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(71) Applicant (for all designated States except US): **XIM-MUNE AB** [SE/SE]; Ole Romers vägen 16, Ideon, S-223 70 Lund (SE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **KALLE, Martina** [DE/SE]; Stilgjutaregatan 2, S-227 36 Lund (SE). **MALMSTEN, Nils Martin** [SE/SE]; Enstavägen 33, S-185 35 Täby (SE). **PAPAREDDY, Praveen** [IN/SE]; Ramesh Tati, Kännärsvägen 2 rum 112, S-226 45 Lund (SE). **RYDENGÅRD, Victoria** [SE/SE]; Fredshögsvägen 28, S-235 91 Vellinge (SE). **SCHMIDTCHEN, Artur** [SE/SE]; Göingegatan 4, S-222 41 Lund (SE). **WALSE, Björn Ulrik** [SE/SE]; Skyttelinjen 130, S-226 49 Lund (SE).

(74) Agent: **SMITH, Stephen**; Potter Clarkson LLP, Park View House, 58 The Ropewalk, Nottingham NG1 5DD (GB).

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(54) Title: POLYPEPTIDES AND USES THEREOF

(57) Abstract: The present invention provides polypeptides comprising or consisting of an amino acid sequence derived from a naturally occurring protein which modulates blood coagulation, or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, for use in the treatment or prevention of inflammation and/or excessive coagulation of the blood. Related aspects of the invention provide isolated polypeptides comprising or consisting of an amino acid sequence of SEQ ID NOs: 1 to 11, or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which exhibit an anti-inflammatory and/or anti-coagulant activity, together with isolated nucleic acid molecules, vectors and host cells for making the same. Additionally provided are pharmaceutical compositions comprising a polypeptide of the invention, as well as methods of use of the same in the treatment and/or prevention of inflammation and/or excessive coagulation.



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POLYPEPTIDES AND USES THEREOFField of the invention

10 The present invention relates to novel polypeptides derived from naturally occurring proteins which modulates blood coagulation and their use in the treatment and prevention of inflammation and/or excessive coagulation. In particular, the invention provides polypeptides comprising or consisting of an amino acid sequence of SEQ ID NOs: 1 to 11, or a fragment, variant, fusion or derivative thereof, or a fusion of said
15 fragment, variant or derivative thereof, for use in medicine, e.g. the treatment or prevention of inflammation and/or excessive coagulation.

Introduction

20

The innate immune system, largely based on antimicrobial peptides, provides a first line of defense against invading microbes (Lehrer, R.I. and T. Ganz, Curr Opin Hematol, 2002. **9**(1): p. 18-22; Harder, J., R. Glaser, and J.M. Schröder, J Endotoxin Res, 2007. **13**(6): p. 317-38; Zasloff, M., Nature, 2002. **415**(6870): p. 389-95; Tossi, A., L. Sandri, and A. Giangaspero, Biopolymers, 2000. **55**(1): p. 4-30; Yount, N.Y., et al., Biopolymers, 2006). During recent years it has become increasingly evident that
25 many cationic and amphipathic antimicrobial peptides, such as defensins and cathelicidins, are multifunctional, also mediating immunomodulatory roles and angiogenesis (Zanetti, M., J Leukoc Biol, 2004. **75**(1): p. 39-48; Elsbach, P., J Clin Invest, 2003. **111**(11): p. 1643-5; Ganz, T., *Defensins: antimicrobial peptides of innate immunity*. Nat Rev Immunol, 2003. **3**(9): p. 710-20), thus motivating the recent and broader definition host defense peptides (HDP) for these members of the innate immune system. The family of HDPs has recently been shown to encompass various
30 bioactive peptides with antimicrobial activities, including proinflammatory and chemotactic chemokines (Cole, A.M., et al., J Immunol, 2001. **167**(2): p. 623-7), neuropeptides (Brogden, K.A., Nat Rev Microbiol, 2005. **3**(3): p. 238-50), peptide

hormones (Kowalska, K., D.B. Carr, and A.W. Lipkowski, Life Sci, 2002. **71**(7): p. 747-50; Mor, A., M. Amiche, and P. Nicolas, Biochemistry, 1994. **33**(21): p. 6642-50), growth factors (Malmsten, M., et al., Growth Factors, 2007. **25**(1): p. 60-70), the anaphylatoxin peptide C3a (Nordahl, E.A., et al., Natl Acad Sci U S A, 2004. **101**(48): p. 16879-84; Pasupuleti, M., et al., J Biol Chem, 2007. **282**(4): p. 2520-8), and kininogen-derived peptides (Frick, I.M., et al., Embo J, 2006. **25**(23): p. 5569-78; Nordahl, E.A., et al., *Domain 5 of high molecular weight kininogen is antibacterial*. J Biol Chem, 2005. **280**(41): p. 34832-9; Rydengard, V., E. Andersson Nordahl, and A. Schmidtchen, *Zinc potentiates the antibacterial effects of histidine-rich peptides against Enterococcus faecalis*. Febs J, 2006. **273**(11): p. 2399-406).

The coagulation cascade also represents a fundamental system activated in response to injury and infection (Davie, E.W. and J.D. Kulman, Semin Thromb Hemost, 2006. **32 Suppl 1**: p. 3-15; Bode, W., Semin Thromb Hemost, 2006. **32 Suppl 1**: p. 16-31). Through a series of cascade-like proteinase activation steps, thrombin is formed, leading to fibrinogen degradation and clot formation. The coagulation cascade is controlled by various regulatory proteins, such as the serine proteinase inhibitors (or "serpins") heparin cofactor II (HCII), antithrombin III (ATIII) and protein C inhibitor, as well as by tissue factor proteinase inhibitor (TFPI). Furthermore, histidine-rich glycoprotein may modulate coagulation by interacting with fibrinogen as well as plasminogen.

The present invention seeks to provide new polypeptide agents, derived from molecules involved in hemostasis, for use in medicine, for example in the treatment or prevention of inflammation and/or excessive coagulation.

Summary of the invention

A first aspect of the invention provides a polypeptide comprising or consisting of an amino acid sequence derived from a naturally occurring protein which modulates blood coagulation (other than heparin cofactor II or thrombin), or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, for use in the treatment or prevention of inflammation and/or excessive coagulation, wherein the fragment, variant, fusion or derivative exhibits an anti-inflammatory and/or anti-coagulant activity.

The invention derives from the unexpected discovery by the inventors that naturally occurring proteins which modulate blood coagulation comprise "cryptic peptides" within their C-terminal, and/or other internal regions, which exhibit anti-inflammatory activity. It is believed that such peptides may be 'released' by cleavage of the parent peptidase holoprotein in response to wounding and other physiological challenges. Thus, the polypeptides of the invention constitute a novel and previously undisclosed class of HDPs, which have therapeutic potential against disorders and conditions associated with inflammation.

By "naturally occurring protein which modulates blood coagulation" we include all naturally occurring proteins which modulates blood coagulation which modulate, either positively or negatively, the blood coagulation process. Such modulatory activity may be determined by methods well known in the art, for example using the activated partial thromboplastin time (aPTT) test, prothrombin time (PT) test or the thrombin clotting time (TCT) test. Furthermore, specific measurements of prekallikrein activation or the activity of Factor X and other coagulation factors may be performed. It will be appreciated by persons skilled in the art that the naturally occurring protein may modulate blood coagulation directly or indirectly.

Advantageously, the naturally occurring protein which modulates blood coagulation is a human protein.

By an amino acid sequence "derived from" a naturally occurring protein which modulates blood coagulation, we mean that the amino acid sequence is found within the amino acid sequence of the naturally occurring protein. For example, in one embodiment the amino acid sequence may be from the C-terminal region of a naturally occurring protein which modulates blood coagulation. By "C-terminal region" we mean that the one hundred amino acids adjacent the C-terminus of the naturally occurring protein. In another embodiment, the amino acid sequence may be from an internal region of up to 100 amino acids of a naturally occurring protein which modulates blood coagulation.

By "anti-inflammatory activity" we mean an ability to reduce or prevent one or more biological processes associated with inflammatory events. Such anti-inflammatory activity of polypeptides may be determined using methods well known in the art, for example by measuring LPS-induced release of pro-inflammatory cytokines from

macrophages (e.g. TNF α , IL-6, IF- γ), or neutrophils (see Examples below). Other relevant assays comprise effects of lipoteichoic acid, zymosan, DNA, RNA, flagellin or peptidoglycan in the above systems as well as determination of regulation at the transcriptional level (e.g. Gene-array, qPCR etc). Furthermore, dendritic cell activation or activation of thrombocytes may also be used as a measure of anti-inflammatory activity.

By "anti-coagulant activity" we mean an ability to increase the prothrombin time (PT), the thrombin clotting time (TCT) and/or the activated partial thromboplastin time (aPTT). Alternatively, peripheral blood mononuclear cells (PBMNC)s can be stimulated by *E. coli* LPS with or without the peptide and tissue factor and clot formation followed after addition of human plasma, or clotting times for whole blood can be measured.

It will be appreciated by persons skilled in the art that the invention encompasses polypeptides comprising or consisting of an amino acid sequence derived from a naturally occurring protein which modulates blood coagulation, as well as fragments, variants, fusions and derivatives of such amino acid sequence which retain an anti-inflammatory activity. Preferably, however, the polypeptide is not a naturally occurring protein, e.g. a holoprotein (although it will, of course, be appreciated that the polypeptide may constitute an incomplete portion or fragment of a naturally occurring protein).

In one embodiment of the polypeptides of the invention, the polypeptide comprises a heparin-binding domain. By "heparin-binding domain" we mean an amino acid sequence within the polypeptide which is capable of binding heparin under physiological conditions. Such sequences often comprise XBBXB and XBBBXXB (where B = basic residue and X = hydrophobic or uncharged residue), or clusters of basic amino acids (XBX, XBBX, XBBBX). Spacing of such clusters with non-basic residues (BXB, BXXB) may also occur. Additionally, a distance of approximately 20 Å between basic amino acids constitutes a prerequisite for heparin-binding.

It will be appreciated by persons skilled in the art that the naturally occurring protein which modulates blood coagulation may be from a human or non-human source. For example, the naturally occurring protein which modulates blood coagulation may be derived (directly or indirectly) from a non-human mammal, such as an ape (e.g.

chimpanzee, bonobo, gorilla, gibbon and orangutan), monkey (e.g. macaque, baboon and colobus), rodent (e.g. mouse, rat) or ungulates (e.g. pig, horse and cow).

In preferred embodiments of the polypeptides of the invention, the naturally occurring protein which modulates blood coagulation is a human protein.

Thus, the naturally occurring protein which modulates blood coagulation may be selected from the group consisting of serine proteinase inhibitors (serpins), tissue factor pathway inhibitors (such as TFPI-1 and TFPI-2) and histidine-rich glycoprotein (HRG).

In one embodiment, the naturally occurring protein which modulates blood coagulation is a serpin.

For example, the serpin may be anti-thrombin III (ATIII; e.g. see Swiss Port Accession No. P01008).

Thus, the polypeptide may comprise or consist of the amino acid sequence of SEQ ID NO:1 or 2:

"FFF21":	FFFAKLNCRLYRKANKSSKLV	[SEQ ID NO:1]
"AKL22":	AKLNCRLYRKANKSSKLVSANR	[SEQ ID NO:2]

or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory activity and/or anti-coagulant of SEQ ID NO:1 or 2.

In an alternative embodiment, the serpin may be protein C inhibitor (PCI; e.g. see Swiss Port Accession No. P05154).

For example, the polypeptide may comprise or consist of the amino acid sequence of SEQ ID NO:3:

"SEK20":	SEKTLRKWLKMFKKRQLELY	[SEQ ID NO:3]
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or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:3.

- 5 In a further embodiment, the naturally occurring protein which modulates blood coagulation is tissue factor pathway inhibitor-1 (TFPI-1; e.g. see Swiss Port Accession No. P10646).

For example, the polypeptide may comprise or consist of the amino acid sequence of
10 any one of SEQ ID NOS:4 to 6:

"GGL27": GGLIKTKRKRKKQRVKIAEYEEIFVKNM [SEQ ID NO:4]

"LIK17": LIKTKRKRKKQRVKIA [SEQ ID NO:5]

"TKR22": TKRKRKKQRVKIAEYEEIFVKNM [SEQ ID NO:6]

15

or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of any one of SEQ ID NOS:4 to 6.

- 20 In a still further alternative embodiment, the naturally occurring protein which modulates blood coagulation is tissue factor pathway inhibitor 2 (TFPI-2; e.g. see Swiss Port Accession No. P48307).

For example, the polypeptide may comprise or consist of the amino acid sequence of
25 SEQ ID NO:7 or 8:

"ALK25": ALKKKKKMPKLRFASRIRKIRKKQF [SEQ ID NO:7]

"EDC34": EDCKRACAKALKKKKKMPKLRFASRIRKIRKKQF [SEQ ID NO:8]

- 30 or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:7 or 8.

In a still further alternative embodiment, the naturally occurring protein which
35 modulates blood coagulation is histidine-rich glycoprotein (HRG; e.g. see Swiss Port Accession No. P04196).

For example, the polypeptide may comprise or consist of the amino acid sequence of SEQ ID NO:9:

$(X_1X_2X_3X_4X_5)_n$ [SEQ ID NO:9],

5

wherein

X_1 and X_4 independently represent G, P, L, I, F, T, V, Y or W;

X_2 , X_3 and X_5 independently represent H, R or K; and

10

'n' is an integer from 2 to 6

or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:9.

15

Thus, the polypeptide may comprise or consist of the amino acid sequence of SEQ ID NO:10:

$(GHHPH)_n$ [SEQ ID NO:10], where 'n' is an integer from 2 to 6

20

or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:10.

25

For example, the polypeptide may comprise or consist of the amino acid sequence of SEQ ID NO:11:

"GHH25": GHHPHGHHPHGHHPHGHHPHGHHPH [SEQ ID NO:11]

30

or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:11.

35

It will be appreciated by persons skilled in the art that the term 'amino acid', as used herein, includes the standard twenty genetically-encoded amino acids and their corresponding stereoisomers in the 'D' form (as compared to the natural 'L' form), omega-amino acids other naturally-occurring amino acids, unconventional amino

acids (e.g., α,α -disubstituted amino acids, N-alkyl amino acids, etc.) and chemically derivatised amino acids (see below).

When an amino acid is being specifically enumerated, such as 'alanine' or 'Ala' or 'A',
5 the term refers to both L-alanine and D-alanine unless explicitly stated otherwise. Other unconventional amino acids may also be suitable components for polypeptides of the present invention, as long as the desired functional property is retained by the polypeptide. For the peptides shown, each encoded amino acid residue, where appropriate, is represented by a single letter designation, corresponding to the trivial
10 name of the conventional amino acid.

In one embodiment, the polypeptides of the invention comprise or consist of L-amino acids.

15 Where the polypeptide comprises an amino acid sequence according to a reference sequence (for example, SEQ ID NOs: 1 to 11), it may comprise additional amino acids at its N- and/or C- terminus beyond those of the reference sequence, for example, the polypeptide may comprise additional amino acids at its N-terminus. Likewise, where the polypeptide comprises a fragment, variant or derivative of an
20 amino acid sequence according to a reference sequence, it may comprise additional amino acids at its N- and/or C- terminus.

In a further embodiment the polypeptide comprises or consists of a fragment of the amino acid sequence according to a reference sequence (for example, SEQ ID NOs:
25 1 to 11). Thus, the polypeptide may comprise or consist of at least 5 contiguous amino acid of the reference sequence, for example at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 contiguous amino acid of SEQ ID NOS: 1 to 11.

In one embodiment the polypeptide fragment commences at an amino acid residue
30 selected from amino acid residues 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 of SEQ ID NO:1. Alternatively/additionally, the polypeptide fragment may terminate at an amino acid residue selected from amino acid residues 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 and of SEQ ID NO:1.

35 It will be appreciated by persons skilled in the art that the polypeptide of the invention may comprise or consist of a variant of the amino acid sequence according to a

reference sequence (for example, SEQ ID NO: 1), or fragment of said variant. Such a variant may be non-naturally occurring.

By 'variants' of the polypeptide we include insertions, deletions and substitutions, either conservative or non-conservative. For example, conservative substitution refers to the substitution of an amino acid within the same general class (e.g. an acidic amino acid, a basic amino acid, a non-polar amino acid, a polar amino acid or an aromatic amino acid) by another amino acid within the same class. Thus, the meaning of a conservative amino acid substitution and non-conservative amino acid substitution is well known in the art. In particular we include variants of the polypeptide which exhibit an anti-inflammatory activity.

In a further embodiment the variant has an amino acid sequence which has at least 50% identity with the amino acid sequence according to a reference sequence (for example, SEQ ID NO: 1) or a fragment thereof, for example at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or at least 99% identity.

The percent sequence identity between two polypeptides may be determined using suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group and it will be appreciated that percent identity is calculated in relation to polypeptides whose sequences have been aligned optimally.

The alignment may alternatively be carried out using the Clustal W program (as described in Thompson *et al.*, 1994, *Nuc. Acid Res.* 22:4673-4680, which is incorporated herein by reference).

The parameters used may be as follows:

Fast pairwise alignment parameters: K-tuple(word) size; 1, window size; 5, gap penalty; 3, number of top diagonals; 5. Scoring method: x percent.

Multiple alignment parameters: gap open penalty; 10, gap extension penalty; 0.05.

Scoring matrix: BLOSUM.

Alternatively, the BESTFIT program may be used to determine local sequence alignments.

In one embodiment, amino acids from the above reference sequences may be mutated in order to reduce proteolytic degradation of the polypeptide, for example by I,F to W modifications (see Strömstedt *et al*, *Antimicrobial Agents Chemother* 2009, 53, 593).

In a further embodiment, the polypeptide comprises or consists of an amino acid sequence from a serpin other than ATIII which corresponds to the sequence of SEQ ID NO: 1 ("FFF21") of ATIII. Examples of such sequences are shown in Table 1 below.

Table 1. Selected regions of human serpin family sequence alignment.
The highlighted region of SEQ ID NO:1 ("FFF21") of anti-thrombin III (P01008) corresponds to amino acids 153-173

20	sp P01008 ANT3_HUMAN	-----KTSDQIH <u>FFFAKLNCRLYR-KANKSSKL</u> VSANRLFGDKSLTFNETYQDISELVYG [SEQ ID NO:12]
	P01011 AACT_HUMAN	-----TSEAEIHQSFQHLRLTL-N-QSSDELQLSMGNAMFVKEQLSLDRFTEDAKRLYG [SEQ ID NO:13]
25	Q86WD7 SPA9_HUMAN	-----TPESAIHQGFQHLVHSL-T-VPSKDLTLKMGSAFLVKKELQLQANFLGNVKRLYE [SEQ ID NO:14]
	tr Q2T9J2 Q2T9J2_HUMAN	-----TPESAIHQGFQHLVHSL-T-VPSKDLTLKMGSAFLVKKELQLQANFLGNVKRLYE [SEQ ID NO:15]
	Q9UIV8 SPB13_HUMAN	EKEVIENTEAVHQQFQKFLTEI-S-KLTNDYELNITNRLFGKTYLFLQKYLDYVEKYHH [SEQ ID NO:16]
30	sp O75635 SPB7_HUMAN	NSS--NSQSGLQSQLKRVSFI-N-ASHKDYDLSIVNGLFAEKVYGFHDKDYIECAEKLYD [SEQ ID NO:17]
	sp O75830 SPI2_HUMAN	-----SAGEEFFVLKSFFSAI-S-EKKQEFTFNLANALYLQEGFTVKEQYLHGNKEFFQ [SEQ ID NO:18]
35	tr Q8TCE1 Q8TCE1_HUMAN	-----KTSDQIHFFFAKLNCRLYR-KANKSSKLVSANRLFGDKSLTF----- [SEQ ID NO:19]
	unk VIRT7736 Blast_submission	-----KTSDQIHFFFAKLNCRLYR-KANKSSKLVSANRLFGDKSLTFNETYQDISELVYG [SEQ ID NO:20]
	sp P01009 A1AT_HUMAN	-----IPEAQIHGEGFQELLRTL-N-QPDSQLQLTTGNGLFLSEGLKLVDFKFLDVKKLYH [SEQ ID NO:21]
40	sp P05120 PAI2_HUMAN	ILQA-QAADKIHSFSLSSAI-N-ASTGNLLESVNKLFGKESASFREEYIRLCQKYYS [SEQ ID NO:22]
	sp P05121 PAI1_HUMAN	-----DKGMAPALRHLYKEL-M-GPWNKDEISTTDAIFVQDLKLVQGFMPHFRLFR [SEQ ID NO:23]
45	sp P05154 IPSP_HUMAN	-----SSEKELHRGFQQLQEL-N-QPRDGFQLSLGNALFTDLVVDLQDTFVSAMKTYL [SEQ ID NO:24]
	sp P05155 IC1_HUMAN	-----TCVHQALKGFTT-----KGVTSVSQIFHSPDLAIRDTFVNASRTLYS [SEQ ID NO:25]
	sp P05543 THBG_HUMAN	-----TPMVEIQHGFQHLICSL-N-FPKKELELQIGNALFIGKHLKPLAKFLNDVKTLYE [SEQ ID NO:26]
50	sp P61640 THBG_PANTR	-----TPMVEIQHGFQHLICSL-N-FPKKELELQIGNALFIGKHLKPLAKFLNDVKTLYE [SEQ ID NO:27]
	tr Q8IVC0 Q8IVC0_HUMAN	-----YEITTIHNLFRKLTHRL-F-RRNFGYTLRSVNDLYIQKQFPILLDFKTKVREYYF [SEQ ID NO:28]
55	sp P07093 GDN_HUMAN	-----GVGKILKKINKAI-V-SKKNKDIVTVANAVFVNASEIEVPFVTRNKDVFFQ [SEQ ID NO:29]
	sp P08697 A2AP_HUMAN	-----SGPCLPHLLSRL-C-QDLGPGAFRLAARMYLQKGFPIKEDFLEQSEQLFG [SEQ ID NO:30]
	tr Q8N5U7 Q8N5U7_HUMAN	-----SGPCLPHLLSRL-C-QDLGPGAFRLAARMYLQKGFPIKEDFLEQSEQLFG [SEQ ID NO:31]
60	sp P20848 A1ATR_HUMAN	-----TPEAKIHECFQVQLQAL-S-RPDTRLQLTTGSSSLFVNKSMKLVDTFLEDTKKLYH [SEQ ID NO:32]

	sp P29508 SPB3_HUMAN	TYHV-DRSGNVHHQFQKLLTEF-N-KSTDAYELKIANKLFGKETYQLQOEYLDAIKKFYQ [SEQ ID NO:33]
	sp P48594 SPB4_HUMAN	TYHV-DRSGNVHHQFQKLLTEF-N-KSTDAYELKIANKLFGKETYQLQOEYLDAIKKFYQ [SEQ ID NO:34]
5	tr Q5K634 Q5K634_HUMAN	TYHV-DRSGNVHHQFQKLLTEF-N-KSTDAYELKIANKLFGKETYQLQOEYLDAIKKFYQ [SEQ ID NO:35]
	tr Q5K684 Q5K684_HUMAN	TYHV-DRSGNVHHQFQKLLTEF-N-KSTDAYELKIANKLFGKETYQLQOEYLDAIKKFYQ [SEQ ID NO:36]
10	tr Q9BYF7 Q9BYF7_HUMAN	TYHV-DRSGNVHHQFQKLLTEF-N-KSTDAYELKIANKLFGKETYQLQOEYLDAIKKFYQ [SEQ ID NO:37]
	sp P29622 KAIN_HUMAN	-----LSESDVHRGFQHLHTL-N-LPGHGLETRVGSALFLSHNLKFLAKFLNDTMAVYE [SEQ ID NO:38]
	sp P30740 ILEU_HUMAN	-----EVHSRFSQSLNADI-N-KRGASYILKLANRLYGEKTYNPLPEFLVSTQKTYG [SEQ ID NO:39]
15	sp P36952 SPB5_HUMAN	-----KDIPFGFQTVTSDV-N-KLSSFYSLKLIKRLYVDKSLNLSTEFISSTKRPYA [SEQ ID NO:40]
	sp P48595 SPB10_HUMAN	EFNL-SNSEEIHSDFTLISEI-L-KPNDYLLKTANAIYGEKTYAFHNKYLEDMKTYFG [SEQ ID NO:41]
20	sp P50453 SPB9_HUMAN	-----EEDIHRAFQSLLETV-N-KAGTQYLLRTANRLFGKCTCQFLSTFKESCQLQFYH [SEQ ID NO:42]
	tr Q5TD03 Q5TD03_HUMAN	-----EEDIHRAFQSLLETV-N-KAGTQYLLRTANRLFGKCTCQFLSTFKESCQLQFYH [SEQ ID NO:43]
	sp P50454 SERPH_HUMAN	-----DEEVHAGLGGELLRLS-SNSTARNVTWKLGSRLYGPSSVSFADDFVRSSKQHYN [SEQ ID NO:44]
25	sp Q6UXR4 SPA13_HUMAN	-----PEEEIQEGFWDLLIRL-R-GQGPRLLLTMDQRRFSGLGARAN----- [SEQ ID NO:45]
	sp Q86U17 SPA11_HUMAN	-----PEADIHQGFRSLHTL-A-LSPKLELKVGNLFLDKRLKPRQHYLDSIKELYG [SEQ ID NO:46]
30	sp Q8IW75 SPA12_HUMAN	-----KDLHEGFHYIIHEL-T-QKTQDLKLSIGNTLFIDQRLQPKRKFLDAKQNFYS [SEQ ID NO:47]
	sp Q96P15 SPB11_HUMAN	SPKC-SQAGRIHSEFGVEFSQI-N-QPDSNCTLSIANRLYGTMTAFHQYLLSCSEKQYQ [SEQ ID NO:48]
	sp Q96P63 SPB12_HUMAN	GSLN-NESGLVSCYFGQLLSKL-D-RIKTDYTLNRLYGEQEPICQEYLDGVIQFYH [SEQ ID NO:49]
35	sp Q99574 NEUS_HUMAN	-----EE--FSFLKEFSN-MV-T-AKESQYVMKIANSLFVQNGFHVNEEFLQMMKKYFN [SEQ ID NO:50]
	sp Q9UK55 ZPT_HUMAN	-----KPGLLPFLFKGLR-ET-L-SRNLELGLTQGSFAFIHKDFDVKETFFNLSKRYFD [SEQ ID NO:51]
40	tr A5Z2A5 A5Z2A5_HUMAN	-----KPGLLPFLFKGLR-ET-L-SRNLELGLTQGSFAFIHKDFDVKETFFNLSKRYFD [SEQ ID NO:52]
	sp P35237 SPB6_HUMAN	-----GGGGDIHQGFQSLLETV-N-KTGTQYLLRVANRLFGKESCDPLSSFRDSCQKQFYQ [SEQ ID NO:53]
	sp P50452 SPB8_HUMAN	-----KDGDHHRGFQSLLETV-N-RTGTQYLLRTANRLFGKCTCDPLPDFKEYCQKQFYQ [SEQ ID NO:54]
45	tr Q8N178 Q8N178_HUMAN	-----KDGDHHRGFQSLLETV-N-RTGTQYLLRTANRLFGKCTCDPLPDFKEYCQKQFYQ [SEQ ID NO:55]

50

In an alternative embodiment, the polypeptide comprises or consists of an amino acid sequence from a serpin other than PCI which corresponds to the sequence of SEQ ID NO: 3 ("SEK20") of PCI. Examples of such sequences are shown in Table 2 below.

