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Trappin-2 Promotes Early Clearance of *Pseudomonas aeruginosa* through CD14-Dependent Macrophage Activation and Neutrophil Recruitment

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Microaspiration of *Pseudomonas aeruginosa* contributes to the pathogenesis of nosocomial pneumonia. Trappin-2 is a host defense peptide that assists with the clearance of P. aeruginosa through undefined mechanisms. A model of macrophage interactions with replicating *P. aeruginosa* (strain PA01) in serum-free conditions was developed, and the influence of subantimicrobial concentrations of trappin-2 was subsequently studied. PA01 that was pre-incubated with trappin-2 (at concentrations that have no direct antimicrobial effects), but not control PA01, was cleared by alveolar and bone marrow-derived macrophages. However, trappin-2-enhanced clearance of PA01 was completely abrogated by CD14-null macrophages. Fluorescence microscopy demonstrated the presence of trappin-2 on the bacterial cell surface of trappin-2-treated PA01. In a murine model of early lung infection, trappin-2-treated PA01 was cleared more efficiently than control PA01 2 hours of intratracheal instillation. Furthermore, trappin-2-treated PA01 up-regulated the murine chemokine CXCL1/KC after 2 hours with a corresponding increase in neutrophil recruitment 1 hour later. These *in vivo* trappin-2-treated PA01 effects were absent in CD14-deficient mice. Trappin-2 appears to opsonize *P. aeruginosa* for more efficient, CD14-dependent clearance by macrophages and contributes to the induction of chemokines that promote neutrophil recruitment. Trappin-2 may therefore play an important role in innate recognition and clearance of pathogens during the very earliest stages of pulmonary infection. ***(Am J Pathol 2009, 174:1338–1346; DOI: 10.2353/ajpath.2009.080746)***

Nosocomial (hospital-acquired) infections are estimated to be responsible for up to 98,000 deaths per annum in the United States, with pneumonia the predominant cause of mortality.[1,2] The opportunistic pathogen *Pseudomonas aeruginosa* is the most commonly isolated microorganism from patients with nosocomial pneumonia in many published series.[3–6]

During hospital admission, the nasopharynx becomes colonized with potential pathogens such as *P. aeruginosa*. The likelihood of acquiring a potentially virulent colonizing organism increases with time.[7] In turn, a significant proportion of nosocomial pneumonias are thought to arise from microaspiration of bacteria from the nasopharynx.[8] Furthermore, aspiration of nasopharyngeal secretions in volumes likely to contain bacterial organisms takes place in normal patients during sleep.[9] Therefore a greater understanding of the innate immune mechanisms operating against the very earliest invasion of the lung by small inocula of aspirated pathogens would aid the design of new preventive and therapeutic strategies for nosocomial pneumonia.

Trappin-2 (also termed pre-elafin) is a 10-kDa cationic host defense peptide produced locally in the lung and in other mucosal sites exposed directly to the environment.[10,11] Trappin-2 has been shown to be directly anti-

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microbial against *P. aeruginosa in vitro*.[12–14] We previously showed that prophylactic overexpression of trappin-2 (which is absent in mice) enhances clearance of *P. aeruginosa* from murine lungs.[15] However, the concentration of trappin-2 in bronchoalveolar lavage (BAL) fluid seemed to be significantly lower than that associated with direct antimicrobial activity.[12–14] These observations have prompted interest in other immunomodulatory activities for trappin-2.

In this context, the immunomodulatory effects of trappin-2 may be divided into those involving innate immune priming and those that are anti-inflammatory. Thus trappin-2 can bind lipopolysaccharide (LPS), up-regulate LPS-induced tumor necrosis factor α (TNF-α) production from macrophages *in vitro*, and enhance LPS-induced leukocyte recruitment *in vivo*.[16,17] On the contrary, trappin-2 has been shown to attenuate nuclear factor kappa B (NF-κB) responses to oxidized low-density lipoprotein, LPS, and TNF-α in macrophages and endothelial cells.[18] Recombinant C-terminal trappin-2 (also termed elafin) has been shown to prevent LPS-induced AP-1 and NF-κB activation through effects on the ubiquitin-proteasome pathway.[19]

The early stages of acute *P. aeruginosa* lung infection are thought mainly to involve interactions between the alveolar macrophage (AM, the patrolling phagocytic cell of the alveolar space) and the microbe. These interactions are thought to be independent of serum opsonins, given the low levels of such opsonins in the lung before injury. Two distinct receptors, complement receptor 3 (CR-3) and CD14, have been shown to mediate nonopsonic phagocytosis of different *P. aeruginosa* strains.[20] Less is known about the ligands that interact with these receptors in the lung during the initial stages of infection when serum-derived ligands in the alveolar space are likely to be present at ineffective concentrations.

We hypothesized that trappin-2 may play a role in the early clearance of *P. aeruginosa* through an influence on macrophages. Using *in vitro* and *in vivo* assays we studied *P. aeruginosa*-macrophage interaction at very early stages of lung infection when bacteria are present at low numbers and the effect of serum and mucosal antibodies are considered to be minimal. Our studies demonstrate for the first time that trappin-2 at subantibacterial concentrations can increase suppression of *P. aeruginosa* growth by macrophages. Our studies in AMs (which are inherently CR-3-deficient) and macrophages genetically engineered to be CD14-deficient suggest that trappin-2-treated *P. aeruginosa* binds to membrane CD14. Infection of mice with low doses of trappin-2-treated *P. aeruginosa* [$\sim 10^5$ colony forming units (CFU)] showed that this effect is maintained *in vivo* and that trappin-2 amplifies host defense by the specific recruitment of neutrophils into the lung during early infection.

