; however, we cannot rule out that additional activities of IDR-1002 not detected in our studies may emerge in vivo as a result of synergy with the action of bacterial compounds or other host mediators, as exemplified by the synergy in the various immunomodulatory properties of natural and synthetic cationic peptides with IL-1β, GM-CSF, and CpG oligonucleotides (38, 39, 46, 47).

Protective activity of IDR-1002 in vivo is dependent on monocytes and macrophages

Because macrophages responded to IDR-1002 by producing chemokines in vitro, and monocytes were recruited to the site of infection by the peptide in vivo, the role of macrophages and monocytes in the protective activity of IDR-1002 was further investigated in vivo. The role of these cells in the protective activity of IDR-1 was previously shown (16). Liposomal clodronate was used to deplete these cell types in vivo as previously described (16). The macrophage/monocyte-depleted mice were challenged with *S. aureus*, with or without IDR-1002 pretreatment (200 μg, i.p.), and sacrificed at 24 h of infection. The protective activity of IDR-1002 was abolished in the mice, indicating the essential role of monocytic cells in the protective activity of the peptide (Fig. 7*F*). In summary, this suggests that the protective activity of peptide IDR-1002 against bacterial challenge was mediated through augmentation of chemokine induction and leukocyte recruitment, with macrophages and/or monocytes being essential contributors to the protective activity.

Discussion

In this study, it was shown that the synthetic cationic peptide IDR-1002, selected from a bactenecin-derived peptide library for its enhanced in vitro chemokine-inducing properties, can provide protection against bacterial challenge in animal models. Importantly, both its in vitro chemokine-inducing potential and its in vivo protective activity were stronger than those of the previously developed synthetic innate defense regulator peptide IDR-1 (16), a variant of which, compound IMX942, recently entered phase I clinical safety trials, with prospective uses in the prevention of infections in chemotherapy-induced immune suppression and other patient groups (www.inimexpharma.com). The activity of IDR-1002 in murine models of *S. aureus* infection was at least 5-fold stronger than that of IDR-1, and an equivalent protection was achieved at significantly lower doses. The protective activity of IDR-1002 was associated with in vivo induction of chemokines and with enhanced leukocyte recruitment to the site of infection, demonstrating a good correlation between the in vitro peptide optimization strategy and its in vivo activities. Thus, the enhanced anti-infective properties of IDR-1002 demonstrated that the in vitro optimization of peptides for enhanced chemokine induction is an effective approach for the development of peptides with enhanced in vivo protective activities.

In addition to the stronger activities of IDR-1002 compared with IDR-1, some functional differences between IDR-1 and IDR-1002 emerged. Although IDR-1 was previously shown to induce IL-10 and increase macrophage numbers at the site of infection (16), IDR-1002 showed enhanced in vivo induction of CCL2, CXCL1, as well as CCL5 and IFN-γ, and recruitment of neutrophils and monocytes. These differences between IDRs raise the possibility that in the future, IDR peptides can be tailored to elicit specific aspects of protective immunity. Importantly, despite some differences in their in vivo activity, the enhanced anti-infective properties of IDR-1002 were achieved without upregulation of proinflammatory cytokines, demonstrating that both IDR-1002 and IDR-1 share the unique property of providing protection against infections without stimulating inflammatory response and without the risks of uncontrolled inflammation seen with other therapies aimed at boosting protective immunity. The immunomodulatory modes of action of IDR-1002 and IDR-1 also share the unique advantage over directly microbicidal therapies of minimizing the risks of pathogen resistance, because the therapies target the immune defenses of the host rather than acting directly on the pathogen.

