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p33 (gC1q Receptor) Prevents Cell Damage by Blocking the Cytolytic Activity of Antimicrobial Peptides

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The innate immune system is the first line of defense against invading microbes. Its specificity relies a great deal on host pattern recognition molecules that sense pathogen-associated molecular patterns of the invading pathogen. However, full protection is not always guaranteed, and some early defense mechanisms involved in bacterial killing, such as the complement system, can also exert cytolytic activity against host cells. Although these cascades are tightly regulated, the host has to take additional precautions to prevent its cell destruction. In this study, we describe that p33, a negatively charged surface protein found on endothelial cells also known as gC1q receptor, protects host cells from a cytolytic attack by antimicrobial peptides (AMPs), such as LL37 and β-defensin 3. To this end, we characterized the interaction of p33 with AMPs by biochemical and functional means. Our data show that p33 forms a doughnut-shaped trimer that can bind up to three AMPs, and we identified a segment in p33 forming a β-sheet that mediates the binding to all AMPs. Moreover, our results show that p33 abolishes the lytic activity of AMPs at an equimolar ratio, and it protects endothelial cells and erythrocytes from AMP-induced lysis. Taken together, our data suggest a novel protective mechanism of p33 in modulating innate immune response by neutralizing cytotoxic AMPs at the host cell surface. The Journal of Immunology, 2013, 191: 5714–5721.

The early recognition and elimination of an invading pathogen are two key features of the innate immune system. To fulfill these tasks, the immune response has to distinguish between self and nonself because this will allow an attack of the foreign invader without causing harm to the host. Pattern recognition molecules play an important role in these processes because they target so-called pathogen-associated molecular patterns, which are exposed on the surface of the intruder. Considering the amplitude of toxicity of some innate defense mechanisms, their selectivity for pathogenic microorganisms is not always guaranteed; thus, there is a risk that some innate immune reactions may also have a deleterious impact on the host. The complement system belongs to this group of early host defense responses that can cause significant tissue injury. To protect themselves from complement-mediated cellular damage, eukaryotic cells express membrane complement regulatory proteins such as membrane cofactor protein, decay-accelerating factor, and membrane inhibitor of reactive lysis, also known as CD55, CD46, and CD59, respectively (for reviews, see Refs. 1, 2). These three proteins are able to interfere with different parts of the complement system and this protects the cells from a complement attack (3).

Antimicrobial peptides (AMPs) are another group of early defense mediators that can target and damage host cells. AMPs are usually short (<100 aa) and cationic molecules. They comprise helical or linear structures, or contain β-sheets stabilized by disulphide bridges. Most AMPs exert broad antibacterial activity against both Gram-positive and -negative bacteria (4) by forming pores in the bacterial cell wall that has a higher negative transmembrane potential than the plasma membrane of eukaryotic cells (5). Moreover, the lack of cholesterol makes the bacterial cell lipid layer more susceptible to an attack by AMPs (6). The specificity of AMPs to target bacterial membranes is concentration dependent, and already low AMP levels are sufficient to cause bacterial cell-wall destruction. However, at high concentrations, AMPs also explore cytolytic activity against eukaryotic cells (7–10). Notably, AMP levels can significantly elevate under inflammatory conditions such as in patients with severe sepsis, and this has been shown to correlate with circulatory derangement (11). It is therefore tempting to speculate that a massive release of AMPs into the bloodstream or at cell–cell interaction sites could contribute to tissue destruction under systemic induction of innate immune reactions, although not much research has been performed to address this issue.

p33 (also referred to as p32 or globular C1q receptor) is a multiligand binding protein that is expressed on the surface of various cell types such as endothelial, neutrophils, lymphocytes, and platelets (12, 13), but it can also be found intracellularly, for instance, in mitochondria or nucleus (14). The protein is synthesized as a precursor protein consisting of 282 aa. At the cell surface it is located in its mature form (aa 74–282) with a highly negative net charge (isoelectric point [pI] of 4.15). p33 was originally identified as a complement protein C1q binding protein that prevents complement activation (15). The binding of p33 to C1q is only of moderate nature (240 ± 10 nM) (12), and other
p33-binding ligands with a higher affinity have been described. For instance, p33 interacts with plasma proteins such as high molecular weight kininogen (HK) and coagulation factor XII (12). An affinity in the lower nanomolar range (9 nM) was determined for this interaction, and the p33 binding site in HK was mapped to a highly cationic sequence located in domain 5 of HK (12). A synthetic 20mer peptide (HKH20) was found to resemble the p33 binding site in HK, and subsequent studies revealed that HKH20 uses antimicrobial activity against *Streptococcus pyogenes* and *Enterococcus coli* (16). In a sepsis model, mice injected with *S. pyogenes* were treated with HKH20, and this resulted in a significant increase in survival (17).