Materials and Methods

Antibodies and Stains

Rabbit anti-PA01 outer membrane protein was provided by Dr. Tyrone Pitt, Centre for Infections, Health Protection Agency, London, UK. Goat anti-human trappin-2 and rat anti-mouse CD14 (clone Sal4-2) were from HyCult Biotechnology (Uden, The Netherlands). Phycoerythrin and cyanin 5 tandem (PE-Cy5)-conjugated rat anti-mouse F4/80 and rat IgG2b isotype control were from Invitrogen (Paisley, UK). Alexa 488-conjugated rat anti-mouse complement receptor 3 (CR-3/CD11b) and rat IgG2b isotype control were from Invitrogen. Alexa 488-conjugated chicken anti-goat IgG, Alexa-488 conjugated chicken anti-rat IgG and Alexa 488-conjugated goat anti-rabbit Fab_2 were from Invitrogen. PKH26-GL red fluorescent cell linker was from Sigma-Aldrich Company Ltd. (Gillingham, UK).

Recombinant Trappin-2

Recombinant trappin-2 was purchased from R&D Systems Europe Ltd. (Abingdon, UK). The trappin-2 molecule contains 117 amino acids, with the mature protein corresponding to residues 23 to 117.[21] A related molecule that occurs in biological samples and termed elafin corresponds to residues 61 to 117, which contains the anti-elastase site.[21]

Bacteria

PA01, a fully sequenced[22] strain of *P. aeruginosa*, was provided by Professor John Govan, Centre for Infectious Diseases, University of Edinburgh. Bacteria were inoculated into Luria-Bertani (LB) broth and incubated at 37°C in an orbital incubator at 200 rpm. After 16 hours, 250 μl of the bacterial culture was subcultured into fresh LB broth for a further 1 to 2 hours at 37°C to allow bacteria to enter logarithmic growth phase. Before experiments, the culture was centrifuged twice at 1000 $\times$ *g* for 15 minutes, each time being resuspended in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen). The bacterial suspension was resuspended to an OD_{595} of 0.1 and diluted 1:1000, resulting in a bacterial cell density of $\sim 5 \times 10^4$ CFU/ml.

Mice

C57BL/6 mice from Charles River Laboratories (Tranent, UK) were maintained in specific pathogen-free conditions at the University of Edinburgh with housing and animal procedures approved by the appropriate local authority. $CD14^{-/-}$ mice produced on a C57BL/6 background,[23] were from Jackson Laboratories (Bar Harbor, ME). All animal work was performed in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Primary Bone Marrow-Derived Macrophages (BMDMs)

Bone marrow was harvested from 8- to 12-week-old female C57BL/6 mice as described previously.[24] In summary, femora were removed and cleaned under sterile conditions. Bone marrow was gently flushed through and plated in Dulbecco's modified Eagle's medium F-12 medium (Invitrogen) supplemented with 2 mmol/L L-glutamine, 10% heat-inactivated fetal bovine serum (PAA, Pasching, Austria), and 10% conditioned supernatant

from L929 cells [as a source of macrophage colony stimulating factor (M-CSF)], without antibiotics. After 6 days in culture cells had acquired a macrophage phenotype[25] and were detached by gentle scraping on ice for 5 minutes using 0.2% bovine serum albumin and 5 mmol/L ethylenediaminetetraacetic acid in Hanks' balanced salt solution without calcium or magnesium. Cells were counted and resuspended at 5×10^5 viable cells/ml in fresh Dulbecco's modified Eagle's medium-supplemented medium. Cell viability was assessed using trypan blue exclusion. Cells were adhered to a 12-well Costar cell culture plate (Fisher Scientific UK Ltd., Loughborough, UK) overnight in 5% CO_2 at 37°C.

MH-S Cells

The MH-S cell line, a simian virus 40 (SV40)-transformed murine AM cell line was obtained from European Collection of Cell Cultures (Wiltshire, UK) and cultured in RPMI 1640 medium containing 10% fetal bovine serum and 50 nmol/L 2-mercaptoethanol. MH-S cells were detached from culture flasks using trypsin-ethylenediaminetetraacetic acid for 10 minutes at 37°C followed by gentle scraping. Cell viability was assessed using trypan blue exclusion. Cells were counted and resuspended at 5×10^5 viable cells/ml in fresh RPMI medium before being left to adhere to a 12-well Costar (Fisher) cell culture plate overnight in 5% CO_2 at 37°C.

Lactate Dehydrogenase Assay

BMDMs or MH-S cells were plated as above. After incubation periods of 2 hours in 10% fetal calf serum (FCS) or serum-free media the supernatants and cell layers were assayed for the levels of the cytoplasmic enzyme lactate dehydrogenase according to the manufacturer's instructions (Roche Diagnostics Ltd., Burgess Hill, UK).

Assay to Assess Direct Antimicrobial Effects of Trappin-2

PA01 colonies were suspended as described above at an estimated bacterial density of 5×10^4 CFU/ml and incubated at 37°C in a rotary mixer at 750 rpm with either trappin-2 in IMDM (at concentrations of 20 nmol/L, 100 nmol/L, or 500 nmol/L), 10 μg/ml gentamicin (Sigma) or IMDM alone. At the start of each experiment ($t = 0$), bacterial samples of 100 μl (in IMDM alone) were plated on LB broth agar. After 150 minutes test samples and control samples were plated on LB agar in triplicate and incubated overnight at 37°C. Bacterial CFU were recorded and the mean of each triplicate calculated.