55

Table 2. Selected regions of human serpin family sequence alignment.
The highlighted region of SEQ ID NO:3 ("SEK20") of anti-thrombin III (P015154) corresponds to amino acids 283-302

60

	sp P05154 IPSP_HUMAN	VPYQGNATALFI-LPSE-----GKMQQVENGLSEKTLRKWLK---MFKK---RQL-ELY [SEQ ID NO:56]
65	P01011 AACT_HUMAN	LKYTGNASALFIL-PDQ-----DKMEEVEAMLLPETLKRWRD---SLEF---REIGELY [SEQ ID NO:57]
	Q86WD7 SPA9_HUMAN	MDYKGDVAFFVL-PSK-----GKMRQLEQALSARTLRKWSH---SIQK---RWI-EVF

			[SEQ ID NO:58]
	tr Q2T9J2 Q2T9J2_HUMAN	MDYKGDVAFFVL-PSK-----GKMRQLEQALSARTLRKWSH---SLQK----RWI-EVF	[SEQ ID NO:59]
5	Q9UIV8 SPB13_HUMAN	IPYKNDLSMFVLLPNDI---DGLEKIIDKISPEKLVEWTS-PS-GHME-----RKV-NLH	[SEQ ID NO:60]
	sp 075635 SPB7_HUMAN	LRYNGG-INMYVLLPE-----NDLSEIENKLTQNLMEWTNP-RRMTS----KYV-EVF	[SEQ ID NO:61]
	sp 075830 SPI2_HUMAN	LSYKGDDEFSLIILPAEG---MDIEEVEKLITAAQILKWLS---EMQE-----EEV-EIS	[SEQ ID NO:62]
10	sp P01008 ANT3_HUMAN	LPFKGDDITMVLILPKPE---KSLAKVEKELTPEVLQEWLD---ELEE-----MML-VVH	[SEQ ID NO:63]
	unk VIRT7736 Blast_submission	LPFKGDDITMVLILPKPE---KSLAKVEKELTPEVLQEWLD---ELEE-----MML-VVH	[SEQ ID NO:64]
	sp P01009 A1AT_HUMAN	MKYLGNATAIF-FLPDE-----GKLQHLLENELTHDIITKFLE---NEDR-----RSA-SLH	[SEQ ID NO:65]
15	sp P05120 PAI2_HUMAN	LPYAGD-VSMFLLLPDEIADVSTGLELLESEITYDKLNKWTSK-DKMAE---DEV-EVY	[SEQ ID NO:66]
	sp P05121 PAI1_HUMAN	LPYHGDLSMFIAAPYEKE---VPLSALTNILSAQLISHWKG---NMTR-----LPR-LLV	[SEQ ID NO:67]
20	sp P05155 IC1_HUMAN	LQLSHN-LSLVILVLPQNLK---HRLDMEQALSPSVFKAIMEKLEMSKF---QPT-LLT	[SEQ ID NO:68]
	sp P05543 THBG_HUMAN	MDYSKNALALFVL-PKE-----GQMESVEAAMSSSKTLKKWNR---LLQK---GWV-DLF	[SEQ ID NO:69]
25	sp P61640 THBG_PANTR	MDYSKNALALFVL-PKE-----GQMESVEAAMSSSKTLKKWNR---LLQK---GWV-DLF	[SEQ ID NO:70]
	tr Q8IVC0 Q8IVC0_HUMAN	LEYVGG-ISMLIVVPHKM---SGMKTLEAQLTPGVVERWQK---SMTN---RTR-EVL	[SEQ ID NO:71]
	sp P07093 GDN_HUMAN	LPYHGESISMLIALPTESS---TPLSAIIPHISTKTIDSWMS---IMVP---KRV-QVI	[SEQ ID NO:72]
30	sp P08697 A2AP_HUMAN	FPPKKNMS-FVVLVPTHFE---WNVSQVLANLSWDTLHP-----PLVWE---RPT-KVR	[SEQ ID NO:73]
	tr Q8N5U7 Q8N5U7_HUMAN	FPPKKNMS-FVVLVPTHFE---WNVSQVLANLSWDTLHP-----PLVWE---RPT-KVR	[SEQ ID NO:74]
	sp P20848 A1ATR_HUMAN	QHYVGNATAFFIL-PDP-----KKMWQLEEKLTYSHLENIQR---AFDI---RSI-NLH	[SEQ ID NO:75]
35	sp P29508 SPB3_HUMAN	IPYKGDLSMIVLLPNEI---DGLQKLEEKLTAEKLMEWTS-L-QNMRE---TRV-DLH	[SEQ ID NO:76]
	sp P48594 SPB4_HUMAN	IPYKGDLSMIVLLPNEI---DGLQKLEEKLTAEKLMEWTS-L-QNMRE---TCV-DLH	[SEQ ID NO:77]
40	tr Q5K634 Q5K634_HUMAN	IPYKGDLSMIVLLPNEI---DGLQKLEEKLTAEKLMEWTS-L-QNMRE---TRV-DLH	[SEQ ID NO:78]
	tr Q5K684 Q5K684_HUMAN	IPYKGDLSMIVLLPNEI---DGLQKLEEKLTAEKLMEWTS-L-QNMRE---TCV-DLH	[SEQ ID NO:79]
45	tr Q9BYF7 Q9BYF7_HUMAN	IPYKGDLSMIVLLPNEI---DGLQKLEEKLTAEKLMEWTS-L-QNMRE---TCV-DLH	[SEQ ID NO:80]
	sp P29622 KAIN_HUMAN	MDYKGDATVFFI-LPNQ-----GKMREIEEVLTPPEMLMRWNN---LLRKRNFYKKL-ELH	[SEQ ID NO:81]
	sp P30740 ILEU_HUMAN	LPYQGEELSMVILLPDDIEDSTGLKKIEEQLTLEKLHEWTKP-ENLDF---IEV-NVS	[SEQ ID NO:82]
50	sp P36952 SPB5_HUMAN	LPFQNKHLSMFILLPKDVEDESTGLEKIEKQLNSESLSQWTPN-STMAN---AKV-KLS	[SEQ ID NO:83]
	sp P48595 SPB10_HUMAN	LYYKSRDLSLLILLPE---DINGLEQLEKAITYEKLNWTS-A-DMMEL---YEV-QLH	[SEQ ID NO:84]
55	sp P50453 SPB9_HUMAN	LPYARKESLLVLLPDD---GVELSTVEKSLTFEKLTAWTKP-DCMKS---TEV-EVL	[SEQ ID NO:85]
	tr Q5TD03 Q5TD03_HUMAN	LPYARKESLLVLLPDD---GVELSTVEKSLTFEKLTAWTKP-DCMKS---TEV-EVL	[SEQ ID NO:86]
	sp P50454 SERPH_HUMAN	MPLAHKLSSLIILMPHH---VEPLERLEKLLTKEQLKIWMG---KMOK---KAV-AIS	[SEQ ID NO:87]
60	sp Q6UXR4 SPA13_HUMAN	MDHAGNTTTFI-FPNR-----GKMRHLEDALLPETLIKWDS---LLRT---REL-DFH	[SEQ ID NO:88]
	sp Q86U17 SPA11_HUMAN	IEYRGNALALLV-LPDP-----GKMKQVEAALQPQTLRKWGQ---LLLP---SLL-DLH	[SEQ ID NO:89]
65	sp Q8IW75 SPA12_HUMAN	IPYQKN-ITAIFILPDE-----GKLKHLEKGLQVDTFSRWKT---LLSR---RVV-DVS	[SEQ ID NO:90]
	sp Q96P15 SPB11_HUMAN	LPYVNNKLSMIILLPV---GIANLKQIEKQLNSGTFHEWTSS-SNMME---REV-EVH	[SEQ ID NO:91]
	sp Q96P63 SPB12_HUMAN	MRYTKGKLSMFVLLPSHSDNLKGLEELERKITYEKMVAWSSS-ENMSE---ESV-VLS	[SEQ ID NO:92]
70	tr Q3SYB5 Q3SYB5_HUMAN	MRYTKGKLSMFVLLPSHSDNLKGLEELERKITYEKMVAWSSS-ENMSE---ESV-VLS	[SEQ ID NO:93]
	sp Q99574 NEUS_HUMAN	IPYEGDEISMML-VLSRQ---EVPLATLEPLVKAQLVEEWAN---SVKK---QKV-EVY	[SEQ ID NO:94]
75	sp Q9UK55 ZPI_HUMAN	LPYQGNATMLVV-LMEKM---GDHLALEDYLTDDLVTETWLR---NMKT---RNM-EVF	[SEQ ID NO:95]
	tr A5Z2A5 A5Z2A5_HUMAN	LPYQGNATMLVV-LMEKM---GDHLALEDYLTDDLVTETWLR---NMKT---RNM-EVF	[SEQ ID NO:96]