Discovering novel aspects of the signaling pathways activated by natural and synthetic IDR peptides will further facilitate the design of peptides with enhanced activities through optimization for interactions with appropriate targets. The current study indicated that despite the lack of sequence similarities between the peptides, the chemokine induction activity of IDR-1002 is mediated through similar signaling pathways to those used by IDR-1 (16, 40), but also revealed a novel signaling response involving Ca^{2+} flux. Pertussis toxin was used to demonstrate the involvement of the G_i-coupled receptor family in the IDR-1002-activity, with possible involvement of the FPRL-1 family member. G_i-coupled receptors are known to mediate a range of immunomodulatory functions of natural cathelicidins, including chemotactic activity toward certain cell types (7, 8), effects on neutrophil survival (10, 11), keratinocyte proliferation, migration, and wound healing (48), as well as the angiogenic activity on the vascular endothelial cells (49). FRPL-1 is one of the receptors mediating these activities; however, it has not been shown to mediate chemokine induction, and other unknown G_i-coupled receptors are required in some cell types (8, 10). This study further showed the involvement of the PI3K, NF-κB, p38, and MEK-1/ERK pathways in the IDR-1002 activity, which mirrors their involvement in the immunomodulatory functions of natural cathelicidins and IDR-1 (16, 38).

LL-37 is actively taken up by human epithelial cells (41) and monocytes (43). The inhibition of the IDR-1002 activity by tubulin and actin depolymerizing drugs indicated a similar requirement for cytoskeletal rearrangements and endocytic uptake for chemokine induction by this peptide. This suggested that IDR-1002 may act on intracellular as well as cell-surface targets, as proposed for several other host defense peptides. For example, porcine cathelicidin PR-39 was reported to bind and act on the adaptor protein p130 (CAS), NADPH-oxidase, and the 26S proteasome (50–52), and we also recently identified GAPDH as an intracellular target of LL-37 (43) and sequestosome-1/p62 as the target of IDR-1 (42). Studies of cell uptake of IDR-1002, its intracellular localization, possible intracellular targets, as well as systems biology approaches to understanding its mechanisms of action using gene-expression and protein-phosphorylation arrays are ongoing and may lead to further optimization of IDR activities.

Identification of the cell types that are the direct targets of peptide activity in vivo is also an important goal for future development of

IDR therapeutics. Macrophages may be one of the cell populations directly responding to IDR-1002 in vivo, consistent with the findings that IDR-1002 had similar in vitro activities on macrophage cell lines and on bone marrow-derived macrophages. The loss of peptide protection with macrophage/monocyte depletion also supported this conclusion and probably reflected the role of macrophages as IDR-1002 sensors and source of IDR-1002 induced chemokines, as well as the contribution of the recruited monocytes in the clearance of infection. Thus, in the i.p. infection models used in this study, resident peritoneal macrophages were likely one of the cell types responsible for the IDR-1002–induced chemokine production, as peritoneal macrophages are known to be an important source of chemokines for leukocyte recruitment during peritoneal infections (53–55). However, a contribution of other cell types to the IDR-1002–stimulated chemokine production cannot be ruled out, and these cell types may include mast cells and peritoneal mesothelial cells, which are also known to be important chemokine producers in some models of peritonitis (55–58). The elevated levels of IFN-γ in the peptide-treated animals during *S. aureus* infection were interesting and unexpected (Fig. 7*B*), as similar signatures were not observed in any of the ex vivo studies of peptide activity. The sources of IFN-γ remain unknown, but may suggest direct or indirect activity of the peptide on NK or γδ T cells. Interestingly, another cytokine upregulated in the peptide-treated mice in this model was CCL5 (Fig. 7*B*), which is known to promote NK and T cell recruitment and activation (59). In the future, further characterization of the target cell populations of IDR-1002 may lead to the optimization of its route of delivery or the development of systems for specifically targeting the peptide to desired cell types to further enhance activity and reduce the protective dose.

In summary, a novel innate defense regulator peptide IDR-1002 was reported in this study. The peptide had enhanced in vitro chemokine induction activities and stronger in vivo anti-infective properties compared with the previously developed peptide IDR-1. This highlighted the in vivo importance of chemokine-induction activity of natural and artificial peptides. Furthermore, it demonstrated that optimization of peptide sequences for chemotactic activity is a promising route for the development of peptides with enhanced in vivo protective activity, allowing rational design of immunomodulatory peptides for clinical intervention.

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Disclosures

R. E. W. H. has founded companies for the exploitation of host defense peptides as antimicrobials (Migenix) and immunomodulators (Inimex). In both, he is a minor shareholder, a member of the Scientific Advisory Board, and consultant.

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