In Vitro *Assay to Determine Effects of Trappin-2 on Clearance of Live PA01 by Macrophages*

PA01 colonies were suspended as described above at an estimated bacterial density of 5×10^4 CFU/ml in the presence of trappin-2 (100 nmol/L) or vehicle alone. Immediately afterward 500 μl of this suspension was added to 5×10^5 macrophages (plated as above) giving a multiplicity of infection of 0.1. The co-culture was incubated for 2 hours before macrophages were lysed by adding 3.5 ml of 0.1% Triton (a concentration that repeatedly did not affect PA01 viability) in IMDM in four sequential washes to harvest total PA01. Serial dilutions were made and 100-μl samples of each treatment plated on LB agar in triplicate and incubated overnight at 37°C. Bacterial CFU were recorded and the mean of the triplicates used to calculate the number of viable PA01 colonies. For measurement of phagocytosis we made cytospin preparations from the assay described, performed in suspension. Cytospins were stained in (Gamidor Technical Services Ltd, Didcot, UK) Quick-Diff according to the manufacturer's protocol. The number of macrophages in which PA01 were clearly visible within phagosomes was counted at light microscopy, and expressed as a proportion of the total number of macrophages. At least 200 macrophages were counted. Finally, in a variation of this system, we used titanium dioxide particles as the target for phagocytosis at a final concentration of 50 μg/ml.

In Vivo *Studies*

The *in vivo* effect of opsonizing PA01 with trappin-2 was investigated by pretreating PA01 with phosphate-buffered saline (PBS) or trappin-2 before intratracheal administration in mice. Eight- to ten-week-old female C57BL/6 mice were anesthetized by intraperitoneal injection of 20 μl/g avertin (composed of 1.25% 2,2,2-tribromoethanol and 2.5% 2-methyl-2-butanol). For intratracheal administration the vocal cords were viewed directly and a blunted 25-gauge needle was passed between and just distal to them. Then $\sim 1 \times 10^5$ CFU of PA01 [pretreated with either PBS or trappin-2 (100 nmol/L)] were administered through the needle. After 2 (or in separate experiments 3) hours mice were sacrificed and BAL performed by injection of 800 μl of PBS three times. Lungs from mice sacrificed at 2 hours were removed and homogenized. Aliquots of lung homogenate were plated on LB agar and incubated at 37°C overnight, before colony counting.

Measurement of Cytokines and Chemokines

Interleukin (IL)-12p70, interferon gamma (IFN)-γ, IL-10, IL-6, TNF-α, and CCL2/MCP-1 were measured by cytometric bead array according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Murine CXCL1/KC was measured by Duoset enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems Europe Ltd.). Murine LPS binding protein (LBP) was measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions (Cambridge Biosciences, Cambridge, UK).

Flow Cytometry

To detect cell surface F4/80 and CR-3 expression, murine BMDMs and MH-S cells at a concentration of 5×10^5

cells/ml were incubated for 60 minutes in 2% rat serum (Biosera, Ringmer, UK) on ice to block nonspecific binding. Cells were incubated with either 0.3 μg of PE-Cy5-conjugated rat anti-mouse F4/80, or 0.3 μg of Alexa 488-conjugated rat anti-mouse CR-3 for 60 minutes. Equivalent concentrations of relevant isotype control antibodies were used to assess nonspecific staining. After washing with PBS, cells were fixed with 5% formalin for 10 minutes. Cells were analyzed in a FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK).

Immunocytochemistry of Macrophages

For detection of cell surface CD14, BMDMs and MH-S cells were allowed to adhere to glass coverslips placed in a 12-well plate for 30 minutes in their respective complete media. Cells were cultured overnight and then fixed in 2% formalin for 10 minutes. The preparations were washed once gently and then incubated with 2% FCS overnight. Cells were then incubated with 10 μg/ml of rat anti-mouse CD14 in 2% FCS for 3 hours. Negative control sample was 2% FCS alone. After three gentle washes in PBS, cells were incubated with Alexa 488-conjugated chicken anti-rat antibody for 2 hours. Cells were washed four times before mounting in 25 μl of Vectashield (Vector Laboratories Ltd., Peterborough, UK). Preparations were visualized by epifluorescent microscopy within 24 hours.

Immunocytochemistry of PA01

For characterization of trappin-2-opsonised PA01 early logarithmic phase PA01 were treated with trappin-2 (100 nmol/L) for 30 minutes in PBS. Bacterial suspensions were then allowed to adhere to glass coverslips for 2 hours. Bacteria were fixed with 2% formalin for 10 minutes, washed once in PBS, followed by overnight incubation in 2% FCS. The next day bacteria were incubated with rabbit anti-PA01 outer membrane protein-specific antiserum (1:100), or goat anti-human trappin-2 antibody (1:10) for 4 hours. Preparations were washed three times before Alexa 488-conjugated Fab_2 goat anti-rabbit antibody (1:200) or Alexa 488-conjugated chicken anti-goat antibody (1:200), respectively, were used as secondary antibodies by incubating for 2 hours at room temperature. Negative controls were prepared that had not been preincubated with trappin-2. These were counterstained with PKH26-GL (1:1000) for nonspecific membrane labeling. Preparations were washed three times before mounting in Vectashield and preparations were visualized by epifluorescent microscopy within 24 hours.