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sp|P35237|SPB6_HUMAN      LPYVGKELNMIIMLPDE---TTDLRTVEKELTYEKFVEWTRL-DMMDE---EEV-EVS
                               {SEQ ID NO:97}
tr|Q8IXH2|Q8IXH2_HUMAN    LPYVGKELNMIIMLPDE---TTDLRTVEKELTYEKFVEWTRL-DMMDE---EEV-EVS
                               {SEQ ID NO:98}
5  sp|P50452|SPB8_HUMAN    LPYVEEELSMVILLPDD---NTDLAVVEKALTYEKFKAWTNS-EKLTQ---SKV-QVF
                               {SEQ ID NO:99}
tr|Q8N178|Q8N178_HUMAN    LPYVEEELSMVILLPDD---NTDLAVKE-----
                               {SEQ ID NO:100}

```

10

By a sequence which "corresponds" to SEQ ID NO: 1 or 3, in relation to Tables 1 and 2 above, we include that the polypeptide corresponds to the equivalent amino acid sequence within a different human serpin, *i.e.* which polypeptide exhibits the maximum sequence identity with SEQ ID NO: 1 or 3 (for example, as measured by a GAP or BLAST sequence comparison). Typically, the corresponding polypeptide will be the same length as the reference sequence (*i.e.* SEQ ID NO: 1 or 3).

15

Variants may be made using the methods of protein engineering and site-directed mutagenesis well known in the art using the recombinant polynucleotides (see example, see *Molecular Cloning: a Laboratory Manual*, 3rd edition, Sambrook & Russell, 2000, Cold Spring Harbor Laboratory Press, which is incorporated herein by reference).

20

In one embodiment, the polypeptide comprises or consists of an amino acid which is a species homologue of any one of the above amino acid sequences (*e.g.* SEQ ID NOS: 1 to 11). By "species homologue" we include that the polypeptide corresponds to the same amino acid sequence within an equivalent protein from a non-human species, *i.e.* which polypeptide exhibits the maximum sequence identity with of any one of SEQ ID NOS: 1 to 11 (for example, as measured by a GAP or BLAST sequence comparison). Typically, the species homologue polypeptide will be the same length as the human reference sequence (*i.e.* SEQ ID NOS: 1 to 11).

25

30

In a still further embodiment, the polypeptide comprises or consists of a fusion protein.

35

By 'fusion' of a polypeptide we include an amino acid sequence corresponding to a reference sequence (for example, SEQ ID NO: 1, or a fragment or variant thereof) fused to any other polypeptide. For example, the said polypeptide may be fused to a polypeptide such as glutathione-S-transferase (GST) or protein A in order to facilitate purification of said polypeptide. Examples of such fusions are well known to those skilled in the art. Similarly, the said polypeptide may be fused to an oligo-histidine

40

tag such as His6 or to an epitope recognised by an antibody such as the well-known Myc tag epitope. In addition, fusions comprising a hydrophobic oligopeptide end-tag may be used. Fusions to any variant or derivative of said polypeptide are also included in the scope of the invention. It will be appreciated that fusions (or variants
5 or derivatives thereof) which retain desirable properties, such as an anti-inflammatory activity, are preferred.

The fusion may comprise a further portion which confers a desirable feature on the said polypeptide of the invention; for example, the portion may be useful in detecting
10 or isolating the polypeptide, or promoting cellular uptake of the polypeptide. The portion may be, for example, a biotin moiety, a streptavidin moiety, a radioactive moiety, a fluorescent moiety, for example a small fluorophore or a green fluorescent protein (GFP) fluorophore, as well known to those skilled in the art. The moiety may be an immunogenic tag, for example a Myc tag, as known to those skilled in the art
15 or may be a lipophilic molecule or polypeptide domain that is capable of promoting cellular uptake of the polypeptide, as known to those skilled in the art.

It will be appreciated by persons skilled in the art that the polypeptide of the invention may comprise one or more amino acids that are modified or derivatised, for example
20 by PEGylation, amidation, esterification, acylation, acetylation and/or alkylation.

As appreciated in the art, pegylated proteins may exhibit a decreased renal clearance and proteolysis, reduced toxicity, reduced immunogenicity and an increased solubility [21, 22]. Pegylation has been employed for several protein-based
25 drugs including the first pegylated molecules asparaginase and adenosine deaminase [22, 23].

In order to obtain a successfully pegylated protein, with a maximally increased half-life and retained biological activity, several parameters that may affect the outcome
30 are of importance and should be taken into consideration. The PEG molecules may differ, and PEG variants that have been used for pegylation of proteins include PEG and monomethoxy-PEG. In addition, they can be either linear or branched [24]. The size of the PEG molecules used may vary and PEG moieties ranging in size between 1 and 40 kDa have been linked to proteins [24-27]. In addition, the number of PEG
35 moieties attached to the protein may vary, and examples of between one and six PEG units being attached to proteins have been reported [24, 26]. Furthermore, the presence or absence of a linker between PEG as well as various reactive groups for

conjugation have been utilised. Thus, PEG may be linked to N-terminal amino groups, or to amino acid residues with reactive amino or hydroxyl groups (Lys, His, Ser, Thr and Tyr) directly or by using γ -amino butyric acid as a linker. In addition, PEG may be coupled to carboxyl (Asp, Glu, C-terminal) or sulfhydryl (Cys) groups.

5 Finally, Gln residues may be specifically pegylated using the enzyme transglutaminase and alkylamine derivatives of PEG has been described [25].

It has been shown that increasing the extent of pegylation results in an increased *in vivo* half-life. However, it will be appreciated by persons skilled in the art that the
10 pegylation process will need to be optimised for a particular protein on an individual basis.

PEG may be coupled at naturally occurring disulphide bonds as described in WO 2005/007197. Disulfide bonds can be stabilised through the addition of a
15 chemical bridge which does not compromise the tertiary structure of the protein. This allows the conjugating thiol selectivity of the two sulphurs comprising a disulfide bond to be utilised to create a bridge for the site-specific attachment of PEG. Thereby, the need to engineer residues into a peptide for attachment of to target molecules is circumvented.

20

A variety of alternative block copolymers may also be covalently conjugated as described in WO 2003/059973. Therapeutic polymeric conjugates can exhibit improved thermal properties, crystallisation, adhesion, swelling, coating, pH dependent conformation and biodistribution. Furthermore, they can achieve
25 prolonged circulation, release of the bioactive in the proteolytic and acidic environment of the secondary lysosome after cellular uptake of the conjugate by pinocytosis and more favourable physicochemical properties due to the characteristics of large molecules (e.g. increased drug solubility in biological fluids). Block copolymers, comprising hydrophilic and hydrophobic blocks, form polymeric
30 micelles in solution. Upon micelle disassociation, the individual block copolymer molecules are safely excreted.

Chemical derivatives of one or more amino acids may also be achieved by reaction with a functional side group. Such derivatised molecules include, for example, those
35 molecules in which free amino groups have been derivatised to form amine hydrochlorides, *p*-toluene sulphonyl groups, carboxybenzoxy groups, *t*-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups

may be derivatised to form salts, methyl and ethyl esters or other types of esters and hydrazides. Free hydroxyl groups may be derivatised to form O-acyl or O-alkyl derivatives. Also included as chemical derivatives are those peptides which contain naturally occurring amino acid derivatives of the twenty standard amino acids. For example: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine and ornithine for lysine. Derivatives also include peptides containing one or more additions or deletions as long as the requisite activity is maintained. Other included modifications are amidation, amino terminal acylation (e.g. acetylation or thioglycolic acid amidation), terminal carboxylamidation (e.g. with ammonia or methylamine), and the like terminal modifications.

It will be further appreciated by persons skilled in the art that peptidomimetic compounds may also be useful. Thus, by 'polypeptide' we include peptidomimetic compounds which have an anti-inflammatory activity. The term 'peptidomimetic' refers to a compound that mimics the conformation and desirable features of a particular peptide as a therapeutic agent.

For example, the polypeptides of the invention include not only molecules in which amino acid residues are joined by peptide (-CO-NH-) linkages but also molecules in which the peptide bond is reversed. Such retro-inverso peptidomimetics may be made using methods known in the art, for example such as those described in Meziere *et al.* (1997) *J. Immunol.* **159**, 3230-3237, which is incorporated herein by reference. This approach involves making pseudopeptides containing changes involving the backbone, and not the orientation of side chains. Retro-inverse peptides, which contain NH-CO bonds instead of CO-NH peptide bonds, are much more resistant to proteolysis. Alternatively, the polypeptide of the invention may be a peptidomimetic compound wherein one or more of the amino acid residues are linked by a -y(CH₂NH)- bond in place of the conventional amide linkage.

In a further alternative, the peptide bond may be dispensed with altogether provided that an appropriate linker moiety which retains the spacing between the carbon atoms of the amino acid residues is used; it may be advantageous for the linker moiety to have substantially the same charge distribution and substantially the same planarity as a peptide bond.

It will be appreciated that the polypeptide may conveniently be blocked at its N- or C-terminal region so as to help reduce susceptibility to exoproteolytic digestion.

A variety of uncoded or modified amino acids such as D-amino acids and N-methyl amino acids have also been used to modify mammalian peptides. In addition, a presumed bioactive conformation may be stabilised by a covalent modification, such as cyclisation or by incorporation of lactam or other types of bridges, for example see Veber *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* **75**:2636 and Thursell *et al.*, 1983, *Biochem. Biophys. Res. Comm.* **111**:166, which are incorporated herein by reference.

A common theme among many of the synthetic strategies has been the introduction of some cyclic moiety into a peptide-based framework. The cyclic moiety restricts the conformational space of the peptide structure and this frequently results in an increased specificity of the peptide for a particular biological receptor. An added advantage of this strategy is that the introduction of a cyclic moiety into a peptide may also result in the peptide having a diminished sensitivity to cellular peptidases.

Thus, exemplary polypeptides of the invention comprise terminal cysteine amino acids. Such a polypeptide may exist in a heterodetic cyclic form by disulphide bond formation of the mercaptide groups in the terminal cysteine amino acids or in a homodetic form by amide peptide bond formation between the terminal amino acids. As indicated above, cyclising small peptides through disulphide or amide bonds between the N- and C-terminal region cysteines may circumvent problems of specificity and half-life sometime observed with linear peptides, by decreasing proteolysis and also increasing the rigidity of the structure, which may yield higher specificity compounds. Polypeptides cyclised by disulphide bonds have free amino and carboxy-termini which still may be susceptible to proteolytic degradation, while peptides cyclised by formation of an amide bond between the N-terminal amine and C-terminal carboxyl and hence no longer contain free amino or carboxy termini. Thus, the peptides of the present invention can be linked either by a C-N linkage or a disulphide linkage.

The present invention is not limited in any way by the method of cyclisation of peptides, but encompasses peptides whose cyclic structure may be achieved by any suitable method of synthesis. Thus, heterodetic linkages may include, but are not limited to formation via disulphide, alkylene or sulphide bridges. Methods of

synthesis of cyclic homodetic peptides and cyclic heterodetic peptides, including disulphide, sulphide and alkylene bridges, are disclosed in US 5,643,872, which is incorporated herein by reference. Other examples of cyclisation methods includes cyclization through click chemistry, epoxides, aldehyde-amine reactions, as well as
5 and the methods disclosed in US 6,008,058, which is incorporated herein by reference.

A further approach to the synthesis of cyclic stabilised peptidomimetic compounds is ring-closing metathesis (RCM). This method involves steps of synthesising a peptide
10 precursor and contacting it with an RCM catalyst to yield a conformationally restricted peptide. Suitable peptide precursors may contain two or more unsaturated C-C bonds. The method may be carried out using solid-phase-peptide-synthesis techniques. In this embodiment, the precursor, which is anchored to a solid support, is contacted with a RCM catalyst and the product is then cleaved from the solid
15 support to yield a conformationally restricted peptide.

Another approach, disclosed by D. H. Rich in *Protease Inhibitors*, Barrett and Selveson, eds., Elsevier (1986), which is incorporated herein by reference, has been to design peptide mimics through the application of the transition state analogue
20 concept in enzyme inhibitor design. For example, it is known that the secondary alcohol of statine mimics the tetrahedral transition state of the scissile amide bond of the pepsin substrate.

In summary, terminal modifications are useful, as is well known, to reduce
25 susceptibility by proteinase digestion and therefore to prolong the half-life of the peptides in solutions, particularly in biological fluids where proteases may be present. Polypeptide cyclisation is also a useful modification because of the stable structures formed by cyclisation and in view of the biological activities observed for cyclic
30 peptides.

Thus, in one embodiment the polypeptide of the first aspect of the invention is linear. However, in an alternative embodiment, the polypeptide is cyclic.

It will be appreciated by persons skilled in the art that the polypeptides of the
35 invention may be of various lengths. Typically, however, the polypeptide is between 10 and 200 amino acids in length, for example between 10 and 150, 15 and 100, 15

and 50, 15 and 30, 17 and 30, or 17 and 28 amino acids in length. For example, the polypeptide may be at least 17 amino acids in length.

As stated at the outset, anti-inflammatory activity is a feature common to the polypeptides of the invention. In one embodiment, the polypeptides are capable of inhibiting the release of one or more pro-inflammatory cytokines from human monocyte-derived macrophages, such as monocyte-derived macrophages, including macrophage inhibitory factor, TNF-alpha, HMGB1, C5a, IL-17, IL-8, MCP-1, IFN-gamma, IL-6, IL-1b, IL-12. Antiinflammatory IL-10 may be unaffected or transiently increased.

Other markers may also be affected: These include tissue factor on monocytes and endothelial cells, procalcitonin, CRP, reactive oxygen species, bradykinin, nitric oxide, PGE1, platelet activating factor, arachidonic acid metabolites, MAP kinase activation.

In particular, the polypeptide may exhibit anti-inflammatory activity in one or more of the following models:

- (i) *in vitro* macrophage models using LPS, LTA, zymosan, flagellin, dust mites, viral or bacterial DNA or RNA, or peptidoglycan as stimulants;
- (ii) *in vivo* mouse models of endotoxin shock;
- (iii) *in vivo* infection models, either in combination with antimicrobial therapy, or given alone.

In a further embodiment of the invention, the polypeptide exhibits anticoagulant activity.

By "anti-coagulant activity" we mean an ability to reduce or prevent coagulation (*i.e.* the clotting of blood) or an associated signal or effect. Such activity may be determined by methods well known in the art, for example using the activated partial thromboplastin time (aPTT) test, prothrombin time (PT) test or the thrombin clotting time (TCT) test. Furthermore, specific measurements of prekallikrein activation or the activity of Factor X and other coagulation factors may be performed. It will be appreciated by skilled persons that the polypeptide may inhibit the extrinsic

coagulation pathway and/or the intrinsic coagulation pathway. However, in a preferred embodiment, the polypeptide inhibits (at least in part) the intrinsic coagulation pathway.

5 In a still further embodiment of the invention, the polypeptide exhibits Toll-like receptor (TLR) blocking activity. Such receptor blocking activity can be measured using methods well known in the art, for example by analysis of suitable down-stream effectors, such as iNOS, nuclear factor kappa B and cytokines.

10 By virtue of possessing an anti-inflammatory activity, the polypeptides of the first aspect of the invention are intended for use in the treatment or prevention of inflammation.

By "treatment or prevention of inflammation" we mean that the polypeptide of the
15 invention is capable of preventing or inhibiting (at least in part) one or more symptom, signal or effect constituting or associated with inflammation.

It will be appreciated by persons skilled in the art that inhibition of inflammation may be in whole or in part. In a preferred embodiment, the polypeptide is capable of
20 inhibiting one or more markers of inflammation by 20% or more compared to cells or individuals which have not been exposed to the polypeptide, for example by at least 30%, 40%, 50%, 60%, 70%, 80%, 90% or more.

Advantageously, the polypeptides of the invention are capable of treating or
25 preventing inflammation selectively.

By 'selectively' we mean that the polypeptide inhibits or prevents inflammation to a greater extent than it modulates other biological functions. Preferably, the polypeptide or fragment, variant, fusion or derivative thereof inhibits or prevents
30 inflammation only.

However, in a further embodiment, the polypeptide also (or alternatively) inhibits or prevents coagulation of the blood. As above, it will be appreciated by persons skilled in the art that inhibition of coagulation may be in whole or in part. In a preferred
35 embodiment, the polypeptide is capable of inhibiting one or more measures and/or markers of coagulation by 20% or more compared to cells or individuals which have

not been exposed to the polypeptide, for example by at least 30%, 40%, 50%, 60%, 70%, 80%, 90% or more.

In one embodiment, the polypeptides are for use in the treatment or prevention of inflammation associated with (*i.e.* caused by or merely co-presenting with) an infection.

In preferred but non-limiting embodiments of the invention, the polypeptides are for use in the treatment or prevention of a disease, condition or indication selected from the following:

- i) Acute systemic inflammatory disease, with or without an infective component, such as systemic inflammatory response syndrome (SIRS), ARDS, sepsis, severe sepsis, and septic shock. Other generalized or localized invasive infective and inflammatory disease, including erysipelas, meningitis, arthritis, toxic shock syndrome, diverticulitis, appendicitis, pancreatitis, cholecystitis, colitis, cellulitis, burn wound infections, pneumonia, urinary tract infections, postoperative infections, and peritonitis.
- ii) Chronic inflammatory and or infective diseases, including cystic fibrosis, COPD and other pulmonary diseases, gastrointestinal disease including chronic skin and stomach ulcerations, other epithelial inflammatory and or infective disease such as atopic dermatitis, oral ulcerations (aphthous ulcers), genital ulcerations and inflammatory changes, parodontitis, eye inflammations including conjunctivitis and keratitis, external otitis, mediaotitis, genitourinary inflammations.
- iii) Postoperative inflammation. Inflammatory and coagulative disorders including thrombosis, DIC, postoperative coagulation disorders, and coagulative disorders related to contact with foreign material, including extracorporeal circulation, and use of biomaterials. Furthermore, vasculitis related inflammatory disease, as well as allergy, including allergic rhinitis and asthma..

iv) Excessive contact activation and/or coagulation in relation to, but not limited to, stroke.

v) Excessive inflammation in combination with antimicrobial treatment.

5 The antimicrobial agents used may be administered by various routes; intravenous (iv), intraarterial, intravitreal, subcutaneous (sc), intramuscular (im), intraperitoneal (ip), intravesical, intrathecal, epidural, enteral (including oral, rectal, gastric, and other enteral routes), or topically, (including dermal, nasal application, application in
10 the eye or ear, eg by drops, and pulmonary inhalation). Examples of agents are penicillins, cephalosporins, carbacephems, cephamycins, carbapenems, monobactams, aminoglycosides, glycopeptides, quinolones, tetracyclines, macrolides, and fluoroquinolones. Antiseptic agents include iodine, silver, copper, chlorhexidine, polyhexanide and
15 other biguanides, chitosan, acetic acid, and hydrogen peroxide.

For example, the polypeptides may be for use in the treatment or prevention of an acute inflammation, sepsis, acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), cystic fibrosis, wounds, asthma, allergic and
20 other types of rhinitis, cutaneous and systemic vasculitis, thrombosis and/or disseminated intravascular coagulation (DIC).

In one embodiment, the polypeptide exhibits both anti-inflammatory *and* anti-coagulant activity and may be used in the concomitant treatment or prevention of
25 inflammation and coagulation. Such polypeptides may be particularly suited to the treatment and prevention of conditions where the combined inhibition of both inflammatory and coagulant processes is desirable, such as sepsis, chronic obstructive pulmonary disorder (COPD), thrombosis, DIC and acute respiratory distress syndrome (ARDS). Furthermore, other diseases associated with excessive
30 inflammation and coagulation changes may benefit from treatment by the polypeptides, such as cystic fibrosis, asthma, allergic and other types of rhinitis, cutaneous and systemic vasculitis.

In a further embodiment, the polypeptides of the invention are for use in combination
35 with one or more additional therapeutic agent. For example, the polypeptides of the invention may be administered in combination with antibiotic agents, anti-inflammatory agents, immunosuppressive agents and/or antiseptic agents, as well as

vasoactive agents and/or receptor-blockers or receptor agonists. The antimicrobial agents used may be applied iv, sc, im, intratechal, per os, or topically. Examples of agents are penicillins, cephalosporins, carbacephems, cephamycins, carbapenems, monobactams, aminoglycosides, glycopeptides, quinolones, tetracyclines, macrolides, and fluoroquinolones. Antiseptic agents include iodine, silver, copper, clorhexidine, polyhexanide and other biguanides, chitosan, acetic acid, and hydrogen peroxide. For example, the peptides of the invention may serve as adjuvants to antiseptic treatments, for example silver/PHMB treatment of wounds to quench LPS effects.

Thus, the peptides of the invention may serve as adjuvants (for blocking inflammation) to complement antibiotic, antiseptic and/or antifungal treatments of internal and external infections (such as erysipelas, lung infections including fungal infections, sepsis, COPD, wounds, and other epithelial infections). Likewise, the peptides of the invention may serve as adjuvants to antiseptic treatments, for example silver/PHMB treatment of wounds to quench LPS effects.

In one embodiment, the polypeptides of the invention are for use in combination with a steroid, for example a glucocorticoid (such as dexamethasone).

A second, related aspect of the invention provides an isolated polypeptide comprising or consisting of an amino acid sequence derived from a naturally occurring protein which modulates blood coagulation, or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which polypeptide exhibits an anti-inflammatory and/or anti-coagulant activity, wherein the naturally occurring protein which modulates blood coagulation is selected from the group consisting of serpins (other than heparin cofactor II), histidine-rich glycoprotein (HRG) and tissue factor pathway inhibitors (such as TFPI-1 and TFPI-2), with the proviso that the polypeptide is not a naturally occurring protein (e.g. holoprotein).

By "naturally occurring protein" in this context we mean that the polypeptide is synthesized *de novo*. However, fragments of such naturally occurring holoproteins generated *in vivo* are not excluded.

It will be appreciated by persons skilled in the art that terms such as fragment, variant, fusion or derivative should be construed as discussed above in relation to the first aspect of the invention.

In one embodiment, the polypeptide comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NOS: 1 to 11, or a fragment, variant, fusion or derivative of said sequence, or a fusion of said fragment, variant or derivative thereof. For example, the polypeptide may comprise or consist of an amino acid sequence selected from the group consisting of SEQ ID NOS: 1 to 11.

It will be appreciated by persons skilled in the art that the optional features discussed above in relation to the polypeptides of the first aspect of the invention are also of relevance to the related polypeptides of the second aspect of the invention.

For example, in one preferred embodiment the polypeptide is capable of inhibiting the release of one or more pro-inflammatory cytokines from human monocyte-derived macrophages (such as IL-6, IFN-gamma, TNF-alpha, IL-12, IL-1 and/or IL-18).

In another preferred embodiment, the polypeptide exhibits anticoagulant activity.

The present invention also includes pharmaceutically acceptable acid or base addition salts of the above described polypeptides. The acids which are used to prepare the pharmaceutically acceptable acid addition salts of the aforementioned base compounds useful in this invention are those which form non-toxic acid addition salts, *i.e.* salts containing pharmacologically acceptable anions, such as the hydrochloride, hydrobromide, hydroiodide, nitrate, sulphate, bisulphate, phosphate, acid phosphate, acetate, lactate, citrate, acid citrate, tartrate, bitartrate, succinate, maleate, fumarate, gluconate, saccharate, benzoate, methanesulphonate, ethanesulphonate, benzenesulphonate, p-toluenesulphonate and pamoate [*i.e.* 1,1'-methylene-bis-(2-hydroxy-3 naphthoate)] salts, among others.

Pharmaceutically acceptable base addition salts may also be used to produce pharmaceutically acceptable salt forms of the polypeptides. The chemical bases that may be used as reagents to prepare pharmaceutically acceptable base salts of the present compounds that are acidic in nature are those that form non-toxic base salts with such compounds. Such non-toxic base salts include, but are not limited to those derived from such pharmacologically acceptable cations such as alkali metal cations (e.g. potassium and sodium) and alkaline earth metal cations (e.g. calcium and magnesium), ammonium or water-soluble amine addition salts such as N-

methylglucamine-(meglumine), and the lower alkanolammonium and other base salts of pharmaceutically acceptable organic amines, among others.

It will be appreciated that the polypeptides of the invention may be lyophilised for storage and reconstituted in a suitable carrier prior to use, e.g. through freeze drying, spray drying, spray cooling, or through use of particle formation (precipitation) from supercritical carbon dioxide. Any suitable lyophilisation method (e.g. freeze-drying, spray drying, cake drying) and/or reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilisation and reconstitution can lead to varying degrees of activity loss and that use levels may have to be adjusted upward to compensate. Preferably, the lyophilised (freeze dried) polypeptide loses no more than about 1% of its activity (prior to lyophilisation) when rehydrated, or no more than about 5%, 10%, 20%, 25%, 30%, 35%, 40%, 45%, or no more than about 50% of its activity (prior to lyophilisation) when rehydrated.

Methods for the production of polypeptides of the invention are well known in the art.

Conveniently, the polypeptide is or comprises a recombinant polypeptide. Suitable methods for the production of such recombinant polypeptides are well known in the art, such as expression in prokaryotic or eukaryotic hosts cells (for example, see Sambrook & Russell, 2000, *Molecular Cloning, A Laboratory Manual*, Third Edition, Cold Spring Harbor, New York, the relevant disclosures in which document are hereby incorporated by reference).

Polypeptides of the invention can also be produced using a commercially available *in vitro* translation system, such as rabbit reticulocyte lysate or wheatgerm lysate (available from Promega). Preferably, the translation system is rabbit reticulocyte lysate. Conveniently, the translation system may be coupled to a transcription system, such as the TNT transcription-translation system (Promega). This system has the advantage of producing suitable mRNA transcript from an encoding DNA polynucleotide in the same reaction as the translation.

It will be appreciated by persons skilled in the art that polypeptides of the invention may alternatively be synthesised artificially, for example using well known liquid-phase or solid phase synthesis techniques (such as *t*-Boc or Fmoc solid-phase peptide synthesis).

Thus, included within the scope of the present invention are the following:

(a) a third aspect of the invention provides an isolated nucleic acid molecule which encodes a polypeptide according to the second aspect of the invention;

5

(b) a fourth aspect of the invention provides a vector (such as an expression vector) comprising a nucleic acid molecule according to the third aspect of the invention;

10

(c) a fifth aspect of the invention provides a host cell comprising a nucleic acid molecule according to the third aspect of the invention or a vector according to the fourth aspect of the invention; and

15

(d) a sixth aspect of the invention provides a method of making a polypeptide according to the second aspect of the invention comprising culturing a population of host cells according to the fifth aspect of the invention under conditions in which said polypeptide is expressed, and isolating the polypeptide therefrom.

20

A seventh aspect of the invention provides a pharmaceutical composition comprising a polypeptide according to the first aspect of the invention together with a pharmaceutically acceptable excipient, diluent or carrier.

25

As used herein, 'pharmaceutical composition' means a therapeutically effective formulation for use in the treatment or prevention of disorders and conditions associated with inflammation.

30

As used herein, 'pharmaceutical composition' means a therapeutically effective formulation for use in the treatment or prevention of disorders and conditions associated with inflammation.

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Additional compounds may also be included in the pharmaceutical compositions, such as other peptides, low molecular weight immunomodulating agents, receptor agonists and antagonists, and antimicrobial agents. Other examples include chelating agents such as EDTA, citrate, EGTA or glutathione.