The interaction between p33 and bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, or *Listeria monocytogenes*, has been described previously (18–20). It has been speculated that these bacteria use p33 to adhere and eventually invade host cells. However, to our knowledge, it has not been reported that p33 is able to dampen a self-destructive host response to infection. In this article, we report a novel mechanism of p33 as modulator of the early innate immune response. We find that the binding of p33 to AMPs, including HKH20, β-defensin 3, and LL37, not only neutralizes their antimicrobial activity, but is also a mechanism that prevents AMP-induced host cell damage.

**Materials and Methods**

**Blood collection**

Peripheral venous blood was collected from healthy human volunteers into 2.7 ml, 0.109 M buffered sodium citrate tubes (Becton Dickinson).

**Bacterial strains**

The *E. coli* ATCC 25922 was from the Department of Bacteriology, Lund University Hospital.

**Purification of recombinant maltose-binding protein–p33**

Recombinant maltose-binding protein (MBP)–p33 was expressed using the pMAL-c2 expression vector (New England Biolabs) and *E. coli* XL1-Blue strain (Strategen, Heidelberg, Germany) as previously described (12). Isopropyl-β-D-thiogalactopyranoside was added to an exponential growing overnight culture of bacteria to a final concentration of 0.3 mM. Bacteria were harvested after 1 h (4000 × g, 10 min, 4°C), and the pellet was resuspended in 30 ml cold 20 mM Tris, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.4 (MBP-buffer). Bacteria were kept on ice and lysed by sonication (3 min in pulses of 15 s). The lysed cell wall was pelleted (15,000 × g, 20 min, 4°C), and the supernatant was removed and transferred (1 ml/ml) to an amylase resin column pre-equilibrated with two column volumes of MBP buffer. After adding the bacterial supernatant, we rinsed the column with 12 column volumes MBP buffer before 4 column volumes 10 mM Tris-HCl, pH 7.4 (Tris-buffer). Bound MBP-p33 was eluted and fractionated with 10 mM Tris-HCl containing 10 mM maltose. The concentrations of MBP-p33 used in this study are calculated based on its monomeric form (75,500 Da).

**Peptides and Abs**

HKH20 and NAT26 were synthesized by Bachem, Feinchemikalien AG (Bubendorf, Switzerland). LL37 and the defensins were from Innovanogen AB (Lund, Sweden), and GGL27 and GGL27(S) were from Biopeptide, San Diego, CA. A list with all AMPs is shown in Table I. Overlapping peptides spanning the entire sequence of p33 (Table II) were generated by a peptide synthesis platform (Sigma-Aldrich PepScreen Directory).

Polyclonal antisera to p33 were raised in rabbits (Innovagen AB). Ascites mouse anti-p33 IgG (60.11) and mouse anti-p33 IgG (74.5.2) were purchased from Nordic BioSite AB (Täby, Sweden). Peroxidase-conjugated goat anti-mouse IgG and peroxidase-conjugated goat anti-rabbit IgG were purchased from Bio-Rad Laboratories.

**ELISA**

Indirect ELISAs were performed by coating microtiter plates (Maxisorb, NUNC) overnight at 4°C with various AMPs (5 μg) in 15.9 mM Na2CO3, 30 mM NaHCO3, pH 9.6 (coating buffer). Plates were washed three times in deionized water, blocked in PBS containing 0.05% Tween 20 and 0.5% BSA (PBST) for 30 min at 37°C, and incubated with 2.5 μg/ml MBP-p33 (1 h, 37°C). Bound MBP-p33 was detected with an mAb against p33 (clone 60.11, MMS-606R; Nordic BioSite AB), diluted 1:3000 for 1 h at 37°C, and visualized by a peroxidase-conjugated secondary Ab against mouse IgG (1:2.500, 1 h, 37°C; Bio-Rad Laboratories). All incubations were followed by three washing steps in PBST. Competitive ELISAs were performed following the same protocol, except that 1 of the 20 aa overlapping p33 peptides (10 μg/ml) was mixed with 2.5 μg/ml p33-MBP before incubation on the microtiter plates.

**Cleavage and purification of native p33**

MBP-p33 was treated with bovine factor Xa (50:1; New England Biolabs) overnight at room temperature (RT). Cleavage was visualized by SDS-PAGE, and a HiLoad 16/600 Superdex 75 pg gel filtration column (GE Healthcare) was used to separate p33 from MBP and factor Xa as described by the manufacturers. Notably, MBP-p33 and cleaved p33 form homo-trimers, and have the same function and yield the same results in binding and functional assays (Supplemental Fig. 1).