Statistics

Results are expressed as the mean ± SD of the number (n) of independent experiments, with each experiment performed in triplicate. Pair-wise comparisons between conditions were made using an unpaired Student's t-test. Analysis of variance with the Bonferroni posthoc test was used to determine differences between multiple groups for *in vivo* experiments. Statistical analysis was performed using GraphPad Prism (Version 5.0; GraphPad Software, Inc., La Jolla, CA) with differences considered significant at $P < 0.05$.

Results

To determine the effect of serum-free conditions on the interaction of BMDMs with PA01, we studied clearance and phagocytosis of bacteria in the presence and absence of serum. In serum-free conditions, macrophages failed to influence the growth of PA01. This observation was associated with very low levels of phagocytosis (3.11 ± 1.30%). If serum was present a different pattern emerged—PA01 proliferated more rapidly than in the absence of serum but macrophages were capable of significantly reducing the growth of serum-opsonized PA01 (39% inhibition of bacterial growth at 2 hours, $P = 0.0168$). This retardation in growth corresponded to an increase in the number of phagocytosed PA01 (21.0 ± 4.0%, $P = 0.0008$ versus $t = 0$ control). The reduced clearance in a serum-free environment was not attributable to cell toxicity because lactate dehydrogenase assays suggested that >95% of this cytoplasmic enzyme remained within the cell whether cultured in serum-rich or serum-free conditions. Similarly, inefficient phagocytosis did not reflect a global functional impairment in the absence of serum because BMDMs in serum-rich and serum-free conditions had no significant difference in their capacity to ingest titanium dioxide particles and to generate a characteristic oxidative burst in response to stimulation with zymosan (data not shown). Thus, we reasoned that macrophages such as AMs have other means of clearing everyday low-grade infections that do not depend on the presence of serum proteins.

Under serum-free conditions we pretreated PA01 with trappin-2. The direct effect of trappin-2 on live actively growing PA01 is shown in Figure 1A. Concentrations of 500 nmol/L significantly decreased PA01 viability throughout 150 minutes. Doses of 20 and 100 nmol/L were found not to affect PA01 viability throughout the same time course. We therefore used the 100 nmol/L dose of trappin-2 to study its opsonizing effects on PA01. Indeed trappin-2 could be detected, forming a punctate staining, on the surface of trappin-2-opsonised PA01 suggesting that it could bind to certain structures on the bacterial cell surface (Figure 1B). In contrast PA01 that was not treated with trappin-2 did not reveal this pattern but could be counterstained with PKH Red (Figure 1C). PA01 probed with a specific anti-serum against O-antigen demonstrated robust staining of the bacterial membrane to demonstrate the integrity of the bacterial membrane at these concentrations of trappin-2 (Figure 1D).

Using co-culture experiments under serum-free conditions, we then pretreated PA01 with 100 nmol/L trappin-2 before adding the bacteria to BMDMs (Figure 2). As demonstrated in Figure 1A trappin-2 had no significant direct effect on PA01 viability when given alone. When trappin-2-treated PA01 were added to BMDMs a significant decrease in viability was observed (Figure 2A). Thus trappin-2-treated PA01 were more efficiently cleared than

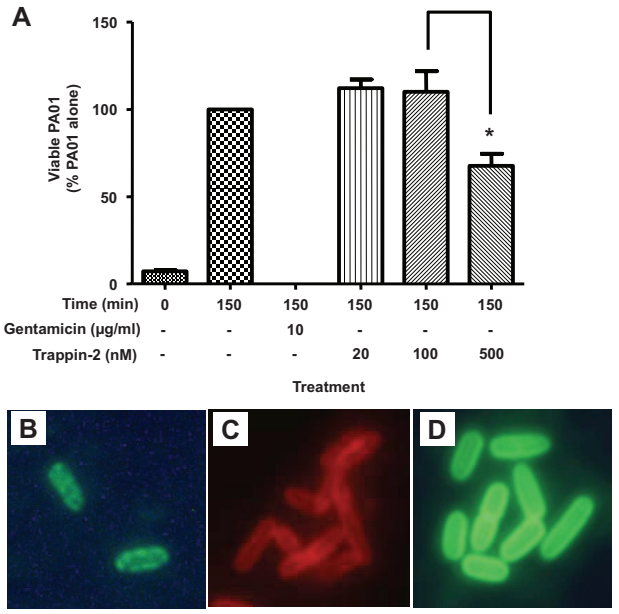

Figure 1. Interaction of trappin-2 with PA01. **A:** Direct anti-microbial effect. Early logarithmic phase PA01 was added to increasing concentrations of trappin-2 and incubated for 150 minutes on an Eppendorf shaker at 750 rpm. Serial dilutions were plated on LB agar in triplicate and allowed to grow overnight. Colonies were counted the following day. Data represent means ± SD of three separate experiments expressed as a percentage of the untreated PA01 at 150 minutes. *$P < 0.05$. **B–D:** Opsonizing effect. PA01 was opsonized with trappin-2 (100 nmol/L) for 30 minutes at 37°C. The PA01 suspension was allowed to adhere to a glass coverslip for 120 minutes. PA01 was fixed with 2% formalin for 15 minutes then blocked overnight in 2% FCS. Opsonized PA01 was then stained: with goat anti-trappin-2 (**B**); without antibody but counterstained with PKH26 (**C**); or with rabbit anti-PA01 outer membrane protein (**D**). Four hours later preparations were washed three times and stained with either chicken anti-goat Alexa 488 or Fab2 goat anti-rabbit 488 for 1 hour. Preparations were washed three times and mounted in Vectashield. Preparations were analyzed by epifluorescent microscopy within 24 hours. Original magnifications, × 1000.