The pharmaceutical compositions may be prepared in a manner known in the art that is sufficiently storage stable and suitable for administration to humans and animals.

The pharmaceutical compositions may be lyophilised, e.g. through freeze drying, spray drying, spray cooling, or through use of particle formation from supercritical particle formation.

- 5 By "pharmaceutically acceptable" we mean a non-toxic material that does not decrease the effectiveness of the biological activity of the active ingredients, *i.e.* the anti-inflammatory polypeptide(s). Such pharmaceutically acceptable buffers, carriers or excipients are well-known in the art (see Remington's Pharmaceutical Sciences, 18th edition, A.R Gennaro, Ed., Mack Publishing Company (1990) and handbook of
10 Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press (2000).

The term "buffer" is intended to mean an aqueous solution containing an acid-base mixture with the purpose of stabilising pH. Examples of buffers are Trizma, Bicine, Tricine, MOPS, MOPSO, MOBS, Tris, Hepes, HEPBS, MES, phosphate, carbonate,
15 acetate, citrate, glycolate, lactate, borate, ACES, ADA, tartrate, AMP, AMPD, AMPSO, BES, CABS, cacodylate, CHES, DIPSO, EPPS, ethanolamine, glycine, HEPPSO, imidazole, imidazolelactic acid, PIPES, SSC, SSPE, POPSO, TAPS, TABS, TAPSO and TES.

20 The term "diluent" is intended to mean an aqueous or non-aqueous solution with the purpose of diluting the peptide in the pharmaceutical preparation. The diluent may be one or more of saline, water, polyethylene glycol, propylene glycol, ethanol or oils (such as safflower oil, corn oil, peanut oil, cottonseed oil or sesame oil).

25 The term "adjuvant" is intended to mean any compound added to the formulation to increase the biological effect of the peptide. The adjuvant may be one or more of colloidal silver or gold, or of zinc, copper or silver salts with different anions, for example, but not limited to fluoride, chloride, bromide, iodide, tiocyanate, sulfite, hydroxide, phosphate, carbonate, lactate, glycolate, citrate, borate, tartrate, and
30 acetates of different acyl composition. The adjuvant may also be cationic polymers such as cationic cellulose ethers, cationic cellulose esters, deacetylated hyaluronic acid, chitosan, cationic dendrimers, cationic synthetic polymers such as poly(vinyl imidazole), and cationic polypeptides such as polyhistidine, polylysine, polyarginine, and peptides containing these amino acids.

35

The excipient may be one or more of carbohydrates, polymers, lipids and minerals. Examples of carbohydrates include lactose, sucrose, mannitol, and cyclodextrines,

which are added to the composition, e.g., for facilitating lyophilisation. Examples of polymers are starch, cellulose ethers, cellulose, carboxymethylcellulose, hydroxypropylmethyl cellulose, hydroxyethyl cellulose, ethylhydroxyethyl cellulose, ethyl cellulose, methyl cellulose, propyl cellulose, alginates, carageenans, hyaluronic acid and derivatives thereof, polyacrylic acid, polysulphonate, polyethyleneglycol/polyethylene oxide, polyethyleneoxide/ polypropylene oxide copolymers, polyvinylalcohol/polyvinylacetate of different degree of hydrolysis, poly(lactic acid), poly(glycolic acid) or copolymers thereof with various composition, and polyvinylpyrrolidone, all of different molecular weight, which are added to the composition, e.g. for viscosity control, for achieving bioadhesion, or for protecting the active ingredient (applies to A-C as well) from chemical and proteolytic degradation. Examples of lipids are fatty acids, phospholipids, mono-, di-, and triglycerides, ceramides, sphingolipids and glycolipids, all of different acyl chain length and saturation, egg lecithin, soy lecithin, hydrogenated egg and soy lecithin, which are added to the composition for reasons similar to those for polymers. Examples of minerals are talc, magnesium oxide, zinc oxide and titanium oxide, which are added to the composition to obtain benefits such as reduction of liquid accumulation or advantageous pigment properties.

The pharmaceutical composition may also contain one or more mono- or disaccharides such as xylitol, sorbitol, mannitol, lactitol, isomalt, maltitol or xylosides, and/or monoacylglycerols, such as monolaurin. The characteristics of the carrier are dependent on the route of administration. One route of administration is topical administration. For example, for topical administrations, a preferred carrier is an emulsified cream comprising the active peptide, but other common carriers such as certain petrolatum/mineral-based and vegetable-based ointments can be used, as well as polymer gels, liquid crystalline phases and microemulsions.

It will be appreciated that the pharmaceutical compositions may comprise one or more polypeptides of the invention, for example one, two, three or four different peptides. By using a combination of different peptides the anti-inflammatory effect may be increased.

As discussed above, the polypeptide may be provided as a salt, for example an acid adduct with inorganic acids, such as hydrochloric acid, sulfuric acid, nitric acid, hydrobromic acid, phosphoric acid, perchloric acid, thiocyanic acid, boric acid etc. or with organic acid such as formic acid, acetic acid, haloacetic acid, propionic acid,

glycolic acid, citric acid, tartaric acid, succinic acid, gluconic acid, lactic acid, malonic acid, fumaric acid, anthranilic acid, benzoic acid, cinnamic acid, p-toluenesulfonic acid, naphthalenesulfonic acid, sulfanilic acid etc. Inorganic salts such as monovalent sodium, potassium or divalent zinc, magnesium, copper calcium, all with a
5 corresponding anion, may be added to improve the biological activity of the antimicrobial composition.

The pharmaceutical compositions of the invention may also be in the form of a liposome, in which the polypeptide is combined, in addition to other pharmaceutically
10 acceptable carriers, with amphipathic agents such as lipids, which exist in aggregated forms as micelles, insoluble monolayers and liquid crystals. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Suitable lipids also include the lipids above modified by poly(ethylene glycol) in the
15 polar headgroup for prolonging bloodstream circulation time. Preparation of such liposomal formulations is can be found in for example US4,235,871.

The pharmaceutical compositions of the invention may also be in the form of biodegradable microspheres. Aliphatic polyesters, such as poly(lactic acid) (PLA),
20 poly(glycolic acid) (PGA), copolymers of PLA and PGA (PLGA) or poly(carpolactone) (PCL), and polyanhydrides have been widely used as biodegradable polymers in the production of microspheres. Preparations of such microspheres can be found in US 5,851,451 and in EP 213 303.

25 The pharmaceutical compositions of the invention may also be formulated with micellar systems formed by surfactants and block copolymers, preferably those containing poly(ethylene oxide) moieties for prolonging bloodstream circulation time.

The pharmaceutical compositions of the invention may also be in the form of polymer
30 gels, where polymers such as starch, cellulose ethers, cellulose, carboxymethylcellulose, hydroxypropylmethyl cellulose, hydroxyethyl cellulose, ethylhydroxyethyl cellulose, ethyl cellulose, methyl cellulose, propyl cellulose, alginates, chitosan, carageenans, hyaluronic acid and derivatives thereof, polyacrylic acid, polyvinyl imidazole, polysulphonate, polyethyleneglycol/polyethylene oxide,
35 polyethylene-oxide/polypropylene oxide copolymers, polyvinylalcohol/polyvinylacetate of different degree of hydrolysis, and polyvinylpyrrolidone are used for thickening of the solution containing the peptide. The polymers may also comprise

gelatin or collagen.

Alternatively, the polypeptides of the invention may be dissolved in saline, water, polyethylene glycol, propylene glycol, ethanol or oils (such as safflower oil, corn oil,
5 peanut oil, cottonseed oil or sesame oil), tragacanth gum, and/or various buffers.

The pharmaceutical composition may also include ions and a defined pH for potentiation of action of anti-inflammatory polypeptides.

10 The compositions of the invention may be subjected to conventional pharmaceutical operations such as sterilisation and/or may contain conventional adjuvants such as preservatives, stabilisers, wetting agents, emulsifiers, buffers, fillers, etc., e.g., as disclosed elsewhere herein.

15 It will be appreciated by persons skilled in the art that the pharmaceutical compositions of the invention may be administered locally or systemically. Routes of administration include topical, ocular, nasal, pulmonary, buccal, parenteral (intravenous, subcutaneous, and intramuscular), oral, vaginal and rectal. Also administration from implants is possible. Suitable preparation forms are, for example
20 granules, powders, tablets, coated tablets, (micro) capsules, suppositories, syrups, emulsions, microemulsions, defined as optically isotropic thermodynamically stable systems consisting of water, oil and surfactant, liquid crystalline phases, defined as systems characterised by long-range order but short-range disorder (examples include lamellar, hexagonal and cubic phases, either water- or oil continuous), or
25 their dispersed counterparts, gels, ointments, dispersions, suspensions, creams, aerosols, droplets or injectable solution in ampoule form and also preparations with protracted release of active compounds, in whose preparation excipients, diluents, adjuvants or carriers are customarily used as described above. The pharmaceutical composition may also be provided in bandages, plasters or in sutures or the like.

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In preferred embodiments, the pharmaceutical composition is suitable for parenteral administration or topical administration.

In alternative preferred embodiments, the pharmaceutical composition is suitable for
35 pulmonary administration or nasal administration.

For example, the pharmaceutical compositions of the invention can be administered intranasally or by inhalation and are conveniently delivered in the form of a dry powder inhaler or an aerosol spray presentation from a pressurised container, pump, spray or nebuliser with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoro-methane, dichlorotetrafluoro-ethane, a hydrofluoroalkane such as 1,1,1,2-tetrafluoroethane (HFA 134A3 or 1,1,1,2,3,3,3-heptafluoropropane (HFA 227EA3), carbon dioxide or other suitable gas. In the case of a pressurised aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurised container, pump, spray or nebuliser may contain a solution or suspension of the active compound, e.g. using a mixture of ethanol and the propellant as the solvent, which may additionally contain a lubricant, e.g. sorbitan trioleate. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder mix of a polypeptide of the invention and a suitable powder base such as lactose or starch.

Aerosol or dry powder formulations are preferably arranged so that each metered dose or 'puff' contains at least 0.1 mg of a polypeptide of the invention for delivery to the patient. It will be appreciated that the overall daily dose with an aerosol will vary from patient to patient, and may be administered in a single dose or, more usually, in divided doses throughout the day.

The pharmaceutical compositions will be administered to a patient in a pharmaceutically effective dose. By "pharmaceutically effective dose" is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose is dependent on the, activity of the compound, manner of administration, nature and severity of the disorder, age and body weight of the patient different doses may be needed. The administration of the dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units and also by multiple administrations of subdivided doses at specific intervals.

The pharmaceutical compositions of the invention may be administered alone or in combination with other therapeutic agents, such as additional antibiotic, anti-inflammatory, immunosuppressive, vasoactive and/or antiseptic agents (such as anti-bacterial agents, anti-fungicides, anti-viral agents, and anti-parasitic agents). Examples of suitable antibiotic agents include penicillins, cephalosporins, carbacephems, cephamycins, carbapenems, monobactams, aminoglycosides,

glycopeptides, quinolones, tetracyclines, macrolides, and fluoroquinolones. Antiseptic agents include iodine, silver, copper, chlorhexidine, polyhexanide and other biguanides, chitosan, acetic acid, and hydrogen peroxide. Likewise, the pharmaceutical compositions may also contain additional anti-inflammatory drugs,
5 such as steroids and macrolactam derivatives.

In one embodiment, the pharmaceutical compositions of the invention are administered in combination with a steroid, for example a glucocorticoid (such as dexamethasone).

10 It will be appreciated by persons skilled in the art that the additional therapeutic agents may be incorporated as part of the same pharmaceutical composition or may be administered separately.

15 In one embodiment of the seventh aspect of the invention, the pharmaceutical composition is associated with a device or material to be used in medicine (either externally or internally). By 'associated with' we include a device or material which is coated, impregnated, covalently bound to or otherwise admixed with a pharmaceutical composition of the invention (or polypeptide thereof).

20 For example, the composition may be coated to a surface of a device that comes into contact with the human body or component thereof (*e.g.* blood), such as a device used in by-pass surgery, extracorporeal circulation, wound care and/or dialysis. Thus, the composition may be coated, painted, sprayed or otherwise applied to or
25 admixed with a suture, prosthesis, implant, wound dressing, catheter, lens, skin graft, skin substitute, fibrin glue or bandage, *etc.* In so doing, the composition may impart improved anti-inflammatory and/or anti-coagulant properties to the device or material.

30 Preferably, the device or material is coated with the pharmaceutical composition of the invention (or the polypeptide component thereof). By 'coated' we mean that the pharmaceutical composition is applied to the surface of the device or material. Thus, the device or material may be painted or sprayed with a solution comprising a pharmaceutical composition of the invention (or polypeptide thereof). Alternatively, the device or material may be dipped in a reservoir of a solution comprising a
35 polypeptide of the invention.

Advantageously, the device or material is impregnated with a pharmaceutical composition of the invention (or polypeptide thereof). By 'impregnated' we mean that the pharmaceutical composition is incorporated or otherwise mixed with the device or material such that it is distributed throughout.

5

For example, the device or material may be incubated overnight at 4°C in a solution comprising a polypeptide of the invention. Alternatively, a pharmaceutical composition of the invention (or polypeptide thereof) may be immobilised on the device or material surface by evaporation or by incubation at room temperature.

10

In an alternative embodiment, a polypeptide of the invention is covalently linked to the device or material, e.g. at the external surface of the device or material. Thus, a covalent bond is formed between an appropriate functional group on the polypeptide and a functional group on the device or material. For example, methods for covalent bonding of polypeptides to polymer supports include covalent linking via a diazonium intermediate, by formation of peptide links, by alkylation of phenolic, amine and sulphydryl groups on the binding protein, by using a poly functional intermediate e.g. glutardialdehyde, and other miscellaneous methods e.g. using silylated glass or quartz where the reaction of trialkoxysilanes permits derivatisation of the glass surface with many different functional groups. For details, see Enzyme immobilisation by Griffin, M., Hammonds, E.J. and Leach, C.K. (1993) In *Technological Applications of Biocatalysts* (BIOTOL SERIES), pp. 75-118, Butterworth-Heinemann. See also the review article entitled 'Biomaterials in Tissue Engineering' by Hubbell, J.A. (1995) *Science* **13**:565-576.

25

In a preferred embodiment, the device or material comprise or consists of a polymer. The polymer may be selected from the group consisting of polyesters (e.g. polylactic acid, polyglycolic acid or poly lactic acid-glycolic acid copolymers of various composition), polyorthoesters, polyacetals, polyureas, polycarbonates, polyurethanes, polyamides) and polysaccharide materials (e.g. cross-linked alginates, hyaluronic acid, carageenans, gelatines, starch, cellulose derivatives).

30

Alternatively, or in addition, the device or material may comprise or consists of metals (e.g. titanium, stainless steel, gold, titanium), metal oxides (silicon oxide, titanium oxide) and/or ceramics (apatite, hydroxyapatite).

35

Such materials may be in the form of macroscopic solids/monoliths, as chemically or physicochemically cross-linked gels, as porous materials, or as particles.

Thus, the present invention additionally provides devices and materials to be used in medicine, to which have been applied a polypeptide of the invention or pharmaceutical composition comprising the same.

Such devices and materials may be made using methods well known in the art.

An eighth aspect of the invention provides a polypeptide according to the second aspect of the invention or a pharmaceutical composition according to the seventh aspect of the invention for use in medicine.

In preferred embodiments, the polypeptide according to the second aspect of the invention or the pharmaceutical composition according to the seventh aspect of the invention are for use:

- (a) the treatment and/prevention of acute and/or chronic inflammation;
- (b) the treatment and/prevention of microbial infection (e.g. bacterial infection);
- (c) the modulation of blood coagulation; and/or
- (d) the treatment of wounds.

For example, the polypeptide according to the second aspect of the invention or the pharmaceutical composition according to the seventh aspect of the invention may be for use in the treatment and/prevention of a disease, condition or indication selected from the following:

- i) Acute systemic inflammatory disease, with or without an infective component, such as systemic inflammatory response syndrome (SIRS), ARDS, sepsis, severe sepsis, and septic shock. Other generalized or localized invasive infective and inflammatory disease, including erysipelas, meningitis, arthritis, toxic shock syndrome, diverticulitis, appendicitis, pancreatitis, cholecystitis, colitis, cellulitis, burn wound infections, pneumonia, urinary tract infections, postoperative infections, and peritonitis.

- 5 ii) Chronic inflammatory and or infective diseases, including cystic fibrosis, COPD and other pulmonary diseases, gastrointestinal disease including chronic skin and stomach ulcerations, other epithelial inflammatory and or infective disease such as atopic dermatitis, oral ulcerations (aphtous ulcers), genital ulcerations and inflammatory changes, parodontitis, eye inflammations including conjunctivitis and keratitis, external otitis, mediaotitis, genitourinary inflammations.
- 10 iii) Postoperative inflammation. Inflammatory and coagulative disorders including thrombosis, DIC, postoperative coagulation disorders, and coagulative disorders related to contact with foreign material, including extracorporeal circulation, and use of biomaterials. Furthermore, vasculitis related inflammatory disease, as well as allergy, including
- 15 allergic rhinitis and asthma..
- iv) Excessive contact activation and/or coagulation in relation to, but not limited to, stroke.
- 20 v) Excessive inflammation in combination with antimicrobial treatment. The antimicrobial agents used may be administred by various routes; intravenous (iv), intraarterial, intravitreal, subcutaneous (sc), intramuscular (im), intraperitoneal (ip), intravesical, intratechal, epidural, enteral (including oral, rectal, gastric, and other enteral
- 25 routes), or topically, (including dermal, nasal application, application in the eye or ear, eg by drops, and pulmonary inhalation). Examples of agents are penicillins, cephalosporins, carbacephems, cephamycins, carbapenems, monobactams, aminoglycosides, glycopeptides, quinolones, tetracyclines, macrolides, and fluoroquinolones. Antiseptic
- 30 agents include iodine, silver, copper, clorhexidine, polyhexanide and other biguanides, chitosan, acetic acid, and hydrogen peroxide.

35 Thus, the polypeptides may be for use in the treatment or prevention of an acute inflammation, sepsis, acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), cystic fibrosis, wounds, asthma, allergic and

other types of rhinitis, cutaneous and systemic vasculitis, thrombosis and/or disseminated intravascular coagulation (DIC).

A related ninth aspect of the invention provides the use of a polypeptide according to the second aspect of the invention or a pharmaceutical composition according to the seventh aspect of the invention in the preparation of a medicament for the treatment or prevention of inflammation and/or excessive coagulation (as described above).

A tenth aspect of the invention provides a method for treating or preventing inflammation and/or coagulation in a patient, the method comprising administering to the patient a therapeutically-effective amount of a polypeptide according to the second aspect of the invention or a pharmaceutical composition according to the seventh aspect of the invention (as described above). In preferred but non-limiting embodiments, the method is for the treatment or prevention of an acute inflammation, sepsis, acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), cystic fibrosis, asthma, allergic and other types of rhinitis, cutaneous and systemic vasculitis, thrombosis and/or disseminated intravascular coagulation (DIC).

Persons skilled in the art will further appreciate that the uses and methods of the present invention have utility in both the medical and veterinary fields. Thus, the polypeptide medicaments may be used in the treatment of both human and non-human animals (such as horses, dogs and cats). Advantageously, however, the patient is human.

Preferred aspects of the invention are described in the following non-limiting examples, with reference to the following figures:

Figure 1: NO-blocking effects of C-terminal peptides of TFPI.

RAW 264.7 macrophages were stimulated with 10 ng/ml *E.coli* LPS, and 10µM of the peptides GGL27, LIK17 and TKR22 were added. NO was measured using Griess reagent.

Figure 2: NO-blocking effects of peptides of heparin cofactor II.

RAW macrophages were stimulated with 10 ng/ml *E.coli* LPS, and the peptides KYE28, NLF20, and KYE21, were added at the indicated doses. LL-37 is presented as positive control. NO was measured using Griess reagent.

5

Figure 3: Anti-inflammatory effects of peptides of heparin cofactor II.

KYE28, KYE21 and NLF20 blocks NO production of RAW264.7 macrophages stimulated with various microbial products. Cells were subjected to the indicated concentrations of *E.coli* LPS, lipoteichoic acid (LTA) and peptidoglycan (PGN) from *S. aureus* as well as zymosan A from *Saccharosmyces cerevisiae*. NO production with or without 10 μ M GKY25 was determined by using the Griess reagent.

10

Figure 4: HRG is LPS-binding.

2 and 5 μ g HRG was applied onto a nitrocellulose membrane, followed by incubation with iodinated LPS in 10 mM Tris, pH 7.4 or 10 mM MES, pH 5.5, with or without 0.15M NaCl. Radioactivity was visualized using a phosphorimager system.

15

Figure 5: HRG increases LPS mediated NO release from murine macrophages.

RAW macrophages were incubated with 100 ng/ml LPS with or without LL-37 and HRG (2 and 10 μ M) for 24 hours. The supernatant was aspirated and NO was measured using Griess method. HRG significantly increased LPS mediated NO release in a dose dependent way (n=6, p=0.001).

20

Figure 6: Antibodies against TLR4 block NO release.

RAW macrophages were incubated with 100 ng/ml LPS with or without HRG (2 μ M) and polyclonal anti mouse TLR4 (5 μ g/ml) for 24 hours. Supernatant was aspirated and NO was measured using Griess method. Anti mouse TLR4 were able to significantly block LPS and HRG mediated NO release. (n=6, p=0.001).

25

Figure 7: Increased survival in LPS induced sepsis in *Hrg*^{-/-} mice.

Increased survival of *Hrg*^{-/-} mice after LPS challenge. Wildtype (dashed line) and *Hrg*^{-/-} (solid line) mice were injected i.p. with *E. coli* LPS and survival of the animals was followed for seven days. Mice lacking HRG (n=8) showed a significantly increased survival compared with wildtype animals (n=9, p=0.002).

30

35

Figure 8: Visual observation scores 18 hours after LPS challenge.

Status of wt and Hrg^{-/-} mice was visual observed (ruffled fur, hunched and pre-mortal) 18 hours post LPS challenge. Wildtype mice were visually significantly sicker Status of wt and Hrg^{-/-} mice was visual observed (ruffled fur [black], hunched [light grey] and pre-mortal [dark grey]) 18 hours post LPS challenge. Wildtype mice were visually significantly sicker compared with Hrg^{-/-} mice, p=0.001 n=10.

Figure 9: Vascular leakage is decreased in lungs of Hrg^{-/-} mice after LPS challenge compared to wildtype animals.

Lungs of wt and Hrg^{-/-} mice, were analyzed by scanning electron microscopy after LPS injection i.p. A) wildtype B) Hrg^{-/-} C) wt, 24 hours post-treatment of LPS D) Hrg^{-/-}, 24 hours post-treatment of LPS. Scale bar represents 50 μ m. A representative lung section is shown.

Figure 10: GHH25 inhibits HRG and LPS induced responses in murine macrophages.

RAW macrophages were incubated with 100 ng/ml LPS with or without HRG (2 μ M) and GHH25 (10 and 100 μ M) for 24 hours. The supernatant was aspirated and NO was measured using Griess method. GHH25 significantly decreased LPS and HRG mediated NO release in a dose dependent way. (n=6, p=0.001).

Figure 11: GHH25 inhibits HRG and LPS induced responses in human macrophages.

Human macrophages were incubated with 10 ng/ml LPS with or without HRG (2 μ M) and GHH25 (10 and 100 μ M) for 24 hours. Supernatant was aspirated and TNF- α was measured.

Figure 12: Improved health status and increased survival in LPS induced sepsis after treatment with GHH25.

Increased survival of wildtype mice after treatment with GHH25. Wildtype animals were injected i.p. with *E. coli* LPS, and 1 mg GHH25 (dashed line) or buffer only (solid line) was injected 30 minutes after. Survival of the animals was followed for seven days. Mice treated with GHH25 (n=13) showed a significantly increased survival when compared with untreated animals (n=12, p=0.03).

Figure 13: Visual observation scores 18 hours after LPS challenge.

Status of wt and Hrg^{-/-} mice was visual observed (ruffled fur, hunched and) 18 hours post LPS challenge. Wildtype mice were visually significantly sicker Status of wt and Hrg^{-/-} mice was visual observed (ruffled fur [black], hunched [light grey] and pre-mortal [dark grey]) 18 hours post LPS challenge. Wildtype mice were visually significantly more sick compared with Hrg^{-/-} mice, p=0.004 n=12.

Figure 14: C-terminal peptides of TFPI block coagulation. (A)

The C-terminal peptides of TFPI; GGL27, LIK17, as well as TKR22 impair the intrinsic pathway of coagulation in normal human plasma. This was determined by measuring the activated partial thromboplastin time (aPTT). GGL27 and LIK17 also affected prothrombin time (PT) monitoring the extrinsic pathway of coagulation. The thrombin clotting time (TCT), measuring thrombin induced fibrin network formation, were not significantly affected by the peptides (B) GGL-27 impairs coagulation in a dose dependent manner monitored by measuring the aPTT, PT and TCT in normal human plasma.

Figure 15: Peptides of heparin-cofactor II block coagulation.

KYE28 and NLF20 impair the intrinsic pathway of coagulation in normal human plasma determined by measuring the activated partial thromboplastin time (aPTT). KYE21 shows only minor blocking of the aPTT. Other parts of the coagulation system, as judged by the prothrombin time (PT) monitoring the extrinsic pathway of coagulation, and the thrombin clotting time (TCT), measuring thrombin induced fibrin network formation, were not significantly affected.

Figure 16: Cartoon illustrating the structure of TFPI.

Cleavage points by enzymes are indicated.

Figure 17: Antimicrobial activities of TFPI-derived peptides.

Antimicrobial activity of selected peptides (at 100 µM in RDA) against the indicated microbes. For determination of antimicrobial activities, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 isolates (4×10^6 cfu) or *C. parapsilosis* ATCC 90018 (1×10^5 cfu) was inoculated in 0.1% TSB agarose gel. Each 4 mm-diameter well was loaded with 6 µl of peptide. The zones of clearance correspond to the inhibitory effect of each peptide after incubation at 37 °C for 18-24 h (mean values are presented, n=3).

Figure 18: Antibacterial effects of TFPI-derived peptides.

Effects of TFPI-derived peptides and LL-37 against *E. coli*, *P. aeruginosa*, and *S. aureus* in viable count assays. 2×10^8 cfu/ml of bacteria were incubated in 50 μ l with peptides at the indicated concentrations in 10 mM Tris, pH 7.4 buffer (Tris), or in 0.15 M NaCl, 10 mM Tris, pH 7.4 containing normal or heat-inactivated 20% human plasma (n=3, SD is indicated).

Figure 19: Kinetic analysis.

The time-dependence of bacterial killing by TFPI-derived peptides (at 6 μ M [left panel] and 30 μ M [right panel]) in 0.15 M NaCl, 10 mM Tris, pH 7.4 containing 20% plasma was analyzed by viable count assays using *E. coli*.

Figure 20: Effects on bacterial membranes.

(A) Permeabilizing effects of peptides on *P. aeruginosa* and *E. coli*. (A) Bacteria were incubated with the indicated peptides and permeabilization was assessed using the impermeant probe FITC. (B) Electron microscopy analysis. *P. aeruginosa* and *S. aureus* bacteria was incubated for 2 h at 37°C with 30 μ M of GKY25 and LL-37 and analysed with electron microscopy. Scale bar represents 1 μ m. Control; Buffer control.

Figure 21: Structure of TFPI peptide LIK17.

Helical content of the TFPI-derived C-terminal peptide in presence of negatively charged liposomes (DOPE/DOPG). LIK17 structure was largely unaffected by the addition of liposomes.

Figure 22: CD spectra of LIK17 in Tris-buffer and in presence of LPS. For control, CD spectra for buffer and LPS alone are also presented.

Figure 23: Effects of the indicated peptides on liposome leakage.

The membrane permeabilizing effect was recorded by measuring fluorescence release of carboxyfluorescein from DOPE/DOPG (negatively charged) liposomes. The experiments were performed in 10 mM Tris-buffer, pH 7.4. Values represents mean of triplicate samples.

Figure 24: Activities on eukaryotic cells

Hemolytic effects of the indicated peptides. The cells were incubated with different concentrations of the peptides, 2% Triton X-100 (Sigma-Aldrich) served as positive

control. The absorbance of hemoglobin release was measured at λ 540 nm and is expressed as % of Triton X-100 induced hemolysis (note the scale of the y-axis). Effects of LL-37 are shown for comparison.

5 **Figure 25:** HaCaT keratinocytes were subjected to the indicated TFPI-peptides as well as LL-37. Cell permeabilizing effects were measured by the LDH based TOX-7 kit. LDH release from the cells was monitored at λ 490 nm and was plotted as % of total LDH release.

10 **Figure 26:** The MTT-assay was used to measure viability of HaCaT keratinocytes in the presence of the indicated peptides (at 60 μ M). In the assay, MTT is modified into a dye, blue formazan, by enzymes associated with metabolic activity. The absorbance of the dye was measured at λ 550 nm.