**Negative staining and transmission electron microscopy**

The binding between p33 and AMPs was visualized by negative staining and electron microscopy as previously described (21). AMPs were conjugated with 5 nm colloidal gold particles according to routine protocols (22). Conjugates were incubated with p33 for 30 min at RT and negatively stained with 0.75% uranyl formate. Specimens were examined in a Philips/FEI CM100 BioTwin transmission electron microscope at a ×100,000 magnification.

**Viable count assay**

The *E. coli* ATCC 25922 strain was grown overnight in 3% (w/v) Tryptic Soy Broth medium at 37°C on rotation. A volume of 350 μl was transferred to 10 ml prewarmed Tryptic Soy Broth medium and grown to midlog phase (A600 nm ~0.4). The bacteria were washed (2300 × g, 10 min, 4°C), resuspended in 10 ml cold 10 mM Tris containing 5 mM glucose, 0.1 mM EDTA, 1 mM DTT, pH 7.4 (MBP-buffer). Bound MBP-p33 was eluted and fractionated with 10 mM Tris-HCl containing 10 mM maltose. The concentrations of MBP-p33 used in this study are calculated based on its monomeric form (75,500 Da).

**Table I. AMPs screened for p33 binding**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>m.w. (Da)</th>
<th>Charge</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HKH20</td>
<td>HKHGIGHGKHKNGKKNQKH</td>
<td>2252</td>
<td>7</td>
<td>Domain 5 of HK</td>
</tr>
<tr>
<td>NAT26</td>
<td>NATFYKIDNKKVQVQVAGKYFI</td>
<td>3049</td>
<td>5</td>
<td>Domain 3 of HK</td>
</tr>
<tr>
<td>GGL27</td>
<td>GGLIKTKRKKQVRQVIAYEEIFVKNM</td>
<td>3263</td>
<td>8</td>
<td>C-terminal of tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>GGL27(S)</td>
<td>GGLISTSSSSSSQVRVKA YEEIFVKMN</td>
<td>2919</td>
<td>1</td>
<td>Charged peptides of GGL27 changed</td>
</tr>
<tr>
<td>LL37</td>
<td>LLGDFFKRKSEKIGFKEF VQRKIDFLRNLYPRTES</td>
<td>4493</td>
<td>6</td>
<td>Neutrophil specific granula</td>
</tr>
<tr>
<td>β defensin 3</td>
<td>DCYCRIPACIAGERRYGTCIYQGRLWAFCC</td>
<td>3486</td>
<td>4</td>
<td>Neutrophil azurophilic granula</td>
</tr>
<tr>
<td>α defensin 5</td>
<td>ATCYCRTGRCATRESLGVCEISGRLYRCLL</td>
<td>3582</td>
<td>4</td>
<td>Neutrophil azurophilic granula</td>
</tr>
<tr>
<td>α defensin 6</td>
<td>AFTCHRCRSCYSTEYSYGCTVGIMNHFKCCL</td>
<td>3708</td>
<td>2</td>
<td>Neutrophil azurophilic granula</td>
</tr>
<tr>
<td>β defensin 1</td>
<td>DHVYCSVQCQFLCSA PFTIKJQCTCGYRGKAKCC</td>
<td>3929</td>
<td>4</td>
<td>Epithelial cells, platelets, monocytes</td>
</tr>
<tr>
<td>β defensin 2</td>
<td>GIGDPYTVCLSKGAI CHPFVCPKRKQIYGTCLPTGKTC CKP</td>
<td>4328</td>
<td>6</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>β defensin 3</td>
<td>GIINTLKyYVCRGGRGCAVLSCLPKEEQGKCTGRKR GCRKRRK</td>
<td>5155</td>
<td>11</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>β defensin 4</td>
<td>ELDRICGYGTARCKRCKRCSQYERICPRNTYACLLRK</td>
<td>4366</td>
<td>7</td>
<td>Epithelial cells</td>
</tr>
</tbody>
</table>
pH 7.5, and further diluted in cold 10 mM Tris containing 5 mM glucose to 2 × 10^6 CFUs/ml. Fifty microliters bacteria were incubated with 50 μl various AMPs (1.5–6 μM), with or without 10 μM MBP-p33 in 10 mM Tris-HCl containing 10 mM maltose (1 h, 37°C, rotation). Antibacterial activity was determined by counting the number of CFUs on serial dilutions of the incubation mixtures plated on Todd Hewitt agar plates.