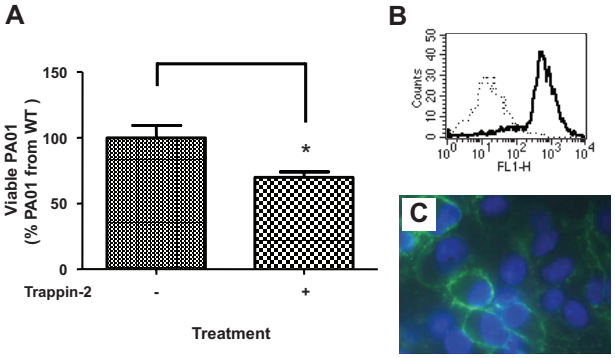

Figure 2. Immunomodulatory effect of trappin-2 on murine wild-type BMDMs. **A:** Bone marrow was harvested from the femora of 8- to 12-week-old wild-type C57BL6 mice and cultured in L929-conditioned media for 6 days, then 5×10^5 macrophages were subcultured into 12-well plates. The next day, PA01 was opsonized with trappin-2 (100 nmol/L) or IMDM for 30 minutes. After this, opsonized or unopsonized PA01 was added to BMDMs for 120 minutes. Total bacteria were estimated by washing the wells four times in 0.1% Triton/IMDM. Colonies were counted the following day. Data represent the mean ± SD of three separate experiments expressed as a percentage of the PA01 counts in PA01-treated wild-type BMDMs at 150 minutes. *$P < 0.05$. BMDMs (5×10^5) were stained for their expression of CR-3 and CD14 and analyzed by flow cytometry and fluorescent microscopy (**B** and **C**, respectively).

untreated PA01 at trappin-2 doses that did not cause direct killing (Figure 2A).

Pseudomonas clearance has been reported to proceed via CR-3 or CD14[20] both of which are expressed on BMDMs derived from wild-type C57BL/6 mice (Figure 2, B and C). To assess whether these membrane-bound receptors played a role in trappin-2-mediated clearance of PA01 we adapted our model for use with the murine AM cell line MH-S (29) and CD14-deficient BMDMs. All three cell types studied (wild-type BMDMs, MH-S cells, and CD14-deficient BMDMs) expressed the murine macrophage-specific marker F4/80, and BMDM preparations were 98% F4/80- and CR-3-positive (data not shown). We found that MH-S cells did not express CR-3 (Figure 3B) but were CD14-positive (Figure 3C). Trappin-2-treated PA01 were more readily cleared by MH-S cells than nonopsonized PA01 (Figure 3A), in keeping with the situation for BMDMs described in Figure 2A. To study the influence of CD14 on this effect we used BMDMs isolated from CD14-deficient mice. These cells expressed CR-3 (Figure 4B) but not CD14 (Figure 4C). Enhanced clearance of trappin-2-treated PA01 was not observed in CD14-deficient BMDMs (Figure 4A), in striking contrast to the situation observed for wild-type BMDMs (Figure 2A) and MH-S cells (Figure 3A). Thus in a serum-free environment trappin-2, when pre-opsonized by PA01, can promote bacterial clearance by macrophages.

To assess the *in vivo* relevance of the trappin-2 opsonization effect we studied the effects of low-dose pulmonary infection with PA01 during the first hours after intratracheal administration (Figures 5 to 7). We postulated that at very early time points (before exudation of neutrophils into the alveolar space) the effect of serum would be minimal and the corresponding effects of trappin-2 would be at their greatest. Time course analysis (data not shown) of infection suggested that lower doses of PA01 ($\leq 4.2 \times 10^6$ CFU) were cleared much more efficiently during the first few hours of infection when compared with higher (1.3×10^7 CFU) doses. We therefore gave wild-type and CD14-deficient mice low-dose PA01 (average

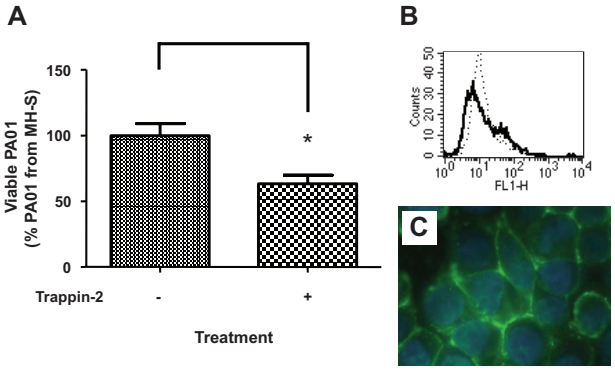

Figure 3. Immunomodulatory effect of trappin-2 on MH-S macrophages. **A:** MH-S macrophages (5×10^5) were subcultured into 12-well plates. The next day, PA01 was opsonized with trappin-2 (100 nmol/L) or IMDM for 30 minutes. After this, opsonized or unopsonized PA01 was added to MH-S cells for 120 minutes. The total bacteria was estimated by washing the wells four times in 0.1% Triton/IMDM. Colonies were counted the following day. Data represent the mean ± SD of three separate experiments expressed as a percentage of the PA01 counts in PA01-treated MH-S at 150 minutes. *$P < 0.05$. MH-S (5×10^5) were stained for their expression of CR-3 and CD14 and analyzed by flow cytometry and fluorescent microscopy (**B** and **C**, respectively).