15 **Figure 27: Antimicrobial activities of heparin cofactor II-derived peptides.** Antimicrobial activity of selected peptides (at 100 μ M) in RDA against the indicated microbes. For determination of antimicrobial activities, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 isolates (4×10^6 cfu) or *C. parapsilosis* ATCC 90018 (1×10^5 cfu) was inoculated in 0.1% TSB agarose gel. Each 4 mm-diameter well was loaded with 6 μ l
20 of peptide. The zones of clearance correspond to the inhibitory effect of each peptide after incubation at 37 °C for 18-24 h (mean values are presented, n=3).

Figure 28: (upper) Antibacterial effects of heparin cofactor II -derived peptides and LL-37 against *E. coli* in viable count assays. 2×10^6 cfu/ml of bacteria were incubated
25 in 50 μ l with peptides at the indicated concentrations in 10 mM Tris, pH 7.4 buffer (Tris), or in 0.15 M NaCl, 10 mM Tris, pH 7.4 containing 20% human plasma (n=3, SD is indicated). (lower) The time-dependence of bacterial killing by heparin cofactor II-derived peptides (at 6 and 30 μ M) in 0.15 M NaCl, 10 mM Tris, pH 7.4 containing 20% plasma was analyzed by viable count assays using *E. coli*. LL-37 (30 μ M) was
30 used for comparison.

Figure 29: Effects on bacterial membranes.

(A) Permeabilizing effects of peptides on *P. aeruginosa* and *E. coli*. (A) Bacteria were incubated with the indicated peptides and permeabilization was assessed using the impermeant probe FITC. (B) Electron microscopy analysis. *P. aeruginosa* and *S. aureus* bacteria was incubated for 2 h at 37°C with 30 μ M of NLF20 and LL-37 and

analysed with electron microscopy. Scale bar represents 1 μm . Control; Buffer control.

Figure 30: Structure and effects on liposomes.

- 5 (A) Helical content of the heparin cofactor II -derived C-terminal peptides in presence of negatively charged liposomes (DOPE/DOPG). (B) Effects of NLF20 on liposome leakage. The membrane permeabilizing effect was recorded by measuring fluorescence release of carboxyfluorescein from DOPE/DOPG (negatively charged) liposomes. (C) CD spectra of NLF20 in Tris-buffer and in presence of LPS. For
10 control, CD spectra for buffer and LPS alone are also presented. The experiments were performed in 10 mM Tris-buffer, pH 7.4. Values represents mean of triplicate samples

Figure 31: Effects of NLF20 in an animal model of *P. aeruginosa* sepsis.

- 15 The thrombin HCII peptide NLF20 significantly increases survival. Mice were i.p. injected with *P. aeruginosa* bacteria, followed by subcutaneous injection of NLF20 or buffer only, after 1 h and then with intervals of 24 h for the three following days. Treatment with the peptide significantly increased survival.

20 **Figure 32: Activities on eukaryotic cells**

- (A) Hemolytic effects of the indicated peptides. The cells were incubated with different concentrations of the peptides, 2% Triton X-100 (Sigma-Aldrich) served as positive control. The absorbance of hemoglobin release was measured at λ 540 nm and is expressed as % of Triton X-100 induced hemolysis (note the scale of the y-
25 axis). Effects of LL-37 are shown for comparison. (B) HaCaT keratinocytes were subjected to the indicated HCII-peptides as well as LL-37. Cell permeabilizing effects were measured by the LDH based TOX-7 kit. LDH release from the cells was monitored at λ 490 nm and was plotted as % of total LDH release. (C) The MTT-
30 assay was used to measure viability of HaCaT keratinocytes in the presence of the indicated peptides (at 60 μM). In the assay, MTT is modified into a dye, blue formazan, by enzymes associated with metabolic activity. The absorbance of the dye was measured at λ 550 nm. Right panel shows results in presence of 20% plasma (hemolysis) or 20% serum (LDH and MTT).

Figure 33: Antithrombin III-derived peptide FFF21 blocks coagulation.

FFF21 impairs the intrinsic pathway of coagulation in normal human plasma determined by measuring the activated partial thromboplastin time (aPTT). Other parts of the coagulation system, as judged by the prothrombin time (PT) monitoring the extrinsic pathway of coagulation, and the thrombin clotting time (TCT), measuring thrombin induced fibrin network formation, were not significantly affected.

Figure 34. Antimicrobial activities of TFPI-derived peptides

(A) Cartoon illustrating the structure of TFPI. Enzymatic cleavage sites are indicated.

(B) Antimicrobial activity (using RDA) of selected peptides against the indicated microbes. For determination of antimicrobial activities, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 or *B. subtilis* ATCC 6633 isolates (4×10^6 cfu) or *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 90018 (1×10^5 cfu) were inoculated in 0.1% TSB agarose gel. Each 4 mm-diameter well was loaded with 6 μ l of peptide (at 100 μ M). The zones of clearance correspond to the inhibitory effect of each peptide after incubation at 37 °C for 18-24 h (mean values are presented, n=3). (C) Antibacterial effects of the TFPI-derived peptides and LL-37 against *E. coli* ATCC 25922 in viable count assays. 2×10^6 cfu/ml of bacteria were incubated in 50 μ l with peptides at the indicated concentrations in 10 mM Tris, pH 7.4 buffer, and the cfu were determined.

Figure 35. Effects on bacterial membranes

(A) Binding of TFPI to bacterial surfaces. The indicated bacteria ($1-2 \times 10^9$ cfu/ml) were incubated with 3 μ M of TAMRA labeled GGL27 peptide in 500 μ l human plasma and samples were analyzed by FACS. (B) Permeabilizing effects of peptides on *E. coli*. Bacteria were incubated with the indicated peptides and permeabilization was assessed using the impermeant probe FITC. (C) Electron microscopy analysis. *P. aeruginosa* bacteria were incubated for 2 h at 37°C with 30 μ M of LIK17 and LL-37 and visualized by negative staining. Scale bar represents 1 μ m. Control; Buffer control.

Figure 36. Structure and effects on liposomes

(A) Effects of the indicated peptides on liposome leakage. The membrane permeabilizing effect was recorded by measuring fluorescence release of carboxyfluorescein from DOPE/DOPG (negatively charged) liposomes. The experiments were performed in 10 mM Tris-buffer. Values represents mean of

triplicate samples. (B) Kinetics of CF release from liposomes. 1 μ M of peptides was used. (C) Helical content of the TFPI-derived C-terminal LIK16 and GGL27 peptides in presence of negatively charged liposomes (DOPE/DOPG). LIK17 and GGL27 structure was largely unaffected by the addition of liposomes. (D) CD spectra of
 5 LIK17 and GGL27 in Tris-buffer and in presence of LPS. For control, CD spectra for buffer and LPS alone are also presented.

Figure 37. Activities on eukaryotic cells

(A) Hemolytic effects of the indicated peptides. The cells were incubated with
 10 different concentrations of the peptides, 2% Triton X-100 (Sigma-Aldrich) served as positive control. The absorbance of hemoglobin release was measured at λ 540 nm and is expressed as % of Triton X-100 induced hemolysis (note the scale of the y-axis). Effects of LL-37 are shown for comparison. (B) Upper panel: HaCaT keratinocytes were subjected to the indicated TFPI-peptides as well as LL-37. Cell
 15 permeabilizing effects were measured by the LDH based TOX-7 kit. LDH release from the cells was monitored at λ 490 nm and was plotted as % of total LDH release. Lower panel: The MTT-assay was used to measure viability of HaCaT keratinocytes in the presence of the indicated peptides (at 60 μ M). In the assay, MTT is modified into a dye, blue formazan, by enzymes associated with metabolic activity. The
 20 absorbance of the dye was measured at λ 550 nm.

Figure 38. Activities of C-terminal TFPI-derived peptides

(A) Antibacterial effects of the TFPI-derived peptides and LL-37 against *E. coli* or *P. aeruginosa* in viable count assays. 2×10^6 cfu/ml of bacteria were incubated in 50 μ l
 25 with peptides at the indicated concentrations in 10 mM Tris, 0.15 M NaCl, pH 7.4 (buffer), or in 0.15 M NaCl, 10 mM Tris, pH 7.4 containing 20% human citrate plasma (CP), or in the same buffer but with heat-inactivated human citrate plasma (HCP) (n=3, SD is indicated). (B) The indicated bacterial isolates were subjected to GGL27 and LIK17 at 3 μ M in buffer, native plasma (CP), or heat-inactivated plasma (HCP)
 30 for 2 h and the number of cfu was determined.

Figure 39. The C-terminal TFPI peptide GGL27 enhances C1q, C3a and MAC binding to *E. coli*

(A) *E. coli* ATCC 25922 and *P. aeruginosa* 15159 bacteria were washed, resuspended, and incubated with citrate plasma either alone or supplemented with
 35 GGL27 (at 3 μ M) for 30 min or 1 h at 37°C. The bacterial cells were collected,

washed with PBS, and bound proteins and corresponding supernatants were subjected to Tris-Tricine SDS-PAGE under reducing conditions, followed by immunoblotting with antibodies recognizing C1q or C5b-9. CP; citrate plasma, S; supernatant or unbound bacterial, P; pellet or material bound to bacterial cells. (B) As in (A), but antibodies against the C3a were used (25). (C) (left panel) Comparison of the mean proportion of bacteria positive for C1q/C3a binding in citrate plasma (control; black columns) and in plasma supplemented with GGL27 (gray columns). (right panel) Comparative degree of C1q and C3a binding to *E. coli* and *P. aeruginosa* strains, expressed as means of the fluorescence index (FI; proportion of bacteria positive for C1q/C3a multiplied by the mean intensity of C1q/C3a binding). (*; $p < 0.05$; **; $p < 0.01$, ***; $p < 0.001$, t-test). (D) Examples of flow cytometry histograms of C1q/C3a binding to *E. coli* and *P. aeruginosa* in citrate plasma and in plasma supplemented with GGL27.

Figure 40. Identification of TFPI in human skin and wounds.

(A) Immunohistochemical identification of TFPI in normal skin, acute wound (AW) and in chronic venous leg ulcer tissue (chronic wounds; CW). Skin biopsies were taken from normal skin (normal skin; $n=3$), acute wounds (AW-1 and AW-2; 5 and 8 days after wounding, respectively) and from the wound edges of patients with chronic venous ulcers (CW; $n=3$). Representative sections are shown. In normal skin TFPI is detected mainly in the basal layers of epidermis. TFPI in AW and CW are ubiquitously found in all epidermal layers. Scale bar is 100 μm . (B) TFPI derived peptides are found in human wounds. Visualization of binding of C-terminal TFPI peptides to bacteria found in fibrin slough from a *P. aeruginosa* infected chronic wound surface. The peptides bind to fibrin (A), bacteria (B, C), and bacteria inside a macrophage (D). In (E) and (F), TFPI and C3a peptides were visualized by immunogold using gold-labeled antibodies of different sizes, specific for C3a (10 nm) and C-termini of TFPI (20 nm), respectively. Evaluation of 50 bacterial profiles showed that ~70% of TFPI-molecules were associated with C3a. See inset for exemplification.

Figure 41. *In vitro* and *in vivo* effects of GGL27

(A) 2×10^6 cfu/ml of *E. coli* were incubated in 50 μl with GGL27 peptide at the indicated concentrations in 10 mM Tris, pH 7.4 buffer (Buffer), or in 0.15 M NaCl, 10 mM Tris, pH 7.4 containing 20% human citrate plasma (CP), or in the same buffer but with mice citrate plasma (Balb/c or C57B/6) ($n=3$, SD is indicated). (B) The GGL27 peptide prolongs survival in *E. coli* and *P. aeruginosa* infected mice. Mice

were injected i.p. with *E. coli* or *P. aeruginosa* bacteria. In the *E. coli* infection model GGL27 (200 µg) or buffer alone was injected i.p. after 30 min. (n=10 in each group, P<0.001, Kaplan-Meier Survival Analysis Log-Rank test). In the *P. aeruginosa* infection model GGL27 (500 µg) or buffer alone was injected s.c. after 1 h (n=8 in each group, P<0.001, Kaplan-Meier Survival Analysis Log-Rank test). (C) GGL27 inhibits NO production. RAW264.7 mouse macrophages were stimulated with LPS from *E. coli* (left panel) or *P. aeruginosa* (right panel) in presence of GGL27 or the two control peptides GGL27(S) and DSE25 at the indicated concentrations. The difference between GGL27 and the control peptides is statistically significant (P<0.01). (D) GGL27 significantly increases survival in LPS-induced shock. Mice were injected with *E. coli* LPS followed by intraperitoneal administration of GGL27 (500 µg). Buffer and the peptides GGL27(S) and DSE25 (500 µg) served as controls. Survival was followed for 7 days. (GGL27; n=16, buffer; n=15, GGL27(S); n=8, DSE25; n=8. The difference between GGL27 and buffer, or the control peptides is significant, P<0.01, Kaplan-Meier Survival Analysis Log-Rank test). * indicates that the lines for GGL27(S), DSE25, and buffer are overlaid. (E) GGL27 attenuates proinflammatory cytokines. In a separate experiment, mice were sacrificed 20 h after i.p. injection of LPS followed by treatment as above with GGL27 (500 µg), buffer or the control peptides GGL27(S) or DSE25, and the indicated cytokines were analyzed in blood (Control; n=8; GGL27, n=12; GGL27(S), n=7; DSE25; n=8). In all cases, the difference between GGL27-treated animals and buffer was significant. P values for the respective cytokines are IL-6; 0.0023, TNF-α; 0.0018, IFN-γ; 0.0002, MCP-1; 0.0002, IL-10; 0.0023. There was a significant difference between controls and GGL27(S) with respect to IFN-γ. (F) Lungs were analyzed 20 h after LPS injection i.p. followed by treatment with GGL27 (500 µg) or buffer. Histochemical analysis shows marked attenuation of inflammatory changes in GGL27-treated lungs (a representative lung section is shown).

Figure 42. Evolution of TFPI

The phylogenetic tree and sequence similarities show that TFPI in *Homo sapiens*, *Pongo abelii* and *Sus scrofa* are closely related.

Figure 43

The peptide GGL27, and the control peptides GGL27(S), having the central K/R residues replaced by S, and the peptide DSE25, from the N-terminus of TFPI were analyzed for antimicrobial activities against the indicated bacteria and fungi. The inhibitory zones of the peptides are indicated. For determination of antimicrobial

activities, bacteria (4×10^6 cfu) or fungi (1×10^5 cfu) were inoculated in 0.1% TSB agarose gel. Each 4 mm-diameter well was loaded with 6 μ l of peptide (at 100 μ M). The zones of clearance correspond to the inhibitory effect of each peptide after incubation at 37 °C for 18-24 h (mean values are presented, n=3). *indicates zero values.

Figure 44

Bacteria were incubated with GGL27 and permeabilization was assessed using the impermeant probe FITC.

Figure 45

The time-dependence of bacterial killing by the indicated TFPI-derived peptides (at 30 μ M) in 0.15 M NaCl, 10 mM Tris, pH 7.4 containing 20% plasma was analyzed by viable count assays using *E. coli* and *P. aeruginosa*.

Figure 46. HRG is LPS-binding

2 and 5 μ g HRG was applied onto a nitrocellulose membrane, followed by incubation with iodinated LPS in 10 mM Tris, pH 7.4 or 10 mM MES, pH 5.5, with or without 0.15M NaCl. Radioactivity was visualized using a phosphorimager system.

Figure 47. HRG increases LPS mediated NO release from murine macrophages.

RAW macrophages were incubated with 100 ng/ml LPS with or without LL-37 and HRG (2 and 10 μ M) for 24 hours. The supernatant was aspirated and NO was measured using Griess method. HRG significantly increased LPS mediated NO release in a dose dependent way (n=6, p=0.001).

Figure 48. Antibodies against TLR4 block NO release

RAW macrophages were incubated with 100 ng/ml LPS with or without HRG (2 μ M) and polyclonal anti mouse TLR4 (5 μ g/ml) for 24 hours. Supernatant was aspirated and NO was measured using Griess method. Anti mouse TLR4 were able to significantly block LPS and HRG mediated NO release. (n=6, p=0.001).

Figure 49. Increased survival in LPS induced sepsis in *Hrg*^{-/-} mice

Increased survival of *Hrg*^{-/-} mice after LPS challenge. Wildtype (dashed line) and *Hrg*^{-/-} (solid line) mice were injected i.p. with *E. coli* LPS and survival of the animals

was followed for seven days. Mice lacking HRG (n=8) showed a significantly increased survival compared with wildtype animals (n=9, p=0.002).

Figure 50. Visual observation scores 18 hours after LPS challenge

5 Status of wt and Hrg^{-/-} mice was visual observed (ruffled fur, hunched and) 18 hours post LPS challenge. Wildtype mice were visually significantly sicker Status of wt and Hrg^{-/-} mice was visual observed (ruffled fur [black], hunched [light grey] and pre-mortal [dark grey]) 18 hours post LPS challenge. Wildtype mice were visually significantly sicker compared with Hrg^{-/-} mice, p=0.001 n=10.

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Figure 51. Vascular leakage is decreased in lungs of Hrg^{-/-} mice after LPS challenge compared to wildtype animals

Lungs of wt and Hrg^{-/-} mice, were analyzed by scanning electron microscopy after LPS injection i.p. A) wildtype B) Hrg^{-/-} C) wt, 24 hours post-treatment of LPS D) Hrg^{-/-}, 24 hours post-treatment of LPS. Scale bar represents 50 µm. A representative lung section is shown.

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Figure 52. GHH25 inhibits HRG and LPS induced responses in murine macrophages

20 RAW macrophages were incubated with 100 ng/ml LPS with or without HRG (2µM) and GHH25 (10 and 100 µM) for 24 hours. The supernatant was aspirated and NO was measured using Griess method. GHH25 significantly decreased LPS and HRG mediated NO release in a dose dependent way. (n=6, p=0.001).

Figure 53. GHH25 inhibits HRG and LPS induced responses in human macrophages

Human macrophages were incubated with 10 ng/ml LPS with or without HRG (2µM) and GHH25 (10 and 100 µM) for 24 hours. Supernatant was aspirated and TNF-α was measured.

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Figure 54. Improved health status and increased survival in LPS induced sepsis after treatment with GHH25

Increased survival of wildtype mice after treatment with GHH25. Wildtype animals were injected i.p. with *E. coli* LPS, and 1 mg GHH25 (dashed line) or buffer only (solid line) was injected 30 minutes after. Survival of the animals was followed for seven days. Mice treated with GHH25 (n=13) showed a significantly increased survival when compared with untreated animals (n=12, p=0.03).

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Figure 55. Visual observation scores 18 hours after LPS challenge

Status of wt and Hrg^{-/-} mice was visual observed (ruffled fur, hunched and) 18 hours post LPS challenge. Wildtype mice were visually significantly sicker Status of wt and Hrg^{-/-} mice was visual observed (ruffled fur [black], hunched [light grey] and pre-mortal [dark grey]) 18 hours post LPS challenge. Wildtype mice were visually significantly more sick compared with Hrg^{-/-} mice, p=0.004 n=12.

Figure 56. Antithrombin III-derived peptide FFF21 blocks coagulation

FFF21 impairs the intrinsic pathway of coagulation in normal human plasma determined by measuring the activated partial thromboplastin time (aPTT). Other parts of the coagulation system, as judged by the prothrombin time (PT) monitoring the extrinsic pathway of coagulation, and the thrombin clotting time (TCT), measuring thrombin induced fibrin network formation, were not significantly affected.

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EXAMPLES

EXAMPLE A

5 Introduction

Heparin cofactor II

Serpins are a group of proteins with similar structures that were first identified as a set of proteins able to inhibit proteases. The acronym serpin was originally coined because many serpins inhibit chymotrypsin-like serine proteases (serine protease inhibitors). The first members of the serpin superfamily to be extensively studied were the human plasma proteins antithrombin and antitrypsin, which play key roles in controlling blood coagulation and inflammation, respectively.

15 Structural studies on serpins have revealed that inhibitory members of the family undergo an unusual conformational change, termed the Stressed to Relaxed (S to R) transition. This conformational mobility of serpins provides a key advantage over static lock-and-key protease inhibitors. In particular, the function of inhibitory serpins can be readily controlled by specific cofactors like heparin. The archetypal example of this situation is antithrombin, which circulates in plasma in a relatively inactive state. Upon binding a high-affinity heparin pentasaccharide sequence within long-chain heparin, antithrombin undergoes a conformational change, exposing key residues important for the mechanism. The heparin pentasaccharide-bound form of antithrombin is, thus, a more effective inhibitor of thrombin and factor Xa. Furthermore, both of these coagulation proteases contain binding sites (called exosites) for heparin. Heparin, therefore, also acts as a template for binding of both protease and serpin, further dramatically accelerating the interaction between the two parties. After the initial interaction, the final serpin complex is formed and the heparin moiety is released.

Peptides corresponding to the heparin binding sites in these proteins possess antibacterial, anti-inflammatory and anti-coagulation properties.

Tissue factor pathway inhibitor (TFPI)

Tissue factor pathway inhibitor (or TFPI) is a Kunitz-type proteinase inhibitor which reversibly inhibits the tissue factor-factor VII (TF-VII) complex in a factor X (FX) dependent manner, leading to inhibition of both FX and FIX activation. TFPI consists of a highly negatively-charged amino-terminus, three tandemly-linked Kunitz-type domains, and a highly positively-charged carboxy-terminus. In plasma, TFPI exists in both full-length and variably C-terminal truncated forms [28]. The first and second Kunitz domains are involved in binding and inhibition of the TF-VII complex and factor Xa, respectively [29]. The third Kunitz domain may via its cationic residues, including amino acid sequences at the C-terminal end, interact with heparin [30]. This C-terminal region has also been implicated in interaction with plasma lipoproteins, thrombospondin-1, clearance receptors ([31]), lipopolysaccharide [32] and may inhibit cell growth [33] as well as blood coagulation [34], [35]. Since various C-terminally truncated forms exist *in vivo*, a potential role of proteolysis of the C-terminus has been implicated, and data indicate that TFPI can be cleaved by various proteinases such as thrombin [36], plasmin [37], and matrix metalloproteinase-8 [38], releasing C-terminal fragments. Upregulators of TFPI expression include endotoxin, IL-1, TNF- α , platelet-derived growth factor, heparin, and basic fibroblast growth factor, all physiological mechanisms involved in infection, inflammation, and growth [31].

The above reported multifunctionality of TFPI, and presence of an exposed cationic and heparin-binding C-terminus made us raise the question whether the C-terminal region of TFPI could exert a direct antimicrobial activity. We here show that C-terminal TFPI peptides may indeed directly kill both Gram-negative and Gram-positive bacteria and fungi. Furthermore, evidence is presented that the peptides may, also exert anticoagulant and antiinflammatory effects.

Histidine-rich glycoprotein and GHH25

The antimicrobial plasma protein Histidine-rich glycoprotein (HRG) protects the host against systemic microbial infections *in vivo*. Here, we show that HRG is a pattern recognition molecule, by binding LPS and increasing LPS mediated TLR-4 response in the host cells.

LPS interacts with CD14, a receptor on macrophages, monocytes and neutrophils, the interaction is increased by plasma protein LBP (LPS-binding protein). The

LPS/CD14 complex leads to activation of toll-like receptor (TLR) 4 which in turn activates the MyD88- dependent or -independent pathways [39]. The LPS-response can also be LBP-independent [40]. There is no difference in TNF- α release *in vivo* between wildtype and LBP-deficient mice after LPS injection, suggesting that a protein with LBP-like capabilities may be responsible [41]. Activation of TLR4 then in turn leads to an increase of phagocytic activity of mononuclear phagocytes, a cascade of released cytokines and nitric oxide (NO), which initiate and support the inflammatory response. NO is produced by a group of enzymes called nitric oxide synthases [42] and involved in many biological processes such as protecting the host against microbes, parasitic worms and tumours and regulate blood pressure, high levels of NO can instead be cytotoxic and destroy endogenous tissue.

The LPS-induced response is essential for the host defence, but an overwhelming response can instead lead to sepsis. Cytokines and NO are identified to be major contributors to the development of septic shock with symptoms as fever, coagulant activity, septic shock, multiple organ failure and in worst cases death of the host [43]. This is supported by the fact that inducible NOS (iNOS) mutant mice where resistant to LPS-induced mortality [44]. The susceptibility of different animal species to the toxicity of LPS is highly variable, for example humans are very sensitive [45, 46] comparing to mice that are fairly resistant [47]. A common misunderstanding is that sepsis is controlled by only pro-inflammatory mediators. The early stages of sepsis is dominated by pro-inflammatory mediators such as TNF- α , NO, bradykinin, thrombin and histamine, whereas anti-inflammatory mediators, such as IL-6, activated protein C, antithrombin and granulocyte colony-stimulating factor, are most widely occurring during the later stages [48].

The treatment of sepsis today includes administration of intravenous fluids, broad-spectrum antibiotics, protective lung ventilation, glucocorticoids, insulin therapy and recombinant human activator protein C (APC). Many attempts have been done to neutralize LPS or inhibit LPS-mediated activation of host immune cells, such as blocking cytokine activity with antibodies, blocking NO production [49] and by using antimicrobial peptides as neutralizers of LPS [50].

The aim of this study was to investigate the effects of an abundant plasma protein, HRG, on LPS-mediated responses. HRG is a 67 kDa plasma protein synthesized in the liver and was first described in 1972 [51, 52]. HRG can also be released upon thrombin activation from α -granules of thrombocytes, and is able to cover the surface

of a fibrin clot. Together with fetuins and kininogens, HRG belongs to the cystatin superfamily. The most distinguishing characteristic of the protein is the histidine-rich domain of the protein contains a conserved GHHPH repeat [53]. HRG has been shown to interact *in vitro* with a diverse group of ligands, like heparin, plasminogen, fibrinogen, thrombospondin, heme, IgG, FcγR and C1q. *In vivo*-studies demonstrates that HRG is an anticoagulant and antifibrinolytic modifier [54], has an inhibitory effects on tumour vascularization [55] and plays an important role in the innate immunity as a antimicrobial protein [56].

Herein, we demonstrate that HRG is a potent pro-inflammatory protein, by enhancing LPS induced NO and cytokines *in vitro*. *In vivo* experiment showed that Hrg-/- mice were markedly resistant against LPS induced sepsis. Our data clearly demonstrate that HRG contributes to LPS toxicity in experimental endotoxemia. However, the data also demonstrate an antiinflammatory effect of the peptides fragments of HRG, such as "GHH25".

Materials and Methods

Histidine-rich glycoprotein.

Synthetic peptide GHH25 (GHHPHGHHPHGHHPHGHHPHGHHPH [SEQ ID NO:11]) was from Biopeptide (San Diego, CA, USA). Polyclonal rabbit anti mouse TRL4 was purchased from GeneTex (Irvine, CA, USA). The cytometric bead array, mouse inflammation kit was from BD Biosciences (Stockholm, Sweden). *Escherichia coli* LPS (0111:B4) was purchased from Sigma (St Louis, MO).

Purification of Human HRG. Serum HRG was purified as described before [57]. Briefly, human serum were gently shaken at 4°C overnight with nickel-nitrilotriacetic acid (Ni-NTA) agarose, washed with phosphate-buffered saline (PBS, pH 7.4). Elution was first performed with PBS containing 80 mM imidazole to elute unspecifically bound proteins, and then with PBS containing 500 mM imidazole to elute purified HRG. The protein was dialyzed, freeze-dried and the concentration was determined using the Bradford method [58].

Radioiodination of heparin and LPS. The radioiodination of heparin (from porcine intestinal mucosa, Sigma-Aldrich) was performed as described previously [59]. The iodination of LPS was performed as described by Ulevitch [60]. 1 mg *Escherichia coli*

0111:B4 LPS was incubated in 50 mM p-OH benzimidate in borate buffer, pH 8, over night at 4°C, and then dialyzed against PBS, pH 7.4. LPS was then radiolabelled with ¹²⁵I using the chloramine T method, and unlabelled ¹²⁵I was then removed by dialysis.

5 *Heparin and LPS-binding assay.* 1, 2 and 5 µg of the synthetic peptides in 100 µl PBS pH 7.4 were applied onto nitrocellulose membranes (Hybond-C, Amersham Biosciences) using a slot-blot apparatus. Membranes were blocked for 1 h at room temperature with 2% bovine serum albumin in PBS pH 7.4 and then incubated with radiolabelled LPS (~40 µg·mL⁻¹, 0.13× 10⁶ cpm·µg⁻¹) or radiolabelled heparin (~10
10 µg·mL⁻¹, 0.4× 10⁶ cpm·µg⁻¹) for 1 h at room temperature in PBS, pH 7.4. Unlabeled heparin (6 mg/ml) was added for competition of binding. The membranes were washed 3 times in PBS, pH 7.4. A Bas 2000 radio-imaging system (Fuji Film, Tokyo, Japan) was used to visualize radioactivity.

15 *Cell culture.* Murine macrophage cell line, RAW 264.7 (kindly provided by Dr. H Björkbacka) were grown in Dulbeccos Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal calf serum (FCS). All experiments were performed under serum free conditions.

20 *Nitric oxide induction in RAW macrophages.* Confluent cells were harvested and transferred to 96-wells plate (3.5 x 10⁵/well). After adhesion cells were washed with phenol red-free DMEM (Gibco). *E. Coli* LPS (100ng/ml), LL-37 (2 or 10 µM) or HRG (2 or 10 µM) was preincubated at 37°C for 30 minutes and then transferred to the cells. For inhibition of NO induction, 5 µg/ml anti mouse TLR4 antibody, 10 or 100
25 µM GHH25 or 100 µg/ml heparin were used. The cells were stimulated for 24 hours and nitric oxide was determined using the Griess chemical method [61].

TNF-α release from human macrophages. Human monocyte-derived macrophages (hMDMs) were obtained from peripheral blood mononuclear cells (PBMCs) obtained
30 from the blood of healthy donors using a Lymphoprep (Axis-Shield PoC AS) density gradient. PBMCs were seeded at concentrations of 3×10⁶ cells/well into 24-well plates and cultured in RPMI1640 medium supplemented with 10% heat-inactivated autologous human plasma, 2 mM L-glutamine, and 50 µl/ml Antibiotic-Antimycotic (Gibco) in a humidified atmosphere of 5% CO₂. After 24 h, non-adherent cells were
35 removed and adherent monocytes were differentiated to macrophages for 10 days, with fresh medium changes every second day. The cells were stimulated for 24 hours with 10ng/ml of LPS with or without HRG (2 µM) and GHH25 (100 µM) under serum-

free conditions. After stimulation the supernatant was aspirated and TNF- α was measured using the TNF- α human ELISA kit (Invitrogen).

Animal experiments. The original knockout mice 129/B6-HRG^{tm1wja1} were crossed with C57BL/6 mice (Taconic) for 14 generations to obtain uniform genetic background. These HRG-deficient mouse strain was called B6-HRG^{tm1wja1} following ILAR (Institute of Laboratory Animal Resources) rules. Wildtype C57BL/6 control mice and C57BL/6 Hrg^{-/-} mice (8-12 weeks, 27 +/- 4g) were bred in the animal facility at Lund University. C57BL/6 Hrg^{-/-}, lacks the translation start point of exon 1 of the Hrg gene [54]. Animals were housed under standard conditions of light and temperature and had free access to standard laboratory chow and water. In order to induce sepsis, 180g/g Escherichia coli 0111:B4 LPS were injected intraperitoneally into C57BL/6 or C57BL/6 Hrg^{-/-} mice, divided into weight and sex matched groups. Survival and status was followed during seven days.