**SDS-PAGE and Western blot analysis**

SDS-PAGE was performed as described by Laemmli (23) using a polyacrylamide concentration of 10%. The separated proteins were transferred to a PVDF membrane (Amersham Biosciences). The membrane was rinsed in PBST before 30-min incubation with blocking buffer (PBST containing 5% milk powder), incubated with primary Ab (clone 60.11, MMS-606R; Nordic BioSite AB) diluted 1:1000 in blocking buffer for 60 min at RT. The membrane was washed three times in PBST, followed by an incubation with a peroxidase-labeled secondary Ab (goat anti-mouse IgG, 1:2,500; Bio-Rad Laboratories) in blocking buffer for 60 min at RT. Membranes were washed three times in PBST and developed using a SuperSignal West Pico Chemiluminescence kit (Thermo Scientific) according to manufacturer’s instructions.

**Fluorescence microscopy**

Endothelial cells (EA.hy926) were grown confluent on coverslips in 12-well plates. Cells were fixed in 4% paraformaldehyde for 15 min on ice and 45 min at RT. After fixation, cells were washed three times in 10 mM PBS before a blocking step with PBST including 2% BSA for 30 min at 37°C. Primary Ab mouse anti-p33 IgG1 (clone 60.11; Abcam) or an isotype control mouse IgG1 (Invitrogen) was added overnight at 4°C. The following day, cells were incubated with secondary goat anti-mouse IgG1 Alexa Fluor 488 (Invitrogen) for 60 min at 37°C. Coverslips were transferred to glass slides with one drop DAPI Prolong Gold Antifade reagent (Invitrogen) and let dry in the dark at RT overnight. One washing step was performed after every incubation step.

**Immunohistochemistry**

Slides were dried at 60°C for 90 min and deparaffinized with Tissue Clear (Histolab). Slides were transferred to a cuvette containing DIVA Decloaker (Biocare Medical) before Ag retrieval. Thereafter, slides were cooled and washed in TBST. Slides were blocked with Background Punisher Blocking reagent (Histolab) and washed again in TBST followed by addition of total IgG from p33 rabbit anti-serum or a rabbit IgG isotype control Ab for 2 h at RT. Slides were washed with TBST and Rabbit-on-Rodent AP polymer (Histolab) was added, followed by another washing step. Staining was visualized with Vulcan Fast Red Chromogen Kit 2 (BMA Biomedicals). Harris hematoxylin (Sigma) was used for counterstaining followed by a wash step in tap water. Slides were dipped in 0.05% HCl diluted in 70% ethanol, washed in tap water, and placed in MilliQ water containing ammonium chloride (4 drops in 500 ml). Slides were washed in tap water, dried, and mounted on coverslips using Pertex (Histolab).

**Hemolysis assay**

One milliliter citrate-blood was centrifuged (800 x g, 10 min, RT), and plasma was removed and replaced by an equal volume of PBS. The washing step was repeated two times, and the washed blood cells were kept in RT. AMPs were diluted to 40 μM in PBS in the absence or presence of 40 μM MBP-p33. Samples were preincubated (37°C, 60 min, on rotation) followed by an addition of 3 μl washed blood cells (5% v/v) and another incubation (60 min at 37°C on rotation). Tox 7-Lysis buffer (1:10) and PBS served as positive and negative control, respectively. Samples were then centrifuged (800 × g, 10 min, RT), and the supernatant was transferred to a 96-well plate. The absorbance of hemoglobin was measured at 540 nm and was expressed as the percentage of Tox-7 lysis-induced hemolysis.

**FIGURE 1.** p33 binds to AMPs. Microtiter plates were coated overnight with 5 μM AMPs and probed with 2.5 μg/ml MBP-p33. Binding was detected with a mAb against p33 and visualized by a peroxidase-conjugated secondary Ab. One representative experiment out of three is shown.

**FIGURE 2.** Negative staining electron microscopy of p33 in complex with AMPs. (upper panel) An overview of p33 is shown. (lower panels) p33 molecules in the absence of a ligand or bound to gold-labeled AMPs are depicted. Arrowheads point to gold-labeled AMPs (last image for each AMP). Scale bars, 100 nm (upper panel), 15 nm (lower panels).
Lactate dehydrogenase assay

EA.hy926 cells were grown confluent in 96-well plates in DMEM containing 10% FBS, 100 mM hypoxanthine, 0.4 mM aminopterin, 16 mM thymidine, 100 mg/ml streptomycin, and 100 U/ml penicillin. Medium was removed and cells were washed with 200 μl phenol-free DMEM. AMPs were diluted in phenol-free DMEM and preincubated with MBP-p33 for 1 h at 37˚C. Samples were transferred to the 96-well plates and incubated for 16 h in 37˚C, 5% CO2. The following day, 90 μl of the supernatant was transferred to a new 96-well plate, and lactate dehydrogenase (LDH) substrate mix was added. LDH release was measured at 490 nm using the Tox-7 kit (Sigma-Aldrich) according to manufacturers’ instructions. LDH release was calculated as percentage of total lysis with Tox-7 Lysis buffer.