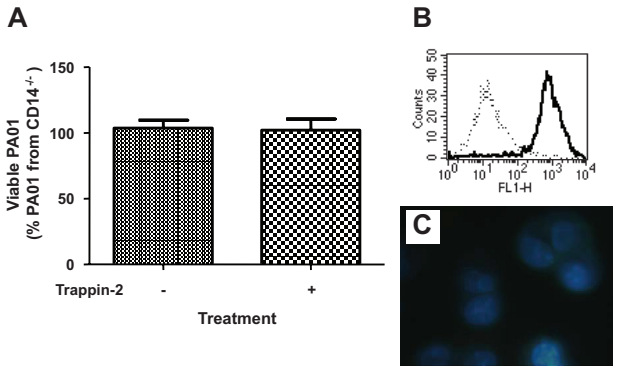

Figure 4. Immunomodulatory effect of trappin-2 on murine *CD14*$^{-/-}$ BMDMs. **A:** Bone marrow was harvested from the femora of 8- to 12-week-old *CD14*$^{-/-}$ C57BL/6 mice and cultured in L929-conditioned media for 6 days, then 5×10^5 macrophages were subcultured into 12-well plates. The next day, PA01 was opsonized with trappin-2 (100 nmol/L) or IMDM for 30 minutes. After this, opsonized or unopsonized PA01 was added to BMDMs for 120 minutes. The total bacteria was estimated by washing the wells four times in 0.1% Triton/IMDM. Colonies were counted the following day. Data represent the mean ± SD of three separate experiments expressed as a percentage of the PA01 in PA01-treated *CD14*$^{-/-}$ BMDMs at 150 minutes. BMDMs (5×10^5) were stained for their expression of CR-3 and CD14 and analyzed by flow cytometry and fluorescent microscopy (**B** and **C**, respectively).

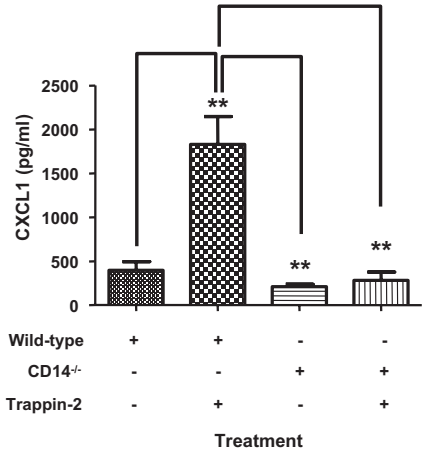

Figure 6. Effect of trappin-2 on CXCL1 levels in BAL during early pulmonary infection in wild-type and *CD14*$^{-/-}$ mice. PA01 was opsonized with trappin-2 (100 nmol/L) or PBS for 30 minutes and then ~1×10^5 bacteria were given intratracheally to wild-type and CD14-deficient mice. Two hours later mice were humanely sacrificed and the concentration of CXCL1 was determined. Data represent the mean ± SD of levels in BAL from seven mice in each treatment group. $^*P < 0.05$, $^{**}P < 0.01$.

dose = $1.03 \times 10^5 \pm 3.2 \times 10^4$ CFU) pretreated with either trappin-2 (100 nmol/L) or vehicle alone via the intratracheal route. The bacterial burden was assessed 2 hours later (Figure 5). Nonopsonized PA01 were cleared to the same degree whether in wild-type mice or CD14-deficient mice. However, wild-type mice given trappin-2-opsonised PA01 showed significantly decreased bacterial burden compared with mice given nonopsonized PA01. In striking contrast CD14-deficient mice were resistant to the PA01-opsonizing effects of trappin-2 and were unable to clear the bacteria as effectively. PA01 treated with trappin-2 or vehicle alone were incubated *in vitro* during the course of the *in vivo* experiment and no significant differences in growth rate were observed.

To further investigate the CD14-dependent clearance of trappin-2-opsonised PA01 *in vivo* we assessed leukocyte and cytokine levels in BAL fluid (Figure 6). After 2 hours the predominant cell in BAL fluid was the AM (~99%) with the remaining cells being neutrophils, as is the case in normal, healthy mice. No differences in leukocyte numbers (or differential cell counts) were observed between animals given PA01 (±trappin-2), PBS alone, or trappin-2 alone (data not shown). Analysis of the cytokines IFN-γ, IL-6, IL-10, IL-12p70, and TNF-α and the chemokines CXCL1 and CCL2 in BAL fluid established that only TNF-α and CXCL1 could be detected at this early time point. We found no differences in TNF-α between the groups studied. In contrast we found significant differences in CXCL1 between the groups. Both wild-type and CD14-deficient mice given trappin-2-opsonised PA01 or nonopsonized PA01 had significantly higher levels of CXCL1 in their lavage than PBS- or trappin-2-treated mice (data not shown). Intriguingly wild-type mice that received trappin-2-opsonised PA01 had a fivefold increase in their CXCL1 levels compared with mice receiving nonopsonized PA01. CD14-deficient mice were resistant to this trappin-2-mediated effect (Figure 6). Importantly we could not detect murine LBP in the same lavage fluid at the 2-hour time point. This suggested that trappin-2 could induce subsequent neutrophil recruitment into the lung. To test this we repeated experiments at the slightly later time of 3 hours to detect newly migrated neutrophils within the lung (Figure 7). As predicted wild-type mice given trappin-2-opsonised PA01 had dramatically increased numbers of neutrophils in their lavage compared with those given nonopsonized PA01. Further, CD14-deficient mice given the same treatments, while having increased neutrophils in the BAL fluid were resistant to the dramatic recruitment seen in wild-type mice given trappin-2-opsonised PA01. Bacterial burden in these trappin-2-treated wild-type mice remained significantly decreased (as in Figure 5) but did not appear to have been cleared further at this time point (data not shown). We also detected levels of murine LBP