For treatment with GHH25 peptide, 1 mg of the peptide (diluted in 10mM Tris, pH 7.4) or buffer only was injected intraperitoneal 30 minutes after LPS-challenge and survival and status was then followed.

TFPI and Heparin cofactor II

Peptides. The TFPI and HCII-derived peptides were synthesized by Biopeptide Co., San Diego, USA, with the exception of LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES [SEQ ID NO: 101]), which was obtained from Innovagen AB, Lund, Sweden. The purity (>95%) of these peptides was confirmed by mass spectral analysis (MALDI-ToF Voyager).

Microorganisms. Bacterial isolates *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 90028 and *Candida parapsilosis* ATCC 90018 and were obtained from the Department of Bacteriology, Lund University Hospital.

Viable count analysis. *E. coli* ATCC 25922 bacteria were grown to mid-logarithmic phase in Todd-Hewitt (TH) medium. Bacteria were washed and diluted in 10 mM Tris, pH 7.4 containing 5 mM glucose. *E. coli* ATCC 25922 (50 μ l; 2 x 10⁶ cfu/ml) were incubated, at 37°C for 2 h with peptides at the indicated concentrations. Other experiments with the TFPI-peptides and LL-37 were performed in 10 mM Tris, pH

7.4, containing also 0.15 M NaCl, with normal or heat inactivated 20% citrate-plasma (PP). Serial dilutions of the incubation mixture were plated on TH agar, followed by incubation at 37°C overnight and cfu determination.

- 5 *Radial diffusion assay.* Essentially as described earlier [62, 63], bacteria were grown to mid-logarithmic phase in 10 ml of full-strength (3% w/v) trypticase soy broth (TSB) (Becton-Dickinson). The microorganisms were then washed once with 10 mM Tris, pH 7.4. Subsequently, 4×10^6 cfu were added to 15 ml of the underlay agarose gel, consisting of 0.03% (w/v) TSB, 1% (w/v) low electroendosmosis type (EEO) agarose
10 (Sigma-Aldrich) and 0.02% (v/v) Tween 20 (Sigma-Aldrich). The underlay was poured into a Ø 144 mm petri dish. After agarose solidification, 4 mm-diameter wells were punched and 6 µl peptide solution of required concentration added to each well. Plates were incubated at 37°C for 3 h to allow peptide diffusion. The underlay gel was then covered with 15 ml of molten overlay (6% TSB and 1% Low-EEO agarose
15 in distilled H₂O). Antimicrobial activity of a peptide was visualized as a zone of clearing around each well after 18-24 h of incubation at 37°C.

Fluorescence microscopy. The impermeant probe FITC (Sigma-Aldrich, St. Louis, USA) was used for monitoring of bacterial membrane permeabilization. *S. aureus*
20 ATCC 29213 bacteria were grown to mid-logarithmic phase in TSB medium. Bacteria were washed and resuspended in buffer (10 mM Tris, pH 7.4, 0.15M NaCl, 5 mM glucose) to yield a suspension of 1×10^7 CFU/ml. 100 µl of the bacterial suspension was incubated with 30 µM of the respective peptides at 30°C for 30 min. Microorganisms were then immobilized on poly (L-lysine)-coated glass slides by
25 incubation for 45 min at 30°C, followed by addition onto the slides of 200 µl of FITC (6 µg/ml) in buffer and a final incubation for 30 min at 30°C. The slides were washed and bacteria fixed by incubation, first on ice for 15 min, then in room temperature for 45 min in 4% paraformaldehyde. The glass slides were subsequently mounted on slides using Prolong Gold antifade reagent mounting medium (Invitrogen, Eugene,
30 USA). Bacteria were visualized using a Nikon Eclipse TE300 (Nikon, Melville, USA) inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled CCD camera (Hamamatsu, Bridgewater, USA) and a Plan Apochromat ×100 objective (Olympus, Orangeburg, USA). Differential interference contrast (Nomarski) imaging was used for visualization of the microbes themselves.

Hemolysis assay. EDTA-blood was centrifuged at 800 g for 10 min, whereafter plasma and buffy coat were removed. The erythrocytes were washed three times and resuspended in PBS, pH 7.4 to get a 5% suspension. The cells were then incubated with end-over-end rotation for 60 min at 37°C in the presence of peptides (60 µM).
5 2% Triton X-100 (Sigma-Aldrich) served as positive control. The samples were then centrifuged at 800 g for 10 min and the supernatant was transferred to a 96 well microtiter plate. The absorbance of hemoglobin release was measured at λ 540 nm and is in the plot expressed as % of TritonX-100 induced hemolysis.

10 *Lactate dehydrogenase (LDH) assay.* HaCaT keratinocytes were grown to confluency in 96 well plates (3000 cells/well) in serum-free keratinocyte medium (SFM) supplemented with bovine pituitary extract and recombinant EGF (BPE-rEGF) (Invitrogen, Eugene, USA). The medium was then removed, and 100 µl of the peptides investigated (at 60 µM, diluted in SFM/BPE-rEGF or in keratinocyte-SFM
15 supplemented with 20% human serum) were added. The LDH-based TOX-7 kit (Sigma-Aldrich, St. Louis, USA) was used for quantification of LDH release from the cells. Results represent mean values from triplicate measurements, and are given as fractional LDH release compared to the positive control consisting of 1% Triton X-100 (yielding 100% LDH release).

20 *Slot-blot assay.* LPS binding ability of the peptides were examined by slot-blot assay. Peptides (2 and 5 µg) were bound to nitrocellulose membrane (Hybond-C, GE Healthcare BioSciences, UK), pre-soaked in PBS, by vacuum. Membranes were then blocked by 2 wt% BSA in PBS, pH 7.4, for 1 h at RT and subsequently incubated
25 with ¹²⁵I-labelled LPS (40 µg/mL; 0.13×10⁶ cpm/µg) or ¹²⁵I-labelled heparin (Sigma) for 1 h at RT in 10 mM Tris, pH 7.4, 0.15 M NaCl, or 10 mM MES, pH 5.5, 0.15 M NaCl. After LPS binding, membranes were washed 3 times, 10 min each time in the above buffers and visualized for radioactivity on Bas 2000 radioimaging system (Fuji, Japan).

30 *Liposome preparation and leakage assay.* The liposomes investigated were either zwitterionic (DOPC/cholesterol 60/40 mol/mol or DOPC without cholesterol) or anionic (DOPE/DOPG 75/25 mol/mol). DOPG (1,2-Dioleoyl-*sn*-Glycero-3-Phosphoglycerol, monosodium salt), DOPC (1,2-dioleoyl-*sn*-Glycero-3-phosphocholine), and DOPE (1,2-dioleoyl-*sn*-Glycero-3-phosphoethanolamine) were all
35 from Avanti Polar Lipids (Alabaster, USA) and of >99% purity, while cholesterol

(>99% purity), was from Sigma-Aldrich (St. Louis, USA). Due to the long, symmetric and unsaturated acyl chains of these phospholipids, several methodological advantages are reached. In particular, membrane cohesion is good, which facilitates very stable, unilamellar, and largely defect-free liposomes (observed from cryo-TEM) and well defined supported lipid bilayers (observed by ellipsometry and AFM), allowing detailed values on leakage and adsorption density to be obtained. The lipid mixtures were dissolved in chloroform, after which solvent was removed by evaporation under vacuum overnight. Subsequently, 10 mM Tris buffer, pH 7.4, was added together with 0.1 M carboxyfluorescein (CF) (Sigma, St. Louis, USA). After hydration, the lipid mixture was subjected to eight freeze-thaw cycles consisting of freezing in liquid nitrogen and heating to 60°C. Unilamellar liposomes of about Ø140 nm were generated by multiple extrusions through polycarbonate filters (pore size 100 nm) mounted in a LipoFast miniextruder (Avestin, Ottawa, Canada) at 22°C. Untrapped CF was removed by two subsequent gel filtrations (Sephadex G-50, GE Healthcare, Uppsala, Sweden) at 22°C, with Tris buffer as eluent. CF release from the liposomes was determined by monitoring the emitted fluorescence at 520 nm from a liposome dispersion (10 mM lipid in 10 mM Tris, pH 7.4). An absolute leakage scale was obtained by disrupting the liposomes at the end of each experiment through addition of 0.8 mM Triton X-100 (Sigma-Aldrich, St. Louis, USA). A SPEX-fluorolog 1650 0.22-m double spectrometer (SPEX Industries, Edison, USA) was used for the liposome leakage assay in Tris buffer in the absence and presence of liposomes under conditions described above. Measurements were performed in triplicate at 37 °C.

CD-spectroscopy. The CD spectra of the peptides in solution were measured on a Jasco J-810 Spectropolarimeter (Jasco, U.K.). The measurements were performed at 37°C in a 10 mm quartz cuvet under stirring and the peptide concentration was 10 µM. The effect on peptide secondary structure of liposomes at a lipid concentration of 100 µM was monitored in the range 200-250 nm. The only peptide conformations observed under the conditions investigated were α-helix and random coil. The fraction of the peptide in α -helical conformation, X_α , was calculated from

$$X_\alpha = (A - A_c) / (A_\alpha - A_c)$$

where A is the recorded CD signal at 225 nm, and A_α and A_c are the CD signal at 225 nm for a reference peptide in 100% α-helix and 100% random coil conformation,

respectively. 100% α -helix and 100% random coil references were obtained from 0.133 mM (monomer concentration) poly-L-lysine in 0.1 M NaOH and 0.1 M HCl, respectively[64, 65]. For determination of effects of lipopolysaccharide on peptide structure, the peptide secondary structure was monitored at a peptide concentration of 10 μ M, both in Tris buffer and in the presence of *E. coli* lipopolysaccharide (0.02 wt%) (*Escherichia coli* 0111:B4, highly purified, less than 1% protein/RNA, Sigma, UK). To account for instrumental differences between measurements the background value (detected at 250 nm, where no peptide signal is present) was subtracted. Signals from the bulk solution were also corrected for.

Effects of various microbial products on macrophages in vitro and anti-inflammatory effects by various peptides of HCII and TFPI. 3.5×10^5 cells were seeded in 96-well tissue culture plates (Nunc, 167008) in phenol red-free DMEM (Gibco) supplemented with 10% FBS and antibiotics. Following 6 hours of incubation to permit adherence, cells were stimulated with 10 ng/mL *E. coli* (0111:B4) LPS (Sigma), lipoteichoic acid, peptidoglycan, or zymosan, with and without peptides at the indicated doses (see figure legends and figures). The levels of NO in culture supernatants were determined after 24 hours from stimulation using the Griess reaction[66]. Briefly, nitrite, a stable product of NO degradation, was measured by mixing 50 μ L of culture supernatants with the same volume of Griess reagent (Sigma, G4410) and reading absorbance at 550 nm after 15 min. Phenol-red free DMEM with FBS and antibiotics were used as a blank. A standard curve was prepared using 0-80 μ M sodium nitrite solutions in ddH₂O.

Clotting assays. All clotting times were measured using an Amelung coagulometer. Activated partial thromboplastin time (aPTT) was measured by incubating the peptides diluted in sterile water at the indicated concentrations, with 100 μ L citrated human plasma for 1 minute followed by the addition of 100 μ L aPTT reagent (aPTT Automate, Diagnostica Stago) for 60 seconds at 37°C. Clotting was initiated by the addition of 100 μ L of a 25-mM CaCl₂ solution. In the prothombrin time assay (PT), clotting was initiated by the addition of 100 μ L Thrombomax with calcium (PT reagent; Sigma-Aldrich). For measuring the thrombin clotting time (TCT), clotting was initiated by the addition of 100 μ L Accuclot thrombin time reagent (TCT reagent; Sigma-Aldrich).

Results and Conclusions

In summary, peptides reported above corresponding cryptic heparin binding sites in serpins such as HCII and ATIII, as well as other proteins including HRG and TFPI possess antibacterial, anti-inflammatory and anti-coagulative properties (Fig. 1-18, 29-33). Of particular importance was the finding that the HCII peptides blocked TLR-mediated LPS responses as well as the intrinsic pathway of coagulation. Furthermore, the TFPI peptides showed unique and previously undisclosed inhibitory activities on both the intrinsic, as well as extrinsic pathways of coagulation. The results thus illustrate that the peptides not only attenuate bacterial infection and the related inflammatory response involving interference with macrophage activation, but importantly also interfere with coagulation, and therefore, show significant therapeutic potential for sepsis, COPD and other multifactorial diseases involving pathogenetic steps including inflammation and coagulation.

References

1. Lehrer, R.I. and T. Ganz, *Cathelicidins: a family of endogenous antimicrobial peptides*. Curr Opin Hematol, 2002. **9**(1): p. 18-22.
2. Harder, J., R. Glaser, and J.M. Schröder, *Review: Human antimicrobial proteins effectors of innate immunity*. J Endotoxin Res, 2007. **13**(6): p. 317-38.
3. Zasloff, M., *Antimicrobial peptides of multicellular organisms*. Nature, 2002. **415**(6870): p. 389-95.
4. Tossi, A., L. Sandri, and A. Giangaspero, *Amphipathic, alpha-helical antimicrobial peptides*. Biopolymers, 2000. **55**(1): p. 4-30.
5. Yount, N.Y., et al., *Advances in antimicrobial peptide immunobiology*. Biopolymers, 2006.
6. Zanetti, M., *Cathelicidins, multifunctional peptides of the innate immunity*. J Leukoc Biol, 2004. **75**(1): p. 39-48.
7. Elsbach, P., *What is the real role of antimicrobial polypeptides that can mediate several other inflammatory responses?* J Clin Invest, 2003. **111**(11): p. 1643-5.
8. Ganz, T., *Defensins: antimicrobial peptides of innate immunity*. Nat Rev Immunol, 2003. **3**(9): p. 710-20.
9. Cole, A.M., et al., *Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity*. J Immunol, 2001. **167**(2): p. 623-7.
10. Brogden, K.A., *Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?* Nat Rev Microbiol, 2005. **3**(3): p. 238-50.
11. Kowalska, K., D.B. Carr, and A.W. Lipkowski, *Direct antimicrobial properties of substance P*. Life Sci, 2002. **71**(7): p. 747-50.
12. Mor, A., M. Amiche, and P. Nicolas, *Structure, synthesis, and activity of dermaseptin b, a novel vertebrate defensive peptide from frog skin: relationship with adenoregulin*. Biochemistry, 1994. **33**(21): p. 6642-50.
13. Malmsten, M., et al., *Antimicrobial peptides derived from growth factors*. Growth Factors, 2007. **25**(1): p. 60-70.

14. Nordahl, E.A., et al., *Activation of the complement system generates antibacterial peptides*. Proc Natl Acad Sci U S A, 2004. **101**(48): p. 16879-84.
15. Pasupuleti, M., et al., *Preservation of antimicrobial properties of complement peptide C3a, from invertebrates to humans*. J Biol Chem, 2007. **282**(4): p. 2520-8.
- 5 16. Frick, I.M., et al., *The contact system—a novel branch of innate immunity generating antibacterial peptides*. Embo J, 2006. **25**(23): p. 5569-78.
17. Nordahl, E.A., et al., *Domain 5 of high molecular weight kininogen is antibacterial*. J Biol Chem, 2005. **280**(41): p. 34832-9.
- 10 18. Rydengard, V., E. Andersson Nordahl, and A. Schmidtchen, *Zinc potentiates the antibacterial effects of histidine-rich peptides against Enterococcus faecalis*. Febs J, 2006. **273**(11): p. 2399-406.
19. Davie, E.W. and J.D. Kulman, *An overview of the structure and function of thrombin*. Semin Thromb Hemost, 2006. **32 Suppl 1**: p. 3-15.
- 15 20. Bode, W., *The structure of thrombin: a janus-headed proteinase*. Semin Thromb Hemost, 2006. **32 Suppl 1**: p. 16-31.
21. Chapman, A.P., *PEGylated antibodies and antibody fragments for improved therapy: a review*. Adv Drug Deliv Rev, 2002. **54**(4): p. 531-45.
22. Veronese, F.M. and J.M. Harris, *Introduction and overview of peptide and protein pegylation*. Adv Drug Deliv Rev, 2002. **54**(4): p. 453-6.
- 20 23. Veronese, F.M. and G. Pasut, *PEGylation, successful approach to drug delivery*. Drug Discov Today, 2005. **10**(21): p. 1451-8.
24. Wang, Y.S., et al., *Structural and biological characterization of pegylated recombinant interferon alpha-2b and its therapeutic implications*. Adv Drug Deliv Rev, 2002. **54**(4): p. 547-70.
- 25 25. Sato, H., *Enzymatic procedure for site-specific pegylation of proteins*. Adv Drug Deliv Rev, 2002. **54**(4): p. 487-504.
26. Bowen, S., et al., *Relationship between molecular mass and duration of activity of polyethylene glycol conjugated granulocyte colony-stimulating factor mutein*. Exp Hematol, 1999. **27**(3): p. 425-32.
- 30 27. Chapman, A.P., et al., *Therapeutic antibody fragments with prolonged in vivo half-lives*. Nat Biotechnol, 1999. **17**(8): p. 780-3.
28. Lwaleed, B.A. and P.S. Bass, *Tissue factor pathway inhibitor: structure, biology and involvement in disease*. J Pathol, 2006. **208**(3): p. 327-39.
- 35 29. Girard, T.J., et al., *Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor*. Nature, 1989. **338**(6215): p. 518-20.
30. Mine, S., et al., *Structural mechanism for heparin-binding of the third Kunitz domain of human tissue factor pathway inhibitor*. Biochemistry, 2002. **41**(1): p. 78-85.
- 40 31. Crawley, J.T. and D.A. Lane, *The haemostatic role of tissue factor pathway inhibitor*. Arterioscler Thromb Vasc Biol, 2008. **28**(2): p. 233-42.
32. Park, C.T., A.A. Creasey, and S.D. Wright, *Tissue factor pathway inhibitor blocks cellular effects of endotoxin by binding to endotoxin and interfering with transfer to CD14*. Blood, 1997. **89**(12): p. 4268-74.
- 45 33. Hembrough, T.A., et al., *Identification and characterization of a very low density lipoprotein receptor-binding peptide from tissue factor pathway inhibitor that has antitumor and antiangiogenic activity*. Blood, 2004. **103**(9): p. 3374-80.
- 50 34. Wesselschmidt, R., et al., *Tissue factor pathway inhibitor: the carboxy-terminus is required for optimal inhibition of factor Xa*. Blood, 1992. **79**(8): p. 2004-10.
35. Ettelaie, C., et al., *The role of the C-terminal domain in the inhibitory functions of tissue factor pathway inhibitor*. FEBS Lett, 1999. **463**(3): p. 341-4.

36. Ohkura, N., et al., *A novel degradation pathway of tissue factor pathway inhibitor: incorporation into fibrin clot and degradation by thrombin*. Blood, 1997. **90**(5): p. 1883-92.
37. Li, A. and T.C. Wun, *Proteolysis of tissue factor pathway inhibitor (TFPI) by plasmin: effect on TFPI activity*. Thromb Haemost, 1998. **80**(3): p. 423-7.
38. Cunningham, A.C., et al., *Structural and functional characterization of tissue factor pathway inhibitor following degradation by matrix metalloproteinase-8*. Biochem J, 2002. **367**(Pt 2): p. 451-8.
39. Lu, Y.C., W.C. Yeh, and P.S. Ohashi, *LPS/TLR4 signal transduction pathway*. Cytokine, 2008. **42**(2): p. 145-51.
40. Nakatomi, K., et al., *Neutrophils responded to immobilized lipopolysaccharide in the absence of lipopolysaccharide-binding protein*. J Leukoc Biol, 1998. **64**(2): p. 177-84.
41. Wurfel, M.M., et al., *Targeted deletion of the lipopolysaccharide (LPS)-binding protein gene leads to profound suppression of LPS responses ex vivo, whereas in vivo responses remain intact*. J Exp Med, 1997. **186**(12): p. 2051-6.
42. Kichler, A., et al., *Histidine-rich amphipathic peptide antibiotics promote efficient delivery of DNA into mammalian cells*. Proc Natl Acad Sci U S A, 2003. **100**(4): p. 1564-8.
43. Karima, R., et al., *The molecular pathogenesis of endotoxic shock and organ failure*. Mol Med Today, 1999. **5**(3): p. 123-32.
44. Wei, X.Q., et al., *Altered immune responses in mice lacking inducible nitric oxide synthase*. Nature, 1995. **375**(6530): p. 408-11.
45. Michie, H.R., et al., *Detection of circulating tumor necrosis factor after endotoxin administration*. N Engl J Med, 1988. **318**(23): p. 1481-6.
46. Sauter, C. and C. Wolfensberger, *Interferon in human serum after injection of endotoxin*. Lancet, 1980. **2**(8199): p. 852-3.
47. Dinges, M.M. and P.M. Schlievert, *Comparative analysis of lipopolysaccharide-induced tumor necrosis factor alpha activity in serum and lethality in mice and rabbits pretreated with the staphylococcal superantigen toxic shock syndrome toxin 1*. Infect Immun, 2001. **69**(11): p. 7169-72.
48. Opal, S.M., *The host response to endotoxin, antilipopolysaccharide strategies, and the management of severe sepsis*. Int J Med Microbiol, 2007. **297**(5): p. 365-77.
49. Schwartz, D. and R.C. Blantz, *Nitric oxide, sepsis, and the kidney*. Semin Nephrol, 1999. **19**(3): p. 272-6.
50. Kirikae, T., et al., *Protective effects of a human 18-kilodalton cationic antimicrobial protein (CAP18)-derived peptide against murine endotoxemia*. Infect Immun, 1998. **66**(5): p. 1861-8.
51. Haupt, H. and N. Heimbürger, *[Human serum proteins with high affinity for carboxymethylcellulose. I. Isolation of lysozyme, C1q and 2 hitherto unknown -globulins]*. Hoppe Seylers Z Physiol Chem, 1972. **353**(7): p. 1125-32.
52. Heimbürger, N., et al., *[Human serum proteins with high affinity to carboxymethylcellulose. II. Physico-chemical and immunological characterization of a histidine-rich 3,8S- 2 -glycoprotein (CM-protein I)]*. Hoppe Seylers Z Physiol Chem, 1972. **353**(7): p. 1133-40.
53. Jones, A.L., M.D. Hulett, and C.R. Parish, *Histidine-rich glycoprotein: A novel adaptor protein in plasma that modulates the immune, vascular and coagulation systems*. Immunol Cell Biol, 2005. **83**(2): p. 106-18.
54. Tsuchida-Straeten, N., et al., *Enhanced blood coagulation and fibrinolysis in mice lacking histidine-rich glycoprotein (HRG)*. J Thromb Haemost, 2005. **3**(5): p. 865-72.
55. Olsson, A.K., et al., *A fragment of histidine-rich glycoprotein is a potent inhibitor of tumor vascularization*. Cancer Res, 2004. **64**(2): p. 599-605.

56. Rydengård, V., et al., *Histidine-rich glycoprotein protects from systemic Candida infection*. PLoS Pathog, 2008. 4(8): p. e1000116.
57. Rydengård, V., et al., *Histidine-rich glycoprotein exerts antibacterial activity*. Febs J, 2007. 274(2): p. 377-89.
- 5 58. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*. Anal Biochem, 1976. 72: p. 248-54.
59. Cheng, F., et al., *A new method for sequence analysis of glycosaminoglycans from heavily substituted proteoglycans reveals non-random positioning of 4- and 6-O-sulphated N-acetylgalactosamine in aggrecan-derived chondroitin sulphate*. Glycobiology, 1992. 2(6): p. 553-61.
- 10 60. Ulevitch, R.J., *The preparation and characterization of a radioiodinated bacterial lipopolysaccharide*. Immunochemistry, 1978. 15(3): p. 157-64.
61. Park, E., et al., *Taurine chloramine inhibits the synthesis of nitric oxide and the release of tumor necrosis factor in activated RAW 264.7 cells*. J Leukoc Biol, 1993. 54(2): p. 119-24.
- 15 62. Andersson, E., et al., *Antimicrobial activities of heparin-binding peptides*. Eur J Biochem, 2004. 271(6): p. 1219-26.
63. Lehrer, R.I., et al., *Ultrasensitive assays for endogenous antimicrobial polypeptides*. J Immunol Methods, 1991. 137(2): p. 167-73.
- 20 64. Greenfield, N. and G.D. Fasman, *Computed circular dichroism spectra for the evaluation of protein conformation*. Biochemistry, 1969. 8(10): p. 4108-16.
65. Sjogren, H. and S. Ulvenlund, *Comparison of the helix-coil transition of a titrating polypeptide in aqueous solutions and at the air-water interface*. Biophys Chem, 2005. 116(1): p. 11-21.
- 25 66. Pollock, J.S., et al., *Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells*. Proc Natl Acad Sci U S A, 1991. 88(23): p. 10480-4.

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EXAMPLE B - C-Terminal Peptides Of Tissue-Factor Pathway Inhibitor Are Novel Host Defense Molecules

Abstract

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Tissue factor pathway inhibitor (TFPI) inhibits tissue factor-induced coagulation, but may, via its C-terminus, also modulate cell surface-, heparin-, and lipopolysaccharide interactions as well as participate in growth inhibition. Here we show that C-terminal TFPI peptide sequences are antimicrobial against the Gram-negative bacteria
10 *Escherichia coli* and *Pseudomonas aeruginosa*, Gram-positive *Bacillus subtilis* and *Staphylococcus aureus*, as well as the fungi *Candida albicans* and *Candida parapsilosis*. Fluorescence studies of peptide-treated bacteria, paired with analysis of peptide effects on liposomes, showed that the peptides exerted membrane-breaking effects similar to those seen for the "classical" human antimicrobial peptide LL-37.
15 The killing of *E. coli*, but not *P. aeruginosa*, by the C-terminal peptide GGLIKTRKRKKQRVKIAEYEEIFVKNM [SEQ ID NO: 4] (GGL27), was enhanced in human plasma and largely abolished in heat-inactivated plasma, a phenomenon linked to generation of antimicrobial C3a and activation of the classical pathway of complement activation. Furthermore, GGL27 displayed anti-endotoxic effects *in vitro*
20 and *in vivo* in a mouse model of LPS-shock. Importantly, TFPI was found to be expressed in the basal layers of normal epidermis, and was markedly up-regulated in acute skin wounds as well as wound edges of chronic leg ulcers. Furthermore, C-terminal fragments of TFPI were associated with bacteria present in human chronic leg ulcers. These findings suggest a new role for TFPI in cutaneous defense against
25 infections.

Introduction

In order to control our microbial flora, humans and virtually all life forms are armored
30 with rapidly acting host defense systems based on various antimicrobial peptides (1-3). The majority of these peptides is characterized by an amphipathic structure, and comprise linear peptides, many of which adopt an α -helical and amphipathic conformation upon bacterial binding, peptides forming cysteine-linked antiparallel β -sheets, as well as cysteine-constrained loop structures. In addition, antimicrobial
35 peptides may be found among peptides not displaying such ordered structures as long as these are characterized by an over-representation of certain amino acids

(1,4-6). Although interactions with bacterial membranes are fundamental for antimicrobial peptide function, the exact modes of action are complex, and can be divided into membrane disruptive and non-membrane disruptive (1,3,7,8). During recent years it has also become increasingly evident that many cationic and
5 amphipathic antimicrobial peptides, such as defensins and cathelicidins, are multifunctional, also mediating immunomodulatory roles and angiogenesis (9-11), thus motivating the recent and broader definition host defense peptides for these members of the innate immune system.

10 Tissue factor pathway inhibitor (or TFPI) is a Kunitz-type proteinase inhibitor which reversibly inhibits the tissue factor-factor VII (TF-VII) complex in a factor X (FX) - dependent manner, leading to inhibition of both FX and FIX activation. TFPI consists of a highly negatively-charged N-terminus, three tandemly-linked Kunitz-type domains, and a highly positively-charged C-terminus. In plasma, TFPI exists in both
15 full-length and various C-terminal truncated forms (12). The first and second Kunitz domains are involved in binding and inhibition of the TF-VII complex and factor Xa, respectively (13). The third Kunitz domain, in turn, may interact with heparin via its cationic C-terminal end (14). This C-terminal region has also been implicated in interactions with plasma lipoproteins, thrombospondin-1, clearance receptors (15),
20 lipopolysaccharide (16), and may also inhibit cell growth (17) and blood coagulation (18,19). Since various C-terminally truncated forms exist *in vivo*, a potential role of proteolysis of the C-terminus has been implicated, and data indicate that TFPI can be cleaved by various proteinases such as thrombin (20), plasmin (21), and matrix metalloproteinase-8 (22), thereby releasing C-terminal fragments. Upregulators of
25 TFPI expression include endotoxin, IL-1, TNF- α , platelet-derived growth factor, heparin, and basic fibroblast growth factor, all molecules involved in infection, inflammation, and growth (15).

30 The above reported multifunctionality of TFPI, and presence of an exposed cationic and heparin-binding C-terminus made us raise the question whether the C-terminal region of TFPI could exert antimicrobial activity. We here show that C-terminal TFPI peptides, found to be expressed in skin wounds, and detected in fibrin of chronic leg ulcers, may indeed function as host defense peptides. Furthermore, killing of the Gram-negative *E. coli*, but not *P. aeruginosa*, was markedly boosted by peptide-mediated complement activation, including formation of the membrane attack complex (MAC) and antimicrobial C3a.
35

Materials & Methods

Peptides – The TFPI-derived peptides (see Fig. 1) and the control peptides GGL27(S) (GGLISTSSSSSSQRVKIAEIEIFVKNM [SEQ ID NO: 102]) and DSE25 (DSEEDDEHTIITDELPLKLMHSF [SEQ ID NO: 103]) were synthesized by Biopeptide Co., San Diego, USA, while LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES [SEQ ID NO: 101]), was obtained from Innovagen AB, Lund, Sweden. The purity (>95%) of these peptides was confirmed by mass spectral analysis (MALDI-ToF Voyager).

Microorganisms – Bacterial isolates *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 90028 and *Candida parapsilosis* ATCC 90018 were obtained from the Department of Bacteriology, Lund University Hospital. Other clinical isolates of *E. coli* and *P. aeruginosa* were from patients with skin infections.

Biological materials – Fibrin slough was collected from chronic venous leg ulcers (chronic wound slough/surface) with a sterile spatula and immediately fixed for electron microscopy. Tissue sections from 3 patients with chronic venous ulcers (duration > 6 months) were analysed. 4 mm biopsies were taken from the edge of the wound and a control area on the thigh. For acute wounds, a 4 mm biopsy was taken from the thigh of one individual, and subsequent biopsies from the wound edges were taken at day 5 and 8. Biopsies from normal skin (n=3, thigh) were also taken for analysis. The research project was approved by the Ethics committee, Lund University Hospital. Written consent was obtained from the patients.

Radial diffusion assay – Essentially as described earlier (23,24), bacteria were grown to mid-logarithmic phase in 10 ml of full-strength (3% w/v) trypticase soy broth (TSB) (Becton-Dickinson). The microorganisms were then washed once with 10 mM Tris, pH 7.4. Subsequently, 4×10^6 cfu were added to 15 ml of the underlay agarose gel, consisting of 0.03% (w/v) TSB, 1% (w/v) low electroendosmosis type (EEO) agarose (Sigma-Aldrich) and 0.02% (v/v) Tween 20 (Sigma-Aldrich). The underlay was poured into a Ø 144 mm petri dish. After agarose solidification, 4 mm-diameter wells were punched and 6 µl peptide solution of required concentration added to each well. Plates were incubated at 37°C for 3 h to allow peptide diffusion. The underlay gel was then covered with 15 ml of molten overlay (6% TSB and 1% Low-EEO agarose in distilled H₂O). Antimicrobial activity of a peptide was visualized as a clearance

zone around each well after 18-24 h of incubation at 37°C.

Viable count analysis – *E. coli* strains were grown to mid-logarithmic phase in Todd-Hewitt (TH). *P. aeruginosa* strains were grown in TH overnight. Bacteria were
5 washed and diluted in 10 mM Tris, pH 7.4 containing 5 mM glucose. 2×10^6 cfu/ml bacteria were incubated in 50 μ l, at 37°C for 2 h with the C-terminal TFPI-derived peptides GGL27, TKR22, or LIK17 at the indicated concentrations. Additional experiments were performed in 10 mM Tris, pH 7.4, containing 0.15 M NaCl, either alone or with 20% normal or heat inactivated citrate-plasma. Serial dilutions of the
10 incubation mixture were plated on TH agar, followed by incubation at 37°C overnight and cfu determination.

Flow cytometry analysis – Fifty μ l of bacteria ($1-2 \times 10^9$ cfu) were incubated with 450 μ l of human plasma either alone or supplemented with GGL27 (at 3 μ M). Samples
15 were incubated for 30 min or 1 h at 37°C, divided into two equal parts, centrifuged, washed with PBS, and resuspended in 100 μ l PBS with rabbit polyclonal antibodies against either LGE27 (25), a C-terminal epitope of human C3a, or rabbit polyclonal antibodies against C1q (both at 1:100). The mixtures were subsequently incubated for 1 h at room temperature. Bacteria were pelleted and washed twice with PBS,
20 incubated in 100 μ l PBS with goat anti rabbit IgG FITC-labeled antibodies (1:500, Sigma) for 30 min at room temperature and washed twice with PBS. In another experiment bacteria were incubated for 1 h in citrate plasma together with 3 μ M TAMRA-labeled GGL27 peptide, then pelleted and washed twice with PBS. Flow cytometry analysis (Becton-Dickinson, Franklin Lakes, NJ) was performed using a