Colocalization of endogenous p33 and AMPs

HUVECs were grown to confluency on 96-well plates in EBM-2 medium with EGM-2 BulletKit, CC-3162 (Lonza). Cells were washed in PBS and incubated with LL37 (2.5 or 20 μM) or β-defensin 3 (2.5 or 20 μM) for 16 h in 37˚C, 5% CO2. CHAPS detergent (1%) was added for 30 min to dissolve the cells into membrane blebs followed by addition of EM-fix. Abs against p33, LL37, and β-defensin 3 were conjugated with gold particles (p33: 5 nm, LL37: 10 nm, and β-defensin 3: 10 nm) and incubated with the cell membrane blebs for 30 min at 4˚C, and negatively stained with uranyl formate before electron microscopy.

Statistical analysis

Statistical analysis was performed using GraphPad Prism, Version 5.00. The p value was determined by using the unpaired t test (comparison of two groups). All experiments were performed at least three times, if not otherwise mentioned. The bars in the figures indicate SEM.

Results

p33 binds to AMPs

Structural analysis revealed that p33 is a doughnut-shaped protein consisting of three monomers with an asymmetric charge distribution and patches with an accumulation of negatively charged amino acids (24). It has been suggested that these domains play an important role in the interaction with p33 ligands (25). Notably, many peptides with antimicrobial activity have like HKH20 (pI 11.2), a positive net charge and a pI, which is in the similar range of p33.

Table II. Overlapping p33 peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Position</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHT20</td>
<td>LHTDGDCAFVDFLSDEIKEE</td>
<td>73–93</td>
<td>−4</td>
</tr>
<tr>
<td>DEI20</td>
<td>DEIEKEIKIQHKTLPKMSG</td>
<td>87–107</td>
<td>+3</td>
</tr>
<tr>
<td>LPK20</td>
<td>LPKMSGGWELELNGTEAKLV</td>
<td>101–121</td>
<td>−1</td>
</tr>
<tr>
<td>TEA20</td>
<td>TEAKLRVAGEKIVTFPN</td>
<td>115–135</td>
<td>+2</td>
</tr>
<tr>
<td>TVT20</td>
<td>TVTFINNSIPPFDGEREEP</td>
<td>129–149</td>
<td>−4</td>
</tr>
<tr>
<td>DGE20</td>
<td>DGEEPSPOQKVREQPET</td>
<td>143–163</td>
<td>−7</td>
</tr>
<tr>
<td>QEP20</td>
<td>QEPETSTNPVFVEIKDD</td>
<td>157–177</td>
<td>−4</td>
</tr>
<tr>
<td>VIK20</td>
<td>VIKNDDGKALVLCHYPEED</td>
<td>171–191</td>
<td>−1</td>
</tr>
<tr>
<td>CHY20</td>
<td>CHYPEDEVGQDEAEASDFS</td>
<td>185–205</td>
<td>−7</td>
</tr>
<tr>
<td>ESD20</td>
<td>ESDFIREVSQFSGEESW</td>
<td>199–219</td>
<td>−4</td>
</tr>
<tr>
<td>TGE20</td>
<td>TGESEWKTNYLNTLDSLW</td>
<td>213–233</td>
<td>−4</td>
</tr>
<tr>
<td>TDS20</td>
<td>TDSLWALYHLMDLFADRG</td>
<td>227–247</td>
<td>−3</td>
</tr>
<tr>
<td>FLA20</td>
<td>FLADRGVDNTPHELVEST</td>
<td>241–261</td>
<td>−4</td>
</tr>
<tr>
<td>LVE20</td>
<td>LVELSTALEQHETYTFLELD</td>
<td>255–275</td>
<td>−4</td>
</tr>
<tr>
<td>LEH20</td>
<td>LEHQEYITFLELDKSFVSQX</td>
<td>262–282</td>
<td>−1</td>
</tr>
</tbody>
</table>