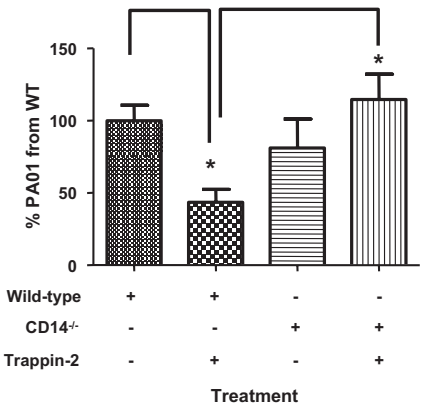

Figure 5. Effect of trappin-2 on PA01 clearance during early pulmonary infection in wild-type and *CD14*$^{-/-}$ mice. PA01 was opsonized with trappin-2 (100 nmol/L) or PBS for 30 minutes and then ~1×10^5 bacteria were given intratracheally to wild-type and CD14-deficient mice. Two hours later mice were humanely sacrificed and the number of viable PA01 in lung homogenates was determined. Data represent the mean ± SD of viable counts in homogenates from seven mice in each treatment group. $^*P < 0.05$.

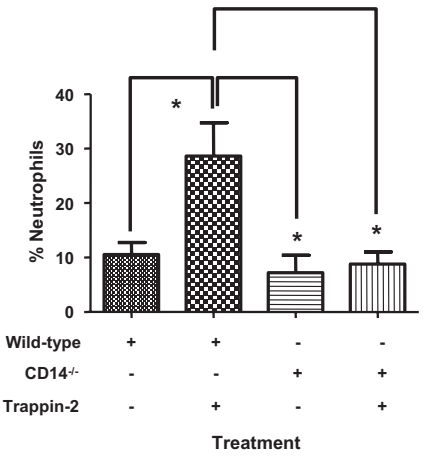

Figure 7. Effect of trappin-2 on neutrophil levels in BAL fluid during early pulmonary infection in wild-type and *CD14*$^{-/-}$ mice. PA01 was opsonized with trappin-2 (100 nmol/L) or PBS for 30 minutes and then $\sim 1 \times 10^5$ bacteria were given intratracheally to wild-type and CD14-deficient mice. Three hours later mice were humanely sacrificed and the number of leukocytes was determined. Data represent the mean ± SD of levels in BAL from five mice in each treatment group. $^*P < 0.05$.

at this 3-hour time point suggesting that this molecule is induced at a later time point than CXCL1.

Discussion

In this study we developed reproducible models of the very earliest interactions between low-dose *P. aeruginosa* and macrophages *in vitro* and *in vivo*, to study the immunomodulatory effects of trappin-2. These conditions have allowed us to mimic the presumed environment encountered in the initial stages of lung infection and to uncover a role for trappin-2 in the amplification of innate host defense. A similar approach has been used to demonstrate that LBP[26] and CD14[23] influence innate immunity. This low dosing regimen is particularly pertinent in the clinical setting of nosocomial pneumonia, in which microaspiration of oropharyngeal secretions is thought to be an independent predictor of subsequent tracheobronchial colonization[8] or pneumonia.[27,28] This type of pathogenesis would suggest a low-dose bacterial burden being taken into the lung repeatedly as has been shown in normal healthy volunteers during sleep.[9]

In vitro infection models using live bacteria/leukocyte co-cultures produce characteristic bacterial growth curves. In serum-containing systems bacteria in co-culture with leukocytes are generally retarded in their growth as compared with bacteria cultured alone, particularly in the early stages of the time course before surviving bacteria overrun the culture system. The specific shape of such growth curves is leukocyte- and bacterium-dependent.[29,30] Far less is known about such interactions in a low-serum environment. Our data show that in a nonopsonic serum-free system bacterial clearance proceeds slowly with macrophages having very little effect on the growth of *P. aeruginosa* strain PA01. This system was used to study the opsonizing effects of trappin-2 in a serum-free environment.

Titration of trappin-2 into the nanomolar range confirmed concentrations that were not directly antimicrobial[12–14] and these concentrations were used for assessing immunomodulatory and opsonin-like effects. We found that trappin-2 enhanced clearance of PA01 by macrophages. This effect was dependent on prior contact between trappin-2 and PA01. The magnitude of this effect is worth considering; although differences in Figures 2A and 3A may not appear striking at first glance, it should be noted that in our serum-free system we have never observed significant clearance of PA01 by macrophages themselves, whether studying murine BMDMs, MH-S cells, or human macrophages (data not shown). Thus trappin-2 drives activation of a system that is otherwise inert under the conditions studied. Nonopsonic interactions between pathogens and phagocytes are believed to take place in a variety of anatomical sites, including the lung and renal medulla, which have low concentrations of serum-derived opsonins. The lung has its own opsonins, which include the collectins such as surfactant protein A and D and mannose binding protein.[31,32]