25 FACS-Calibur flow cytometry system equipped with a 15 mW argon laser turned a 488 nm. The bacterial population was selected by gating with appropriate settings of forward scatter (FSC) and sideward scatter (SSC).

Fluorescence microscopy – Fluorescein isothiocyanate (FITC; Sigma-Aldrich, St. Louis, USA) was used for monitoring of bacterial membrane permeabilization. *E. coli*
30 ATCC 25922 bacteria were grown to mid-logarithmic phase in TSB medium. Bacteria were washed and resuspended in buffer (10 mM Tris, pH 7.4, 0.15M NaCl, 5 mM glucose) to yield a suspension of 1×10^7 cfu/ml. 100 μ l of the bacterial suspension was incubated with 30 μ M of the respective peptides at 30°C for 30 min.
35 Microorganisms were then immobilized on poly (L-lysine)-coated glass slides by incubation for 45 min at 30°C, followed by addition onto the slides of 200 μ l of FITC

(6 µg/ml) in buffer and a final incubation for 30 min at 30°C. The slides were washed and bacteria fixed by incubation, first on ice for 15 min, then in room temperature for 45 min in 4% paraformaldehyde. The glass slides were subsequently mounted on slides using Prolong Gold antifade reagent mounting medium (Invitrogen, Eugene, USA). Bacteria were visualized using a Nikon Eclipse TE300 (Nikon, Melville, USA) inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled CCD camera (Hamamatsu, Bridgewater, USA) and a Plan Apochromat ×100 objective (Olympus, Orangeburg, USA). Differential interference contrast (Nomarski) imaging was used for visualization of the microbes themselves.

Histochemistry – For immunostaining, wound biopsies were fixed in 10% formalin, rehydrated and embedded in paraffin. Sections of 5 µm thickness were placed on poly-lysine coated glass slides, deparaffinized in xylene and rehydrated in graded alcohols. The slides were then treated with Dako antigen retrieval solution (Dako) for 40 min at 97°C, and incubated for 24 h at room temperature in a 1:50 dilution of polyclonal antibodies against TFPI (Sigma-Aldrich, in TBS with 1% BSA, 5% goat serum, 0.05% Tween 20). After three 20 min washes in TBS with 0.05% Tween 20, the sections were incubated with alkaline phosphatase conjugated secondary goat anti-rabbit IgG (Dako) diluted 1:1000 in the same buffer as the primary antibody and incubated for another 24 hours, followed by three 20 min washes. Sections were developed with Vulcan Fast Red chromogen (Biocare Medical, Concord, CA) and the slides were counterstained with Harris Hematoxylin (EM Science, Gibbstown, NJ). For histological evaluation of lungs derived from the *in vivo* LPS-models in mice, tissues were embedded as above, sectioned and stained with hematoxylin and eosin by routine procedures (Histocenter, Gothenburg, Sweden).

Electron Microscopy – For transmission electron microscopy and visualization of peptide effects on bacteria, *P. aeruginosa* ATCC 27853 ($1-2 \times 10^8$ cfu/sample) was incubated for 2 h at 37°C with the peptide GGL27 at 30 µM. LL-37 (30 µM) was included as a control. *P. aeruginosa* sample suspensions were adsorbed onto carbon-coated copper grids for 2 min, washed briefly on two drops of water, and negatively stained on two drops of 0.75 % uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. *In-vivo* experiment; Fibrin slough from patients with chronic venous ulcers (CWS) was fixed (1.5% PFA, 0.5% GA in 0.1 M phosphate buffer, pH 7.4) for 1 h at room temperature, followed by washing with 0.1 M phosphate buffer, pH 7.4. The fixed and washed samples were

subsequently dehydrated in ethanol and further processed for Lowicryl embedding (26). Sections were cut with a LKB ultratome and mounted on gold grids. For immunostaining, the grids were floated on top of drops of immune reagents displayed on a sheet of parafilm. Free aldehyde groups were blocked with 50 mM glycine, and the grids were then incubated with 5% (vol/vol) goat serum in incubation buffer (0.2% BSA-c in PBS, pH 7.6) for 15 minutes. This blocking procedure was followed by overnight incubation at 4°C with C-terminal TFPI goat polyclonal antibodies (1 µg/ml) (Abcam, UK) alone, or in combination with rabbit polyclonal antibodies against the C-terminal part of C3a (LGE27 antibodies) (1 µg/ml) (Innovagen AB) (25). Controls without primary antibodies were included. The grids were washed in a large volume (200 ml) of incubation buffer, floating on drops containing the gold conjugate reagents. For detection of TFPI-peptides, 1 µg/ml EM rabbit anti-goat IgG 10 nm Au (BBI) in incubation buffer was added and incubation performed for 2 h at 4°C. For simultaneous detection of TFPI and C3a, 1 µg/ml EM rabbit anti-goat IgG 20 nm Au (BBI) and 1 µg/ml EM goat anti-rabbit IgG 10 nm Au (BBI) were used. After further washes by an excess volume of incubation buffer, the sections were postfixed in 2% glutaraldehyde. Finally, sections were washed with distilled water and poststained with 2% uranyl acetate and lead citrate. All samples were examined with a Jeol JEM 1230 electron microscope operated at 80 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 charge-coupled device camera.

SDS-PAGE and immunoblotting – Human citrate plasma (450 µl) was incubated with 50 µl bacteria ($1-2 \times 10^9$ cfu) either alone or supplemented with the peptide GGL27 at 3 µM. The mixture was incubated for 30 min or 1 h at 37°C, centrifuged, and supernatants and bacteria collected. The bacterial pellet was washed with PBS and bound proteins eluted with 0.1 M glycine-HCl, pH 2.0. The pH of the eluted material was raised to 7.5 with 1 M Tris. Eluted proteins were precipitated by addition of 1 volume of trichloroacetic acid (TCA) to 4 volumes of sample, followed by incubation for 30 min on ice and centrifugation at 15 000g (4°C for 20 min). Precipitated material and supernatants were dissolved in SDS sample buffer and analyzed under reducing conditions by SDS-PAGE on 16.5% Tris-tricine gels (Clear PAGE™, C.B.S Scientific). Proteins and peptides were transferred to nitrocellulose membranes (Hybond-C). Membranes were blocked by 3% (w/v) skimmed milk, washed, and incubated for 1 h with rabbit polyclonal antibodies against the C-terminal part of C3a (LGE27 antibodies) (1:1000), rabbit polyclonal antibodies to C1q (1:1000) (Dako), goat polyclonal antibodies recognizing the C-terminal TFPI sequence

VKIAEEEEIFVKNM [SEQ ID NO: 104] (Abcam), or rabbit polyclonal antibodies to C5b-9 (1:1000) (Abcam, England). The membranes were washed three times for 10 min, and incubated (1 h) with HRP-conjugated secondary antibodies (1:2000) (Dako), and washed (3 x 10 min). Proteins were visualized by an enhanced chemiluminescent substrate (LumiGLO®) developing system (Upstate cell signaling solutions).

LPS effects on macrophages in vitro – 3.5×10^5 cells were seeded in 96-well tissue culture plates (Nunc, 167008) in phenol red-free DMEM (Gibco) supplemented with 10% FBS containing 1% Anti-Anti (Invitrogen). Following 20 hours of incubation to permit adherence, cells were washed and stimulated with 10 ng/ml *E. coli* (0111:B4) or *P. aeruginosa* LPS (Sigma), with and without the peptides GGL27, GGL27(S), and DSE25 of various doses. The levels of NO in culture supernatants were determined after 24 hours from stimulation using the Griess reaction (27). Briefly, nitrite, a stable product of NO degradation, was measured by mixing 50 μ l of culture supernatants with the same volume of Griess reagent (Sigma, G4410) and reading absorbance at 550 nm after 15 min. Phenol-red free DMEM with FBS and antibiotics were used as a blank. A standard curve was prepared using 0-80 μ M sodium nitrite solutions in ddH₂O.

Animal infection model – Animals were housed under standard conditions of light and temperature and had free access to standard laboratory chow and water. *P. aeruginosa* 15159 bacteria were grown to logarithmic phase ($OD_{620} \sim 0.5$), harvested, washed in PBS, diluted in the same buffer to 2×10^8 cfu/ml, and kept on ice until injection. Hundred microliter of the bacterial suspension was injected intraperitoneally (i.p.) into female Balb/c mice. Sixty minutes after the bacterial injection, 0.5 mg GGL27 or buffer alone was injected subcutaneously (s.c.) into the mice. In a corresponding *E. coli* infection model, bacteria were grown to early logarithmic phase ($OD_{620} \sim 0.4$), harvested, washed in PBS, diluted in the same buffer to 1×10^8 cfu/ml, and kept on ice until injection. Hundred microliter of the bacterial suspension was injected i.p. into female Balb/c mice. Thirty minutes after the bacterial injection, 0.2 mg of GGL27 peptide or buffer alone was injected i.p. Data from two independent experiments were pooled.

LPS model in vivo – Male C57BL/6 mice (8-10 weeks, 22 $\pm$ 5g), were injected intraperitoneally with 18 mg *E. coli* 0111:B4 LPS (Sigma) per kg of body weight. Thirty minutes after LPS injection, 0.5 mg GGL27, GGL27(S), DSE25 or buffer alone

was injected intraperitoneally into the mice. Survival and status was followed during seven days. For blood collection and histochemistry, mice were sacrificed 20 h after LPS challenge, and lungs were removed and fixed. These experiments were approved by the Laboratory Animal Ethics Committee of Malmö/Lund.

5

Cytokine assay – The cytokines IL-6, IL-10, MCP-1, INF- γ , and TNF- α were measured in plasma from mice subjected to LPS (with or without peptide treatment) using the Cytometric bead array; mouse inflammation kit (Becton Dickinson AB) according to the manufacturer's instructions.

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Hemolysis assay – EDTA-blood was centrifuged at 800 g for 10 min, whereafter plasma and buffy coat were removed. The erythrocytes were washed three times and resuspended in PBS, pH 7.4 to get a 5% suspension. The cells were then incubated with end-over-end rotation for 60 min at 37°C in the presence of peptides (60 μ M).

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2% Triton X-100 (Sigma-Aldrich) served as positive control. The samples were then centrifuged at 800 g for 10 min and the supernatant was transferred to a 96 well microtiter plate. The absorbance of hemoglobin release was measured at 540 nm and expressed as % of Triton X-100 induced hemolysis.

20

Lactate dehydrogenase (LDH) assay – HaCaT keratinocytes were grown to confluency in 96 well plates (3000 cells/well) in serum-free keratinocyte medium (SFM) supplemented with bovine pituitary extract and recombinant EGF (BPE-rEGF) (Invitrogen, Eugene, USA). The medium was then removed, and 100 μ l of the peptides investigated (at 60 μ M, diluted in SFM/BPE-rEGF or in keratinocyte-SFM supplemented with 20% human serum) were added. The LDH-based TOX-7 kit (Sigma-Aldrich, St. Louis, USA) was used for quantification of LDH release from the cells. Results represent mean values from triplicate measurements, and are given as fractional LDH release compared to the positive control consisting of 1% Triton X-100 (yielding 100% LDH release).

25

Liposome preparation and leakage assay – The liposomes investigated were anionic (DOPE/DOPG 75/25 mol/mol). DOPG (1,2-Dioleoyl-*sn*-Glycero-3-Phosphoglycerol, monosodium salt) and DOPE (1,2-dioleoyl-*sn*-Glycero-3-phosphoethanolamine) were both from Avanti Polar Lipids (Alabaster, USA) and of >99% purity. Due to the long, symmetric and unsaturated acyl chains of these phospholipids, several methodological advantages are reached. In particular, membrane cohesion is good, which facilitates very stable, unilamellar, and largely defect-free liposomes (observed

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from cryo-TEM), allowing detailed studies on liposome leakage. The lipid mixtures were dissolved in chloroform, after which solvent was removed by evaporation under vacuum overnight. Subsequently, 10 mM Tris buffer, pH 7.4, was added together with 0.1 M carboxyfluorescein (CF) (Sigma, St. Louis, USA). After hydration, the lipid mixture was subjected to eight freeze-thaw cycles consisting of freezing in liquid nitrogen and heating to 60°C. Unilamellar liposomes of about Ø140 nm were generated by multiple extrusions through polycarbonate filters (pore size 100 nm) mounted in a LipoFast miniextruder (Avestin, Ottawa, Canada) at 22°C. Untrapped CF was removed by two subsequent gel filtrations (Sephadex G-50, GE Healthcare, Uppsala, Sweden) at 22°C, with Tris buffer as eluent. CF release from the liposomes was determined by monitoring the emitted fluorescence at 520 nm from a liposome dispersion (10 µM lipid in 10 mM Tris, pH 7.4). An absolute leakage scale was obtained by disrupting the liposomes at the end of each experiment through addition of 0.8 mM Triton X-100 (Sigma-Aldrich, St. Louis, USA). A SPEX-fluorolog 1650 0.22-m double spectrometer (SPEX Industries, Edison, USA) was used for the liposome leakage assay. Measurements were performed in triplicate at 37 °C.

CD-spectroscopy – CD spectra of the peptides were measured on a Jasco J-810 Spectropolarimeter (Jasco, U.K.). The measurements were performed at 37°C in a 10 mm quartz cuvet under stirring and the peptide concentration was 10 µM. The effect on peptide secondary structure of liposomes at a lipid concentration of 100 µM was monitored in the range 200-250 nm. The fraction of the peptide in α-helical conformation, X_{α} , was calculated from

$$X_{\alpha} = (A - A_c) / (A_{\alpha} - A_c)$$

where A is the recorded CD signal at 225 nm, and A_{α} and A_c are the CD signal at 225 nm for a reference peptide in 100% α-helix and 100% random coil conformation, respectively. 100% α-helix and 100% random coil references were obtained from 0.133 mM (monomer concentration) poly-L-lysine in 0.1 M NaOH and 0.1 M HCl, respectively (28,29). For determination of effects of lipopolysaccharide on peptide structure, the peptide secondary structure was monitored at a peptide concentration of 10 µM, both in Tris buffer and in the presence of *E. coli* lipopolysaccharide (0.02 wt%) (*Escherichia coli* 0111:B4, highly purified, less than 1% protein/RNA, Sigma, UK). To account for instrumental differences between measurements the background value (detected at 250 nm, where no peptide signal is present) was subtracted. Signals from the bulk solution were also corrected for.

Phylogenetic analyses of TFPI – The TFPI amino acid sequence was retrieved from the NCBI site. Each sequence was analyzed with Psi-Blast (NCBI) to find the ortholog and paralog sequences. Sequences that showed structural homology >70% were selected. These sequences were aligned using ClustalW using Blosom 69 protein weight matrix settings. Internal adjustments were made taking the structural alignment into account utilizing the ClustalW interface. The level of consistency of each position within the alignment was estimated by using the alignment-evaluating software Tcoffee.

Statistical analysis – Bar diagrams (RDA, VCA) are presented as mean and standard deviation, from at least three independent experiments.

Results

To elucidate whether C-terminal peptides of TFPI possess antimicrobial activity, we investigated the effects of defined regions of TFPI previously reported to be generated by proteolytic action (plasmin and thrombin), as well as the peptide LIKTKRKRKKQRVKIAY [SEQ ID NO: 5] (LIK17) comprising the C-terminal heparin-binding epitope of TFPI (see Fig. 34A for sequences). The results showed that the peptides were indeed antimicrobial in radial diffusion assays (RDA) against Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*, Gram-positive *Bacillus subtilis* and *Staphylococcus aureus*, as well as the fungi *Candida albicans* and *Candida parapsilosis* (Fig. 34B). It is of note that the peptides displayed activities comparable to that of the "classical" AMP human cathelicidin LL-37 (Fig. 34B). In contrast, the control peptides GGL27(S), having the central K/R residues replaced by S, and the peptide DSE25, from the N-terminus of TFPI (see Experimental Procedures for sequences), yielded no antimicrobial effects against the above microbes (Fig. 43). The antibacterial results above were further substantiated by matrix-free viable count assays. The results from these dose-response experiments utilizing *E. coli* confirmed that particularly GGL27 and LIK17 displayed significant antibacterial activity (Fig. 34C).

FACS analyses showed that plasmin-generated GGL27 avidly bound to *E. coli* and *P. aeruginosa* in human plasma (Fig. 35A). Studies employing the impermeant probe FITC showed that LIK17 (Fig. 35B) and GGL27 (Fig. 44) permeabilized bacterial membranes of *E. coli* similarly to those seen after treatment with LL-37 (30,31) (Fig.

35B). Electron microscopy utilizing *P. aeruginosa* demonstrated extensive membrane damage, with cell envelopes of *P. aeruginosa* devoid of their cytoplasmic contents, and intracellular material found extracellularly (Fig. 35C). Again, similar findings were obtained with LL-37. These data suggest that the TFPI-derived peptides act on
5 bacterial membranes, however they do not demonstrate the exact mechanistic events following peptide addition to bacteria, as secondary metabolic effects on bacteria also may trigger bacterial death and membrane destabilization. Therefore, a liposome model was employed to study membrane permeabilization of LIK17 and GGL27. The peptides caused CF release (Fig. 36A), thus indicating a direct effect on
10 lipid membranes. Kinetic analysis showed that ~80% of the maximal release occurred within 5-10 minutes, comparable to results obtained with LL-37 (Fig. 36B). It is noteworthy that LIK17 and GGL27 displayed no significant conformational changes associated with binding to liposomes (Fig. 36C) and only relatively minor ones together with *E. coli* LPS (Fig. 36D), the latter originating from peptide and/or LPS,
15 contrasting to LL-37, which showed a significant increase in helicity on liposome binding. AMPs that kill bacteria may also exhibit hemolytic and membrane permeabilizing activities against eukaryotic cells. The results showed, however, that there was no hemolytic activity of the TFPI-derived peptides at doses of 3-60 μ M (Fig. 37A). This contrasted to LL-37, which readily permeabilized erythrocytes at
20 doses >6 μ M. Likewise, the TFPI-derived peptides did not permeabilize HaCaT cells, nor did they display any significant toxicity at the concentrations studied (Fig. 37B).

Since the activity of many antimicrobial peptides is abrogated by the presence of physiological salt as well as presence of "biomatrices" such as plasma or serum
25 (32,33), we also investigated the influence of salt and human plasma on peptide activity. As demonstrated in Figure 38A, the bactericidal activity of all three TFPI-derived peptides (GGL27, TKR22, and LIK17), particularly against *E. coli*, was not only retained in presence of human plasma, but significantly enhanced. It should here be noted that killing of *E. coli* by C-terminal TFPI peptides was recently shown
30 to be mediated via the classical pathway and linked to formation of the membrane attack complex (MAC) (34). Consequently, heat-inactivation of plasma abolished the potentiation, findings compatible with previous results showing that the presence of an intact complement system promotes the killing of this microbe. Additionally, it should be noted that the TFPI-derived peptides also mediated killing in heat-
35 inactivated plasma, although at higher concentrations (Fig. 38A), compatible with a direct peptide-mediated antimicrobial effect, as demonstrated in Figure 35 and 36. Interestingly, in contrast to the findings with *E. coli*, killing of *P. aeruginosa* was not

enhanced in human plasma when compared with physiological buffer. Furthermore, the potentiating effect of native plasma, when compared with heat-inactivated plasma, was less significant (notably for GGL27 and LIK17). The above findings were generalized using a panel of *E. coli* and *P. aeruginosa* isolates (Fig. 38B). In addition, kinetic studies demonstrated that the bacterial killing by the peptides occurred within 5-20 min indicating a fast direct action compatible with many antimicrobial peptides (Fig. 45).

Western blot experiments (Fig. 39A and B) and FACS analyses (Fig. 39C and D), showed that GGL27 enhanced binding of C1q to *E. coli*, resulting in increased formation of MAC (Fig. 39A, C and D). It also induced a significant generation of C3a (Fig. 39B, C and D), an anaphylatoxin previously shown to exert antimicrobial effects under physiological conditions against various bacteria, mediated by bacterial binding and membrane lysis (25). Taken together, these observations provide a novel mechanism for bacterial killing, based on initial bacterial binding of GGL27 (Fig. 35A), followed by boosting of formation and bacterial binding of antimicrobial C3a (Fig. 39B, C, and D), which therefore should further add to the total antimicrobial effect induced by GGL27 (Fig. 38B). In contrast to these results, no significant C1q/MAC alterations were induced after subjecting *P. aeruginosa* to GGL27 (Fig. 39A, C and D). Considering C3a in relation to *P. aeruginosa*, the western blots of bacteria-bound peptides identified minor C3a bands, as well as peptides of higher molecular weight, containing the C-terminal epitope of C3a. FACS analysis showed increased generation and binding of these C3a-containing fragments to the bacterial surface (Fig. 39 C and D).

TFPI is mostly considered a plasma protein, but is also expressed by endothelial cells, monocytes and macrophages (12). Immunohistochemistry analyses showed that TFPI was expressed in normal human skin, particularly in the basal epidermal layers (Fig. 40A). Furthermore, the molecule showed a ubiquitous expression at the wound edges of both acute wounds and chronic leg ulcers. Chronic leg ulcers are characterized by an excessive chronic inflammatory state, high levels of proteinases (such as plasmin) and frequent bacterial colonization with *P. aeruginosa* (35). Hence, we analyzed fibrin slough from such an ulcer, infected with *P. aeruginosa*. As demonstrated by electron microscopy and immunogold-labeled antibodies against the C-terminal part of TFPI, these peptide epitopes were found in association with bacterial membranes, as well as fibrin fibers. Importantly, using double staining, C3a was found to be particularly associated with these TFPI-peptides (Fig. 40B). Hence,

these *in vivo* data are compatible with previous results (Fig. 39) on microbial binding of GGL27, and its relation to enhanced generation of antimicrobial C3a in plasma *in vitro*.

5 It is of note that the human TFPI peptides did not enhance bacterial killing in mouse plasma (Fig. 41A). Nevertheless, a prolongation of survival in the mouse infection models was observed (Fig. 41B). In order to delineate a possible mechanism underlying this observation, and considering the previously reported LPS-binding property of TFPI (16), we investigated whether GGL27 could exert anti-endotoxin effects *in vitro* and *in vivo*. The results showed indeed that the peptide inhibited LPS-mediated NO-release from mouse-derived macrophages (RAW 264.7 cells) (Fig. 41C), and also significantly increased survival in a mouse model of LPS shock (Fig. 41D). Analyses of cytokines 20 hours after LPS injection, showed significant reductions of proinflammatory IL-6, IFN- γ , TNF- α , and MCP-1, whereas anti-inflammatory IL-10 was increased (Fig. 41E). Furthermore, a marked reduction of inflammation and vascular leakage in the lungs of the GGL27-treated animals was observed (Fig. 41F). In contrast, the two control peptides DSE25 from the N-terminal region of TFPI, and the peptide GGL27(S), having the "core" K/R residues substituted with S, did neither block NO release *in vitro*, nor significantly alter overall cytokine release, as well as survival *in vivo* (Fig. 41C, D, E). These data demonstrate a specific anti-endotoxic role of GGL27, compatible with the observed improvement in the *E. coli* and *P. aeruginosa* mouse infection models. Furthermore, the abrogated anti-endotoxic activity of GGL27(S) also highlights the importance of the central cationic K/R residues in this molecule. Taken together, the results are indicative of differential and host-related effects of GGL27; a host-independent anti-endotoxic effect, and a host-dependent and complement-based antimicrobial function, likely a logical consequence of different structural prerequisites for LPS- and complement-interactions. In line with this reasoning, Fig. 42 illustrates the evolution of this region of TFPI and highlights the conservation of the cationic "core", but also significant sequence changes between mice and men in this C-terminal region of TFPI.

Discussion

35 The key findings in this study are the identification of a dual antimicrobial activity of C-terminal TFPI peptides, based on direct and complement-mediated bactericidal effects, the demonstration of an anti-endotoxic effect, combined with the identification of TFPI in skin, its upregulation during wounding, and presence in wounds.

The presently disclosed direct antibacterial action of C-terminal peptides of TFPI is in line with observations indicating that heparin-binding proteins, such as complement C3 (25), kininogen (36,37), heparin-binding protein (38), heparin-binding epidermal growth factor and other growth factors (39), heparin/heparan sulfate interacting protein (40), β 2-glycoprotein (41), histidine-rich glycoprotein (42), and human thrombin (43) may, either as holoproteins or after fragmentation, exert antimicrobial activities *in vitro*, and in several cases, also *in vivo* (36,37,42). In addition, peptide-mediated C3a generation, along with enhanced MAC formation, represents a mechanism by which a host defense peptide may selectively enhance microbial killing by generation of additional complement-derived AMPs. Recent evidence showing a significant cross-talk between the coagulation and complement systems (44) further adds biological relevance to the facilitated generation of C3a by the TFPI-peptides.

Considering the C-terminal region of TFPI, peptides derived from this region resemble other linear peptides of low helical content. For example, antimicrobial peptides derived from growth factors also display a low helical content in presence of membranes, reflecting their low content of features typical of "classical" helical peptides, such as regularly interspersed hydrophobic residues (39). Furthermore, studies on a kininogen-derived antimicrobial peptide, HKH20 (HKHGHGKHGKHKNKGKNGKH [SEQ ID NO: 105]) (45) showed that the HKH20 peptide displays predominantly random coil conformation in buffer and at lipid bilayers, the interactions dominated by electrostatics (45). It is noteworthy that like the TFPI-derived peptides, both HKH20 (36) and GKR22 (GKRKKKGKGLGKKRDPCLRKYK [SEQ ID NO: 106]) (39), a peptide derived from heparin-binding growth factor, retain their antibacterial activity at physiological conditions. In relation to the above, it is interesting that recent studies indicate that certain low amphipathic peptides exhibit activity mainly by strong electrostatic interactions, e.g., leading to negative curvature strains, to membrane thinning, or to the formation of lipid domains, sometimes resulting in bacterial membrane fluidity changes affecting biological function (46).

As previously mentioned, many antimicrobial peptides, including LL-37 (31), C3a (25), thrombin- and kininogen-derived peptides (36,37,43) are released during proteolysis. Considering TFPI, enzymes such as plasmin and thrombin release distinct C-terminal fragments of relevance for physiological events, such as

wounding. Therefore, TFPI-derived peptides generated in fibrin clots by thrombin (20), may contribute to antimicrobial activity during wound healing. Furthermore, subsequent proteolysis by plasmin (generating GGL27) (21) may further add to the spectrum of host defense peptides released. As mentioned above, it is of note that

5 TFPI, in addition to its synthesis in microvascular endothelial cells and subsequent occurrence in the endothelium, plasma, and platelets, is also produced by the liver, and found in monocytes and macrophages (12). Production of TFPI has also been demonstrated in capillaries, megakaryocytes, several cell lines, as well as neoplastic cells. This evidence combined with a marked upregulation of TFPI during wounding

10 of human skin, as well as its occurrence in fibrin and on bacteria, suggests that high local levels of endogenous TFPI peptides may occur *in vivo*. The finding that the peptides were particularly active in human plasma against *E. coli*, and to a lesser extent against *P. aeruginosa*, is particularly relevant and interesting, in the light of the ubiquitous occurrence of *P. aeruginosa* in chronic leg ulcers, contrasting to the very

15 sparse *E. coli* colonization in these wounds (35,47). Furthermore, based on the demonstration that bacterial omptins (such as OmpT from *E. coli*) may release GGL27 fragments from TFPI, it was recently proposed that TFPI has evolved sensitivity to omptin-mediated proteolytic inactivation to potentiate procoagulant responses to *E. coli* infection in certain conditions (48). Our data further substantiate

20 this hypothesis by adding another host-response mechanism to *E. coli* infection; OmpT-mediated generation of the host defense peptide GGL27.

Simultaneous control of inflammation and coagulation plays a key role in maintaining homeostasis, and it is notable that trials utilizing recombinant TFPI indicate that the

25 protein protects from *E. coli* induced severe sepsis (49), and furthermore, TFPI is also under evaluation in a phase III clinical trial involving patients with severe community acquired pneumonia. In this context, it is interesting to note that the here observed anti-endotoxic effect of GGL27 *in vitro* and *in vivo* may explain, at least partly, the previously observed protective effects of TFPI during *E. coli* sepsis (49).

30 The current findings showing direct and indirect, MAC and C3a-mediated, antimicrobial effects of the C-terminal epitope of TFPI, GGL27, may have implications for future attempts in designing and developing peptide-based therapeutics combating severe infection. Finally, the finding that the TFPI-peptide did not significantly enhance bacterial killing in mouse plasma *ex vivo* as well as increase

35 total survival during bacterial sepsis (although a prolonged survival was observed), illustrates that mouse models might be disadvantageous when it comes to assessing certain aspects of the physiological roles of human C-terminal TFPI peptides, such

as those involving complement-mediated bacterial clearance. Interestingly, whereas the TFPI- α form (having the C-terminal cationic sequence) is the predominant form in humans, mice appear to largely produce TFPI- β (a form lacking the C-terminal sequence) (50), an observation compatible with the above mentioned evolution of unique C-terminal and OmpT-releasable peptides in humans.

References

1. Yount, N. Y., Bayer, A. S., Xiong, Y. Q., and Yeaman, M. R. (2006) *Biopolymers* **84**, 435-458
2. Zelezetsky, I., and Tossi, A. (2006) *Biochim Biophys Acta* **1758**, 1436-1449
3. Tossi, A., and Sandri, L. (2002) *Curr Pharm Des* **8**, 743-761
4. Powers, J. P., and Hancock, R. E. (2003) *Peptides* **24**, 1681-1691
5. Bulet, P., Stocklin, R., and Menin, L. (2004) *Immunol Rev* **198**, 169-184
6. Durr, U. H., Sudheendra, U. S., and Ramamoorthy, A. (2006) *Biochim Biophys Acta* **1758**, 1408-1425
7. Brogden, K. A. (2005) *Nat Rev Microbiol* **3**, 238-250
8. Lohner, K., and Blondelle, S. E. (2005) *Comb Chem High Throughput Screen* **8**, 241-256
9. Zanetti, M. (2004) *J Leukoc Biol* **75**, 39-48
10. Elsbach, P. (2003) *J Clin Invest* **111**, 1643-1645
11. Ganz, T. (2003) *Nat Rev Immunol* **3**, 710-720
12. Lwaleed, B. A., and Bass, P. S. (2006) *J Pathol* **208**, 327-339