![FIGURE 3.](http://www.jimmunol.org/Downloaded.png) Mapping of the AMP binding sites in p33. (A) Microtiter plates were coated with 5 μM AMPs, followed by incubation with MBP-p33 and a panel of p33-derived peptide (10 μM; see Table II). Binding was detected by using a specific Ab against p33 and a secondary peroxidase-conjugated Ab. Values are described as percentage of absorbance compared with MBP-p33 alone. ***,<25% absorbance, ***,25–50% absorbance, *,50–75% absorbance. (B) The three-dimensional structure of p33 is shown, and the AMP-binding peptides are indicated: TEA20 in red, CHY20 in blue, and TGE20 in green (Jmol: an open-source Java viewer for chemical structures in 3D; http://www.jmol.org).
or even higher (26). We therefore wished to test whether it is a

*Figure 4 shows that all peptides tested, except the*

CHY20 (aa 185–205 and net charge of

TEA20 (aa 115–135) with a positive net charge of +2, inhibited

p33 to the AMPs. Figure 3A and Supplemental Fig. 2 show that

a competitive ELISA for their ability to displace the binding of

the entire p33 sequence (Table II). These peptides were tested in

peptides was synthesized, each consisting of 20 aa and spanning

binding site(s) in p33. For this purpose, a panel of 15 overlapping

designated positive amino acids were replaced by a serine. This mod-

ification led to a change of the net charge from +8 to +1 and a com-

plete loss of GGL27(S)’s affinity for p33 (Fig. 1). Together, these data
demonstrate that p33 binds to some but not all AMPs tested.

MBP-p33 has an apparent m.w. of 75.5 kDa (pI 4.6) as deter-

mined by SDS-PAGE analysis. After removal of the MBP tag

duced by Xa cleavage, recombinant p33 migrates as a 33-kDa

monomer when examined by SDS-PAGE under denaturing and

nondenaturing conditions (data not shown). However, additional
gel filtration experiments suggest that p33 forms homotrimers (data

not shown), which was confirmed when the protein was negatively

stained and visualized by transmission electron microscopy (Fig.

2). For further electron microscopy analysis, AMPs were labeled

with colloid 5 nm gold particles and incubated with p33. Fig. 2
shows that p33 can bind up to three peptides (HKH20, GGL27,

LL37, and the β-defensins 2 and 3, respectively). No binding was
detected when α-defensin 3 was tested.

In the next series of experiments, we sought to map the AMPs
binding site(s) in p33. For this purpose, a panel of 15 overlapping
peptides was synthesized, each consisting of 20 aa and spanning
the entire p33 sequence (Table II). These peptides were tested in

a competitive ELISA for their ability to displace the binding of
p33 to the AMPs. Fig. 3A and Supplemental Fig. 2 show that
TEA20 (aa 115–135) with a positive net charge of +2, inhibited
the binding of p33 to all AMPs. DGE20 (aa 143–163) with a net
charge of −7, in contrast, did not have any inhibitory activity, and
CHY20 (aa 185–205 and net charge of −7) was able to block
the binding of some, but not all, AMPs. Other peptides, such as
TGE20 (aa 213–233), TDS20 (aa 227–247), and LEH20 (aa 262–
282), respectively, had an effect on one or two AMPs. These
findings may indicate that the AMP binding site in p33 is formed
by a discontinuous epitope involving only parts of the binding
peptides that are negatively charged (so-called binding spots),
and indeed, Fig. 3B revealed that the three major binding epitopes
are located in close vicinity (Fig. 3B).

**p33 inhibits the antimicrobial activity of AMPs**

The AMPs used in this study have been shown to explore a broad
spectrum of antimicrobial activity (27–30). Thus, a set of ex-
periments was designed to determine whether p33 is able to

counteract the ability of the AMPs to kill the *E. coli* strain ATCC
25922. Fig. 4 shows that all peptides tested, except the α-defensins
and GGL27(S) (data not shown), showed antimicrobial activity

against the ATCC 25922 strain, which was in the same range as
described by others. Fig. 4 also depicts that bacterial killing was
almost completely blocked when equimolar concentrations of p33
were applied to the reaction mixture. p33 did not affect bacterial
survival in the absence of AMPs, and no binding of p33 to the ATCC
25922 strain was detected that could theoretically provide an alter-
native explanation for a protective effect that does not require a binding
of p33 to AMPs (data not shown). Taken together, these data implicate
that binding of p33 to AMPs neutralizes their antimicrobial activity.