Our opsonizing studies with trappin-2 on PA01 showed a unique punctate staining pattern on the bacterial cell surface. In support of this others have used scanning electron microscopy to show that trappin-2-treated *P. aeruginosa* have altered membranes characterized by wrinkling, crumpling, and blebbing.[14] These observations are probably the result of membrane disruption at trappin-2 concentrations above those used in our study. More specifically Bellemare and co-workers[33] localized fluorescently tagged trappin-2 to the surface of *P. aeruginosa* and suggested that the N-terminal cementoin domain binds through ionic interaction whereas the C-terminal elafin domain binds hydrophobically. Indeed there is a general acceptance that the initial interaction between anti-microbial peptides and pathogens is electrostatic because molecules such as LPS and lipoteichoic acid are negatively charged and the host defense peptide is positively charged.[34] Indeed trappin-2 can bind purified LPS from *Escherichia coli*[17] but we are unaware of studies assessing its binding to other specific structures of the bacterial cell wall to date.

To our knowledge no cell surface receptor has been identified that participates in mediating trappin-2 activity. Here for the first time we demonstrate that CD14, a promiscuous pattern recognition receptor, is involved in mediating the effect of trappin-2 as has been suggested for a related molecule, secretory leukocyte protease inhibitor (SLPI).[35] In contrast we could find no role for CR-3 in trappin-2-mediated enhancement of *P. aeruginosa* clearance by macrophages. Our study suggests that trappin-2 acts in a manner analogous to LBP by opsonizing PA01 and then presenting the bacteria to CD14 on the macrophage cell surface.

McMichael and co-workers[17] have demonstrated that under serum-free conditions trappin-2 is capable of binding both smooth and rough forms of LPS at the lipid A portion of the molecule. Interestingly their study showed that LBP and polymyxin B, two lipid A core binding proteins, can displace trappin-2 suggesting a weaker inter-

action with the latter (kDa 1 nmol/L versus 2.2 to 7.9 nmol/L). LBP is known to convert oligomeric micelles of LPS to monomeric form for delivery to CD14.[36,37] LBP and trappin-2 appear to share a number of overlapping properties including chromosomal location, clusters of positively charged amino acids, and an ability to mediate CD14-dependent interactions.[38–40] It is therefore tempting to speculate that trappin-2 might complement pulmonary LBP during Gram-negative lung infection, considering it is produced locally by alveolar epithelial cells[41] and is found in bronchial secretions.[10] Interestingly, in our *in vivo* studies, we could detect LBP in BAL but only at later time points suggesting that its impact would become apparent later during infection. Furthermore CXCL1 (and CXCL2) are increased in LBP-deficient mice after administration of *Klebsiella pneumoniae*.[42] Thus it appears that trappin-2 and LBP perform distinct and independent roles in our model. Further study is required to determine whether these roles are complementary, suggesting that LBP may act to decrease these neutrophil chemokines. Indeed others have suggested that LBP is required for an intact respiratory burst in neutrophils and is required to contain Gram-negative infections.[26,43,44] On the contrary trappin-2 may offer the lung added protection through its activity against Gram-positive organisms against which LBP is inert.[42,45]

CD14 has a broad ligand specificity allowing it to bind Gram-positive,[46] Gram-negative,[36] and viral pathogens.[47] CD14 can also participate in noninflammatory or anti-inflammatory responses by acting as a macrophage receptor for engulfment of apoptotic cells.[48] Trappin-2 has also been shown to be pro-inflammatory[16,49] or anti-inflammatory[50–52] in models of LPS-induced injury. Furthermore it can enhance clearance of *P. aeruginosa* and prevent neutrophil-mediated lung injury in a model of significant murine pneumonia.[15] These differences in trappin-2 function are not completely understood but may be significantly influenced by the mode of delivery (for example recombinant protein versus genetic augmentation), the cellular compartment to which trappin-2 is delivered, the presence of serum, and the timing of administration relative to the inflammatory insult induced. We propose that accessibility to CD14 may now be added to this list.

The finding that trappin-2 could augment clearance of PA01 from the murine lung at 2 hours, before recruitment of neutrophils, clearly suggests a potential contribution to the very early clearance of pathogens entering the distal respiratory tract. However we also found that trappin-2-opsonised PA01 simultaneously promoted a fivefold increase in CXCL1 compared with nonopsonized PA01, and that this effect also appeared to be CD14-dependent. This effect preceded a rapid recruitment of neutrophils an hour later. Thus trappin-2 also appears to amplify host defense by recruiting neutrophils to the site of infection. Our previous studies using adenoviral delivery of trappin-2 to the murine lung broadly support this finding in that trappin-2 significantly enhanced LPS-mediated CXCL2 generation in BAL fluid.[49] Both CXCL1 and CXCL2 act through the chemokine receptor CXCR2, which has been shown to be essential for host protection against *P. aeruginosa* pneumonia.[53] These results therefore raise the intriguing possibility that in the early stages of infection trappin-2 simultaneously delivers pathogens to resident AMs, while contributing to activation of the neutrophil/CXCR2 axis should the bacterial inoculum appear sufficient to drive significant infection.

In summary the models of early lung infection developed in this study have identified novel roles for the cationic host defense peptide trappin-2 in innate immune recognition. We propose that trappin-2 may be involved in the everyday host defense of the lung against inhaled particles, such as low concentrations of inhaled microorganisms. In turn these findings suggest that properties inherent to trappin-2 and related host defense molecules may be worthy of attention in designing strategies aimed at the prevention (or early treatment) of nosocomial respiratory infection.

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