**Localization of p33**

The ability of p33 to dampen the antimicrobial effect of AMPs
seems at first glance counterproductive. We therefore wished to test
whether this feature of p33 is of pathophysiological importance.
Notably, the concentration of AMPs in plasma can increase under

inflammatory conditions (28, 29, 31, 32); thus, we measured
whether this will also lead to increased plasma levels of p33. To
this end, we used ELISA and Western blot analysis, but we were
unable to detect p33 with both assays in plasma samples from
healthy donors or sepsis patients (detection limit 6 ng/ml, data

not shown). We noted, however, that human endothelial cells (EA.
hy926 cells, an umbilical vein cell line), but not erythrocytes,
express p33 express as determined by Western blot analysis (Fig.
5A). Further immunohistological examination revealed that p33 is
located at the cell membrane of EA.hy926 cells (Fig. 5B). These
findings are in line with a report by Guo and colleagues (32), who
used bone marrow endothelial cells in their study. To test whether
p33 is also expressed in vivo, we immunostained lung tissue from
BALB/c mice and also here we saw specific expression of p33 in
the endothelial outer lining of the blood vessels (Fig. 5C). These
findings suggest that the interaction between p33 and AMPs takes
place at cellular surfaces rather than in the circulation.

**p33 protects host cells from lysis by AMPs**

It has been reported that AMPs at high concentrations can exert
cytotoxic activities against host cells such as keratinocytes, lung
epithelial cells, and endothelial cells, and intratracheal adminis-
tration of β-defensin 3 into C57BL/6 mice has been shown to
cause pulmonary inflammation and tissue injury (7, 33). To test
whether p33 can prevent AMP-induced damage of eukaryotic

cells, we performed hemolysis assay with human erythrocytes,
because these cells have not been reported to express endogenous

p33, which was also seen in our assays (Fig. 5A). Purified human
erythrocytes were incubated with AMPs, cells were then pelleted,
and the release of hemoglobin from lysed erythrocytes was mea-
sured in the supernatants. Fig. 6A shows that LL37 and β-defensin
3 had hemolytic activity, whereas HKH20, NAT26, GGL27, and
the β-defensins 2 and 4 showed no effect. Importantly, the activity
of the two hemolytic AMPs was decreased to baseline levels when preincubated with p33, and it was found that the effect of AMPs and p33 was dose dependent (data not shown). Similar results were obtained when the effect of AMPs on endothelial cells was studied. EA.hy926 cells were incubated with different AMPs, and membrane disruption was monitored by measurement of LDH leakage into the cell medium. As shown in Fig. 6B, LL37 and β-defensin 3 caused significant cell damage; also in these experiments, preincubation with p33 abolished the cytotoxic effect of these peptides.

Endogenous p33 colocalizes with LL37 and β-defensin 3 on the surface of endothelial cells

To test whether endogenous p33 on endothelial cells interacts with LL37 and β-defensin 3, we prepared membrane fractions as described in Materials and Methods. These fractions were then incubated with low concentrations of LL37 and β-defensin 3 (2.5 μM) followed by immunodetection with gold-labeled Abs against the two peptides (10 nm) and p33 (5 nm). Ab binding was visualized by negative staining electron microscopy. Fig. 7A and 7B il-
lustrate that at low concentration, LL37 and β-defensin 3 bind in close proximity to p33, and that this interaction did not cause membrane damage. When using higher concentrations (20 μM) of LL37 and β-defensin 3, we noticed that the peptides could also attach to the membrane without binding to p33 (Fig. 7C, 7D). This interaction led to membrane perforation leading to released blebs, suggesting that once the levels of LL37 and β-defensin 3 exceed the concentration of endogenous p33, endothelial cells are not any longer protected from a cytolytic attack and started to become perforated.

Taken together, our data suggest that p33 has an important regulatory function in the early immune response to infection because it prevents the otherwise self-destructive activity of AMPs against host cells.

**Discussion**

Today, it is generally believed that many, if not most, peptides with antimicrobial activity are multifunctional, and they are therefore also referred to as host defense peptides (34). LL37, as well as α- and β-defensins, are probably the best characterized AMPs and apart from their ability to kill a huge variety of bacterial species, they have been shown to be proinflammatory and anti-inflammatory mediators, promote wound healing, and play important roles in chemotaxis, fertility, and cancer (for reviews, see Refs. 35–37). Under pathological conditions, AMP levels can become upregulated and their overexpression has been correlated with clinical complications such as psoriasis, circulatory derangement in severe infectious diseases, chronic obstructive pulmonary disease, and tumorigenesis (11, 38–40). However, thus far, not much is known about whether the human host has established mechanisms that can counteract these deleterious side effects.

In this study, we show that p33, a multicompartmental negatively charged protein, binds to a series of AMPs and is able to neutralize their lytic activity, thereby protecting eukaryotic cells from AMP-mediated lysis. To study the interaction between p33 and AMPs, we characterized the protein–protein interaction at the molecular level and correlated these findings with the physiological function of p33. To this end, we performed ELISA, competitive binding studies, and electron microscopy analyses to identify p33-binding AMPs and map AMP-binding sites in p33. Our results show that p33 binds to many, but not all, AMPs tested. By using synthetic peptides, we found that the main interaction site in p33 is located at an epitope that is close to the N terminus of the protein that forms a β-sheet structure, but other binding epitopes also were identified. Interestingly, all interaction sites are located in close proximity when looking at the three-dimensional structure of p33 (Fig. 3B), suggesting that the AMP binding site is formed by a discontinuous epitope. Our results further revealed that p33 is efficient in neutralizing the lytic effect of AMPs toward bacteria and eukaryotic cells, which raises the question about p33’s physiological function. The neutralization of the antimicrobial effect of AMPs would be counterproductive and seems therefore unlikely. This is also further supported by our findings that we were unable to detect soluble p33 in plasma samples from healthy donors or sepsis patients. We therefore believe that p33 has an important function as membrane-bound protein where it protects eukaryotic cells from an AMP attack. For example, LL37 and β-defensin 3 were able to lyse endothelial cells and erythrocytes in our experiments, and the addition of p33 was able to prevent this damage. Both AMPs (LL37 in form of its cathelicidin precursor) are stored in blood cells including neutrophils, monocytes, and thrombocytes, and can be released upon stimulation (40, 41).

Our results show that p33 is expressed on endothelial cells and in the endothelial lining of mice lungs, which is a common site for

**FIGURE 7.** Endogenous p33 colocalizes with LL37 and β-defensin 3.
Electron microscopic analysis of cell membranes treated with low dose (2.5 μM; A) or high dose (20 μM; B) of LL37. Membrane damage after incubation with high-dose LL37 is indicated by the formation of blebs, which are secreted from the plasma membrane. (C and D) Membrane damage caused by treatment with low (2.5 μM) or high dose (20 μM) of β-defensin 3 is shown. Endogenous p33 was immunostained by using a gold-labeled (5 nm gold) polyclonal Ab, whereas LL37 (A, B) and β-defensin 3 (C, D) were labeled with 10 nm gold particles. Scale bar, 50 nm.
bacterial infection and increased antimicrobial activity. Under in-
fec tious conditions, neutrophils attach to endothelial cells to breach
the vascular barrier and reach the site of infection. This is a critical
step, because adhesion and transmigration trigger activated neu-
tr ophils to degranulate and release their content in close proximity
to the endothelial cells. As a consequence, endothelial cells will be
exposed to high levels of AMPs including, for instance, LL37, which
can upregulate p33 on their surface under inflammatory conditions. It is therefore tempting to speculate
that this presents a self-defense mechanism to protect cell damage
caused by adhering neutrophils or platelets. Taken together, our
results suggest that p33 has an important function in the early
immune response to infection because it helps AMPs to attack
the invading pathogen without causing harm to host cells.

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Disclosures

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Supplemental data

Figure 1. **(panel A)** **MBP-p33 forms a trimer and binds to up to three AMPs.** p33 molecules in the absence of a ligand or bound to gold-labeled AMPs are depicted. Arrowheads point to gold labeled AMPs (last image for each AMP). Scale bar 15 nm. **(panel B)** **Native p33 binds to AMPs.** Microtiter plates were coated overnight with 5 μM AMPs and probed with 15 nM native p33. Binding was detected with a polyclonal antibody against MBP-p33 and visualized by a peroxidase-conjugated secondary antibody. One representative experiment out of three is shown. **(panel C)** **Native p33 inhibits the cytolytic activity of LL37 against erythrocytes.** AMPs (40 μM) were incubated (60 min at 37°C) with washed and diluted human erythrocytes (5% v/v) in the absence or presence of 10 μM p33. Samples were centrifuged and the release of hemoglobin was measured in the supernatant at 540 nm. Hemolysis was calculated as percentage of cells treated with Tox-7 Lysis buffer. ***, p < 0.001.** Data are mean ± SE values from three separate experiments.

Figure 2. **Mapping of the AMP binding sites in p33.** Microtiterplates were coated with 5 μM AMPs followed by an incubation with MBP-p33 in the presence of one p33-derived peptide (10 μM) (see table II). Binding of MBP-p33 to the AMP was detected with a specific antibody against p33 and a secondary peroxidase-conjugated antibody. Inhibition by p33-derived peptide is shown as “% decrease” of absorbance compared to MBP-p33 alone. Signals above 100% are limited to 100%.