13. Girard, T. J., Warren, L. A., Novotny, W. F., Likert, K. M., Brown, S. G., Miletich, J. P., and Broze, G. J., Jr. (1989) *Nature* **338**, 518-520
14. Mine, S., Yamazaki, T., Miyata, T., Hara, S., and Kato, H. (2002) *Biochemistry* **41**, 78-85
15. Crawley, J. T., and Lane, D. A. (2008) *Arterioscler Thromb Vasc Biol* **28**, 233-242
16. Park, C. T., Creasey, A. A., and Wright, S. D. (1997) *Blood* **89**, 4268-4274
17. Hembrough, T. A., Ruiz, J. F., Swerdlow, B. M., Swartz, G. M., Hammers, H. J., Zhang, L., Plum, S. M., Williams, M. S., Strickland, D. K., and Pribluda, V. S. (2004) *Blood* **103**, 3374-3380
18. Wesselschmidt, R., Likert, K., Girard, T., Wun, T. C., and Broze, G. J., Jr. (1992) *Blood* **79**, 2004-2010
19. Ettelaie, C., Adam, J. M., James, N. J., Oke, A. O., Harrison, J. A., Bunce, T. D., and Bruckdorfer, K. R. (1999) *FEBS Lett* **463**, 341-344
20. Ohkura, N., Enjyoji, K., Kamikubo, Y., and Kato, H. (1997) *Blood* **90**, 1883-1892
21. Li, A., and Wun, T. C. (1998) *Thromb Haemost* **80**, 423-427
22. Cunningham, A. C., Hasty, K. A., Enghild, J. J., and Mast, A. E. (2002) *Biochem J* **367**, 451-458
23. Andersson, E., Rydengård, V., Sonesson, A., Mörgelin, M., Björck, L., and Schmidtchen, A. (2004) *Eur J Biochem* **271**, 1219-1226
24. Lehrer, R. I., Rosenman, M., Harwig, S. S., Jackson, R., and Eisenhauer, P. (1991) *J Immunol Methods* **137**, 167-173
25. Nordahl, E. A., Rydengard, V., Nyberg, P., Nitsche, D. P., Morgelin, M., Malmsten, M., Björck, L., and Schmidtchen, A. (2004) *Proc Natl Acad Sci U S A* **101**, 16879-16884

26. Carlemalm, E., Villiger, W., Hobot, J. A., Acetarin, J. D., and Kellenberger, E. (1985) *J Microsc* **140**, 55-63
27. Pollock, J. S., Forstermann, U., Mitchell, J. A., Warner, T. D., Schmidt, H. H., Nakane, M., and Murad, F. (1991) *Proc Natl Acad Sci U S A* **88**, 10480-10484
- 5 28. Greenfield, N., and Fasman, G. D. (1969) *Biochemistry* **8**, 4108-4116
29. Sjogren, H., and Ulvenlund, S. (2005) *Biophys Chem* **116**, 11-21
30. Tossi, A., Sandri, L., and Giangaspero, A. (2000) *Biopolymers* **55**, 4-30
31. Zasloff, M. (2002) *Nature* **415**, 389-395.
32. Ganz, T. (2001) *Semin Respir Infect* **16**, 4-10.
- 10 33. Wang, Y., Agerberth, B., Lothgren, A., Almstedt, A., and Johansson, J. (1998) *J Biol Chem* **273**, 33115-33118.
34. Schirm, S., Liu, X., Jennings, L. L., Jedrzejewski, P., Dai, Y., and Hardy, S. (2009) *J Infect Dis* **199**, 1807-1815
35. Lundqvist, K., Herwald, H., Sonesson, A., and Schmidtchen, A. (2004) *Thromb Haemost* **92**, 281-287
- 15 36. Nordahl, E. A., Rydengard, V., Morgelin, M., and Schmidtchen, A. (2005) *J Biol Chem* **280**, 34832-34839
37. Frick, I. M., Akesson, P., Herwald, H., Morgelin, M., Malmsten, M., Nagler, D. K., and Bjorck, L. (2006) *Embo J* **25**, 5569-5578
- 20 38. Pereira, H. A. (1995) *J Leukoc Biol* **57**, 805-812
39. Malmsten, M., Davoudi, M., Walse, B., Rydengard, V., Pasupuleti, M., Morgelin, M., and Schmidtchen, A. (2007) *Growth Factors* **25**, 60-70
40. Meyer-Hoffert, U., Hornef, M., Henriques-Normark, B., Normark, S., Andersson, M., and Putsep, K. (2008) *FASEB J* **22**, 2427-2434
- 25 41. Nilsson, M., Wasylik, S., Morgelin, M., Olin, A. I., Meijers, J. C., Derksen, R. H., de Groot, P. G., and Herwald, H. (2008) *Mol Microbiol* **67**, 482-492
42. Rydengard, V., Shannon, O., Lundqvist, K., Kacprzyk, L., Chalupka, A., Olsson, A. K., Morgelin, M., Jahnen-Dechent, W., Malmsten, M., and Schmidtchen, A. (2008) *PLoS Pathog* **4**, e1000116
- 30 43. Papareddy, P., Rydengard, V., Pasupuleti, M., Walse, B., Morgelin, M., Chalupka, A., Malmsten, M., and Schmidtchen, A. *PLoS Pathog* **6**, e1000857
44. Amara, U., Rittirsch, D., Flierl, M., Bruckner, U., Klos, A., Gebhard, F., Lambris, J. D., and Huber-Lang, M. (2008) *Adv Exp Med Biol* **632**, 71-79
45. Ringstad, L., Andersson Nordahl, E., Schmidtchen, A., and Malmsten, M. (2007) *Biophys J* **92**, 87-98
- 35 46. Yamamoto, N., and Tamura, A. (2010) *Peptides* **31**, 794-805
47. Schmidtchen, A., Wolff, H., and Hansson, C. (2001) *Acta Derm Venereol* **81**, 406-409.
48. Yun, T. H., Cott, J. E., Tapping, R. I., Slauch, J. M., and Morrissey, J. H. (2009) *Blood* **113**, 1139-1148
- 40 49. Creasey, A. A., Chang, A. C., Feigen, L., Wun, T. C., Taylor, F. B., Jr., and Hinshaw, L. B. (1993) *J Clin Invest* **91**, 2850-2860
50. Maroney, S. A., Ellery, P. E., and Mast, A. E. *Thromb Res* **125 Suppl 1**, S52-56

EXAMPLE C - Anticoagulative and anti-inflammatory effects of exemplary peptides derived from HRG and ATIII

Histidine-rich glycoprotein (HRG)

5

Introduction

Histidine-rich glycoprotein and GHH25

10 The antimicrobial plasma protein Histidine-rich glycoprotein (HRG) protects the host against systemic microbial infections *in vivo*. Here, we show that HRG is a pattern recognition molecule, by binding LPS and increasing LPS mediated TLR-4 response in the host cells.

15 LPS interacts with CD14, a receptor on macrophages, monocytes and neutrophils, the interaction is increased by plasma protein LBP (LPS-binding protein). The LPS/CD14 complex leads to activation of toll-like receptor (TLR) 4 which in turn activates the MyD88- dependent or -independent pathways [1]. The LPS-response can also be LBP-independent [2]. There is no difference in TNF- α release *in vivo*
20 between wildtype and LBP-deficient mice after LPS injection, suggesting that a protein with LBP-like capabilities may be responsible [3]. Activation of TLR4 then in turn leads to an increase of phagocytic activity of mononuclear phagocytes, a cascade of released cytokines and nitric oxide (NO), which initiate and support the inflammatory response. NO is produced by a group of enzymes called nitric oxide
25 synthases [4] and involved in many biological processes such as protecting the host against microbes, parasitic worms and tumours and regulate blood pressure, high levels of NO can instead be cytotoxic and destroy endogenous tissue.

The LPS-induced response is essential for the host defence, but an overwhelming
30 response can instead lead to sepsis. Cytokines and NO are identified to be major contributors to the development of septic shock with symptoms as fever, coagulant activity, septic shock, multiple organ failure and in worst cases death of the host [5]. This is supported by the fact that inducibleNOS (iNOS) mutant mice where resistant to LPS-induced mortality [6]. The susceptibility of different animal species to the
35 toxicity of LPS is highly variable, for example humans are very sensitive [7,8] comparing to mice that are fairly resistant [9]. A common misunderstanding is that sepsis is controlled by only pro-inflammatory mediators. The early stages of sepsis is

dominated by pro-inflammatory mediators such as TNF- α , NO, bradykinin, thrombin and histamine, whereas anti-inflammatory mediators, such as IL-6, activated protein C, antithrombin and granulocyte colony-stimulating factor, are most widely occurring during the later stages [10].

5

The treatment of sepsis today includes administration of intravenous fluids, broad-spectrum antibiotics, protective lung ventilation, glucocorticoids, insulin therapy and recombinant human activator protein C (APC). Many attempts have been done to neutralize LPS or inhibit LPS-mediated activation of host immune cells, such as
10 blocking cytokine activity with antibodies, blocking NO production [11] and by using antimicrobial peptides as neutralizers of LPS [12].

The aim of this study was to investigate the effects of an abundant plasma protein, HRG, on LPS-mediated responses. HRG is a 67 kDa plasma protein synthesized in
15 the liver and was first described in 1972 [13,14]. HRG can also be released upon thrombin activation from α -granules of thrombocytes, and is able to cover the surface of a fibrin clot. Together with fetuins and kininogens, HRG belongs to the cystatin superfamily. The most distinguishing characteristic of the protein is the histidine-rich domain of the protein contains a conserved GHHPH repeat [15]. HRG has been
20 shown to interact *in vitro* with a diverse group of ligands, like heparin, plasminogen, fibrinogen, thrombospondin, heme, IgG, Fc γ R and C1q. *In vivo*-studies demonstrates that HRG is an anticoagulant and antifibrinolytic modifier [16], has an inhibitory effects on tumour vascularization [17] and plays an important role in the innate immunity as a antimicrobial protein [18].

25

Herein, we demonstrate that HRG is a potent pro-inflammatory protein, by enhancing LPS induced NO and cytokines *in vitro*. *In vivo* experiment showed that Hrg-/- mice were markedly resistant against LPS induced sepsis. Our data clearly demonstrate that HRG contributes to LPS toxicity in experimental endotoxemia. The data also
30 demonstrate an antiinflammatory effect of the peptide GHH25.

Materials & Methods

Synthetic peptide GHH25 (GHHPHGHHPHGHHPHGHHPHGHHPH [SEQ ID NO:
35 11]) was from Biopeptide (San Diego, CA, USA). Polyclonal rabbit anti mouse TRL4 was purchased from GeneTex (Irvine, CA, USA). The cytometric bead array, mouse inflammation kit was from BD Biosciences (Stockholm, Sweden). *Escherichia coli*

LPS (0111:B4) was purchased from Sigma (St Louis, MO).

Purification of Human HRG. Serum HRG was purified as described before [19]. Briefly, human serum were gently shaken at 4°C overnight with nickel-nitrilotriacetic acid (Ni-NTA) agarose, washed with phosphate-buffered saline (PBS, pH 7.4). Elution was first performed with PBS containing 80 mM imidazole to elute unspecifically bound proteins, and then with PBS containing 500 mM imidazole to elute purified HRG. The protein was dialyzed, freeze-dried and the concentration was determined using the Bradford method [20].

Radioiodination of heparin and LPS. The radioiodination of heparin (from porcine intestinal mucosa, Sigma-Aldrich) was performed as described previously [21]. The iodination of LPS was performed as described by Ulevitch [22]. 1 mg *Escherichia coli* 0111:B4 LPS was incubated in 50 mM p-OH benzimidate in borate buffer, pH 8, overnight at 4°C, and then dialyzed against PBS, pH 7.4. LPS was then radiolabelled with ^{125}I using the chloramine T method, and unlabelled ^{125}I was then removed by dialysis.

Heparin and LPS-binding assay. 1, 2 and 5 µg of the synthetic peptides in 100 µl PBS pH 7.4 were applied onto nitrocellulose membranes (Hybond-C, Amersham Biosciences) using a slot-blot apparatus. Membranes were blocked for 1 h at room temperature with 2% bovine serum albumin in PBS pH 7.4 and then incubated with radiolabelled LPS ($\sim 40 \mu\text{g}\cdot\text{mL}^{-1}$, $0.13 \times 10^6 \text{ cpm}\cdot\mu\text{g}^{-1}$) or radiolabelled heparin ($\sim 10 \mu\text{g}\cdot\text{mL}^{-1}$, $0.4 \times 10^6 \text{ cpm}\cdot\mu\text{g}^{-1}$) for 1 h at room temperature in PBS, pH 7.4. Unlabeled heparin (6 mg/ml) was added for competition of binding. The membranes were washed 3 times in PBS, pH 7.4. A Bas 2000 radio-imaging system (Fuji Film, Tokyo, Japan) was used to visualize radioactivity.

Cell culture. Murine macrophage cell line, RAW 264.7 (kindly provided by Dr. H Björkbacka) were grown in Dulbeccos Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal calf serum (FCS). All experiments were performed under serum free conditions.

Nitric oxide induction in RAW macrophages. Confluent cells were harvested and transferred to 96-wells plate ($3.5 \times 10^5/\text{well}$). After adhesion cells were washed with phenol red-free DMEM (Gibco). *E. Coli* LPS (100ng/ml), LL-37 (2 or 10 µM) or HRG (2 or 10 µM) was preincubated at 37°C for 30 minutes and then transferred to the cells. For inhibition of NO induction, 5µg/ml anti mouse TLR4 antibody, 10 or 100 µM

GHH25 or 100µg/ml heparin were used. The cells were stimulated for 24 hours and nitric oxide was determined using the Griess chemical method [23].

TNF-α release from human macrophages. Human monocyte-derived macrophages (hMDMs) were obtained from peripheral blood mononuclear cells (PBMCs) obtained from the blood of healthy donors using a Lymphoprep (Axis-Shield PoC AS) density gradient. PBMCs were seeded at concentrations of 3×10^6 cells/well into 24-well plates and cultured in RPMI1640 medium supplemented with 10% heat-inactivated autologous human plasma, 2 mM L-glutamine, and 50 µl/ml Antibiotic-Antimycotic (Gibco) in a humidified atmosphere of 5% CO₂. After 24 h, non-adherent cells were removed and adherent monocytes were differentiated to macrophages for 10 days, with fresh medium changes every second day. The cells were stimulated for 24 hours with 10ng/ml of LPS with or without HRG (2 µM) and GHH25 (100 µM) under serum-free conditions. After stimulation the supernatant was aspirated and TNF-α was measured using the TNF- α human ELISA kit (Invitrogen).

Animal experiments. The original knockout mice 129/B6-HRG^{tm1wja1} were crossed with C57BL/6 mice (Taconic) for 14 generations to obtain uniform genetic background. These HRG-deficient mouse strain was called B6-HRG^{tm1wja1} following ILAR (Institute of Laboratory Animal Resources) rules. Wildtype C57BL/6 control mice and C57BL/6 Hrg^{-/-} mice (8-12 weeks, 27 +/- 4g) were bred in the animal facility at Lund University. C57BL/6 Hrg^{-/-}, lacks the translation start point of exon 1 of the Hrg gene [16]. Animals were housed under standard conditions of light and temperature and had free access to standard laboratory chow and water. In order to induce sepsis, 18µg/g Escherichia coli 0111:B4 LPS were injected intraperitoneally into C57BL/6 or C57BL/6 Hrg^{-/-} mice, divided into weight and sex matched groups. Survival and status was followed during seven days.

For treatment with GHH25 peptide, 1 mg of the peptide (diluted in 10mM Tris, pH 7.4) or buffer only was injected intraperitoneal 30 minutes after LPS-challenge and survival and status was then followed.

Antithrombin III

Serpins are a group of proteins with similar structures that were first identified as a set of proteins able to inhibit proteases. The acronym serpin was originally coined because many serpins inhibit chymotrypsin-like serine proteases (serine protease inhibitors). The first members of the serpin superfamily to be extensively studied were the human plasma proteins antithrombin and antitrypsin, which play key roles in controlling blood coagulation and inflammation, respectively.

Structural studies on serpins have revealed that inhibitory members of the family undergo an unusual conformational change, termed the Stressed to Relaxed (S to R) transition. This conformational mobility of serpins provides a key advantage over static lock-and-key protease inhibitors. In particular, the function of inhibitory serpins can be readily controlled by specific cofactors like heparin. The archetypal example of this situation is antithrombin, which circulates in plasma in a relatively inactive state. Upon binding a high-affinity heparin pentasaccharide sequence within long-chain heparin, antithrombin undergoes a conformational change, exposing key residues important for the mechanism. The heparin pentasaccharide-bound form of antithrombin is, thus, a more effective inhibitor of thrombin and factor Xa. Furthermore, both of these coagulation proteases contain binding sites (called exosites) for heparin. Heparin, therefore, also acts as a template for binding of both protease and serpin, further dramatically accelerating the interaction between the two parties. After the initial interaction, the final serpin complex is formed and the heparin moiety is released.

Materials and Methods

(as above)

Results

The FFF21 peptide (FFFAKLNCRLYRKANKSSKLV [SEQ ID NO: 1], aa 153-173 of antithrombin III, accession number P01008) displays antibacterial and antiinflammatory properties (see Figure 56).

CLAIMS

1. A polypeptide comprising or consisting of an amino acid sequence derived
from a naturally occurring protein which modulates blood coagulation (other
5 than heparin cofactor II or thrombin), or a fragment, variant, fusion or
derivative thereof, or a fusion of said fragment, variant or derivative thereof,

for use in the treatment or prevention of inflammation and/or excessive
coagulation
10

wherein the fragment, variant, fusion or derivative exhibits an anti-
inflammatory and/or anti-coagulant activity.
2. A polypeptide according to Claim 1 wherein the polypeptide is not a naturally
15 occurring protein.
3. A polypeptide according to any one of the preceding claims wherein the
naturally occurring protein which modulates blood coagulation is a human
protein.
20
4. A polypeptide according to any one of the preceding claims wherein the
naturally occurring protein which modulates blood coagulation is selected from
the group consisting of serine proteinase inhibitors (serpins), tissue factor
pathway inhibitors (such as TFPI-1 and TFPI-2) and histidine-rich glycoprotein
25 (HRG).
5. A polypeptide according to Claim 4 wherein the naturally occurring protein
which modulates blood coagulation is a serpin.
- 30 6. A polypeptide according to Claim 5 wherein the serpin is anti-thrombin III.
7. A polypeptide according to Claim 6 wherein the anti-thrombin III is Swiss Port
Accession No. P01008.
- 35 8. A polypeptide according to Claim 6 or 7 comprising or consisting of the amino
acid sequence of SEQ ID NO:1 or 2:

"FFF21": FFFAKLNCRLYRKANKSSKLV [SEQ ID NO:1]

"AKL22": AKLNCRLYRKANKSSKLVSANR [SEQ ID NO:2]

or a fragment, variant, fusion or derivative thereof, or a fusion of said
fragment, variant or derivative thereof, which retains an anti-inflammatory
and/or anticoagulant activity of SEQ ID NO:1 or 2.

9. A polypeptide according to Claim 8 comprising or consisting of the amino acid
sequence of SEQ ID NO: 1 or 2.

10. A polypeptide according to Claim 5 wherein the serpin is protein C inhibitor
(PCI).

11. A polypeptide according to Claim 5 wherein the protein C inhibitor is Swiss
Port Accession No. P05154.

12. A polypeptide according to Claim 10 or 11 comprising or consisting of the
amino acid sequence of SEQ ID NO:8:

"SEK20": SEKTLRKWLKMFKKRQLELY [SEQ ID NO:3]

or a fragment, variant, fusion or derivative thereof, or a fusion of said
fragment, variant or derivative thereof, which retains an anti-inflammatory
and/or anti-coagulant activity of SEQ ID NO:3.

13. A polypeptide according to Claim 12 comprising or consisting of the amino
acid sequence of SEQ ID NO:3.

14. A polypeptide according to Claim 4 wherein the naturally occurring protein
which modulates blood coagulation is a tissue factor pathway inhibitor (TFPI).

15. A polypeptide according to Claim 14 wherein the tissue factor pathway
inhibitor is TFPI-1.

16. A polypeptide according to Claim 14 wherein the TFPI-1 is Swiss Port
Accession No. P10646.

17. A polypeptide according to Claim 15 or 16 comprising or consisting of the amino acid sequence of any one of SEQ ID NOS:4 to 6:

"GGL27": GGLIKTKRKRKKQRVKIAEYEEIFVKNM [SEQ ID NO:4]

5 "LIK17": LIKTKRKRKKQRVKIAY [SEQ ID NO:5]

"TKR22": TKRKRKKQRVKIAEYEEIFVKNM [SEQ ID NO:6]

10 or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of any one of SEQ ID NOS:4 to 6.

18. A polypeptide according to Claim 17 comprising or consisting of the amino acid sequence of any one of SEQ ID NOS:4 to 6.

- 15 19. A polypeptide according to Claim 14 wherein the tissue factor pathway inhibitor is TFPI-2.

20. A polypeptide according to Claim 19 wherein the TFPI-2 is Swiss Port Accession No. P48307.

20

21. A polypeptide according to Claim 19 or 20 comprising or consisting of the amino acid sequence of SEQ ID NO:7 or 8:

"ALK25": ALK KKKKMPKLR FASRIRKIRKKQF [SEQ ID NO:7]

25 "EDC34": EDCKRACAKALKKKKKMPKLR FASRIRKIRKKQF

[SEQ ID NO:8]

30 or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:7 or 8.

22. A polypeptide according to Claim 19 comprising or consisting of the amino acid sequence of SEQ ID NO:7 or 8.

- 35 23. A polypeptide according to Claim 4 wherein the naturally occurring protein which modulates blood coagulation is histidine-rich glycoprotein (HRG).

24. A polypeptide according to Claim 4 wherein the HRG is Swiss Port Accession No. P04196.

25. A polypeptide according to Claim 23 or 24 comprising or consisting of the amino acid sequence of SEQ ID NO:9:

$(X_1X_2X_3X_4X_5)_n$ [SEQ ID NO:9],

wherein

X_1 and X_4 independently represent G, P, L, I, F, T, V, Y or W;

X_2 , X_3 and X_5 independently represent H, R or K; and

'n' is an integer from 2 to 6

or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:9.

26. A polypeptide according to any one of Claims 23 to 25 comprising or consisting of the amino acid sequence of SEQ ID NO:10:

$(GHHPH)_n$ [SEQ ID NO:10], where 'n' is an integer from 2 to 6

or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:10.

27. A polypeptide according to any one of Claims 23 to 26 comprising or consisting of the amino acid sequence of SEQ ID NO:11:

"GHH25": GHHPHGHHPHGHHPHGHHPHGHHPH [SEQ ID NO:11]

or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:11.

28. A polypeptide according to any one of Claims 23 to 27 comprising or consisting of the amino acid sequence of SEQ ID NO:9, 10 or 11.
29. A polypeptide according to any one of the preceding claims wherein the polypeptide, or fragment, variant, fusion or derivative thereof, comprises or consists of L-amino acids.
30. A polypeptide according to any one of the preceding claims wherein the polypeptide, or fragment, variant, fusion or derivative thereof, comprises one or more amino acids that are modified or derivatised.
31. A polypeptide according to Claim 30 wherein the one or more amino acids are modified or derivatised by PEGylation, amidation, esterification, acylation, acetylation and/or alkylation.
32. A polypeptide according to any one of the preceding claims wherein the polypeptide comprises or consists of a fragment of any one of the amino acid sequences of SEQ ID NOS: 1 to 11.
33. A polypeptide according to Claim 32 wherein the fragment comprises or consists of at least 5 contiguous amino acids of SEQ ID NO: 3, for example at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 or 27 contiguous amino acids.
34. A polypeptide according to any one of the preceding claims wherein the polypeptide comprises or consists of a variant of any one of the amino acid sequence of any one of SEQ ID NOS: 1 to 11.
35. A polypeptide according to Claim 34 wherein the variant has at least 50% identity with the amino acid sequence amino acid sequence of any one of SEQ ID NOS: 1 to 11, for example at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or at least 99% identity.
36. A polypeptide according to any one of the preceding claims wherein the polypeptide is between 10 and 200 amino acids in length, for example between 10 and 150, 15 and 100, 15 and 50, 15 and 30, 17 and 30, or 17 and 28 amino acids in length.

37. A polypeptide according to Claim 36 wherein the polypeptide is at least 17 amino acids in length.

5 38. A polypeptide according to any one of the preceding claims wherein the polypeptide is linear.

39. A polypeptide according to any one of the preceding claims wherein the polypeptide is a recombinant polypeptide.

10

40. A polypeptide according to any one of the preceding claims wherein the polypeptide is capable of inhibiting the release of one or more pro-inflammatory cytokines from human monocyte-derived macrophages.

15 41. A polypeptide according to Claim 33 wherein the pro-inflammatory cytokines are selected from the group consisting of macrophage inhibitory factor, TNF-alpha, HMGB1, C5a, IL-17, IL-8, MCP-1, IFN-gamma, IL-6, IL-1b, IL-12.

20 42. A polypeptide according to any one of the preceding claims wherein the polypeptide is capable of blocking platelet activation.

43. A polypeptide according to any one of the preceding claims wherein the polypeptide is capable of interfering with Toll-like receptor (TLR)-signalling in leukocytes, epithelial cells (including keratinocytes) and/or mesenchymal cells (including fibroblasts).

25

44. A polypeptide according to any one of the preceding claims wherein the polypeptide exhibits anti-inflammatory activity in one or more of the following models:

30

(a) *in vitro* macrophage models using LPS, LTA, zymosan, flagellin, dust mites, viral or bacterial DNA or RNA, or peptidoglycan as stimulants;

(b) *in vivo* mouse models of endotoxin shock; and/or

35

(c) *in vivo* infection models, either in combination with antimicrobial therapy, or given alone.

45. A polypeptide according to any one of the preceding claims wherein the polypeptide exhibits anticoagulant activity.

5 46. A polypeptide according to Claim 45 for use in the concomitant treatment or prevention of inflammation and coagulation.

47. A polypeptide according to any one of the preceding claims wherein the polypeptide exhibits Toll-like receptor (TLR) blocking activity.

10

48. A polypeptide according to any one of the preceding claims wherein the polypeptide is for use in the treatment or prevention of inflammation associated with an infection

15 49. A polypeptide according to any one of the preceding claims for use in the treatment or prevention of a disease, condition or indication selected from the following:

20 i) Acute systemic inflammatory disease, with or without an infective component, such as systemic inflammatory response syndrome (SIRS), ARDS, sepsis, severe sepsis, and septic shock. Other generalized or localized invasive infective and inflammatory disease, including erysipelas, meningitis, arthritis, toxic shock syndrome, diverticulitis, appendicitis, pancreatitis, cholecystitis, colitis, cellulitis,
25 burn wound infections, pneumonia, urinary tract infections, postoperative infections, and peritonitis.

30 ii) Chronic inflammatory and or infective diseases, including cystic fibrosis, COPD and other pulmonary diseases, gastrointestinal disease including chronic skin and stomach ulcerations, other epithelial inflammatory and or infective disease such as atopic dermatitis, oral ulcerations (aphthous ulcers), genital ulcerations and inflammatory changes, parodontitis, eye inflammations including conjunctivitis and keratitis, external otitis, mediaotitis, genitourinary
35 inflammations.

iii) Postoperative inflammation. Inflammatory and coagulative disorders including thrombosis, DIC, postoperative coagulation disorders, and coagulative disorders related to contact with foreign material, including extracorporeal circulation, and use of biomaterials. Furthermore, vasculitis related inflammatory disease, as well as allergy, including allergic rhinitis and asthma..

iv) Excessive contact activation and/or coagulation in relation to, but not limited to, stroke.

v) Excessive inflammation in combination with antimicrobial treatment. The antimicrobial agents used may be administered by various routes; intravenous (iv), intraarterial, intravitreal, subcutaneous (sc), intramuscular (im), intraperitoneal (ip), intravesical, intratechal, epidural, enteral (including oral, rectal, gastric, and other enteral routes), or topically, (including dermal, nasal application, application in the eye or ear, eg by drops, and pulmonary inhalation). Examples of agents are penicillins, cephalosporins, carbacephems, cephamycins, carbapenems, monobactams, aminoglycosides, glycopeptides, quinolones, tetracyclines, macrolides, and fluoroquinolones. Antiseptic agents include iodine, silver, copper, chlorhexidine, polyhexanide and other biguanides, chitosan, acetic acid, and hydrogen peroxide.

50. A polypeptide according to any one of the preceding claims for use in the treatment or prevention of acute inflammation, sepsis, acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), cystic fibrosis, asthma, allergic and other types of rhinitis, cutaneous and systemic vasculitis, thrombosis and disseminated intravascular coagulation (DIC).

51. A polypeptide according to Claim 50 for use in the treatment or prevention of sepsis.

52. A polypeptide according to Claim 50 for use in the treatment or prevention of chronic obstructive pulmonary disease (COPD).

53. A polypeptide according to any one of the preceding claims for use in combination with one or more additional therapeutic agent.
54. A polypeptide according to Claim 53 wherein the additional therapeutic agent
5 is selected from the group consisting of antibiotic agents, anti-fungal agents, antiseptic agents, anti-inflammatory agents, immunosuppressive agents, vasoactive agents, receptor blockers, receptor agonists and antiseptic agents.
55. A polypeptide according to Claim 54 wherein the antibiotic agents are selected
10 from the groups consisting of anti-bacterial agents, anti-fungicides, anti-viral agents and anti-parasitic agents.
56. An isolated polypeptide comprising or consisting of an amino acid sequence
15 derived from a naturally occurring protein which modulates blood coagulation, or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which polypeptide exhibits an anti-inflammatory and/or anti-coagulant activity,
- 20 wherein the naturally occurring protein which modulates blood coagulation is selected from the group consisting of serpins (other than heparin cofactor II), histidine-rich glycoprotein (HRG) and tissue factor pathway inhibitors (such as TFPI-1 and TFPI-2).
- with the proviso that the polypeptide is not a naturally occurring protein.
- 25
57. A polypeptide according to Claim 56 wherein the naturally occurring protein which modulates blood coagulation is a human protein.
58. A polypeptide according to Claim 56 or 57 wherein the naturally occurring
30 protein which modulates blood coagulation is selected from the group consisting of serine proteinase inhibitors (serpins), tissue factor pathway inhibitors (such as TFPI-1 and TFPI-2) and histidine-rich glycoprotein (HRG).
59. A polypeptide according to Claim 58 wherein the naturally occurring protein
35 which modulates blood coagulation is a serpin.
60. A polypeptide according to Claim 59 wherein the serpin is anti-thrombin III.

61. A polypeptide according to Claim 60 wherein the anti-thrombin III is Swiss Port Accession No. P01008.

5 62. A polypeptide according to Claim 60 or 61 comprising or consisting of the amino acid sequence of SEQ ID NO:1 or 2:

"FFF21": FFFAKLNCRLYRKANKSSKLV [SEQ ID NO:1]

"AKL22": AKLNCRLYRKANKSSKLVSANR [SEQ ID NO:2]

10

or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anticoagulant activity of SEQ ID NO:1 or 2.

15 63. A polypeptide according to Claim 62 comprising or consisting of the amino acid sequence of SEQ ID NO: 1 or 2.

64. A polypeptide according to Claim 59 wherein the serpin is protein C inhibitor (PCI).

20

65. A polypeptide according to Claim 64 wherein the protein C inhibitor is Swiss Port Accession No. P05154.

25 66. A polypeptide according to Claim 64 or 65 comprising or consisting of the amino acid sequence of SEQ ID NO:3:

"SEK20": SEKTLRKWLKMFKKRQLELY [SEQ ID NO:3]

30

or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:3.

67. A polypeptide according to Claim 66 comprising or consisting of the amino acid sequence of SEQ ID NO:3.

35

68. A polypeptide according to Claim 58 wherein the naturally occurring protein which modulates blood coagulation is a tissue factor pathway inhibitor (TFPI).

69. A polypeptide according to Claim 68 wherein the tissue factor pathway inhibitor is TFPI-1.

5 70. A polypeptide according to Claim 69 wherein the TFPI-1 is Swiss Port Accession No. P10646.

71. A polypeptide according to Claim 68 or 69 comprising or consisting of the amino acid sequence of any one of SEQ ID NOS:4 to 6:

10

"GGL27":	GGLIKTKRKRKKQRVKIAEYEEIFVKNM	[SEQ ID NO:4]
"LIK17":	LIKTKRKRKKQRVKIAY	[SEQ ID NO:5]
"TKR22":	TKRKRKKQRVKIAEYEEIFVKNM	[SEQ ID NO:6]

15 or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of any one of SEQ ID NOS:4 to 6.

20 72. A polypeptide according to Claim 71 comprising or consisting of the amino acid sequence of any one of SEQ ID NOS:4 to 6.

73. A polypeptide according to Claim 68 wherein the tissue factor pathway inhibitor is TFPI-2.

25 74. A polypeptide according to Claim 73 wherein the TFPI-2 is Swiss Port Accession No. P48307.

75. A polypeptide according to Claim 73 or 74 comprising or consisting of the amino acid sequence of SEQ ID NO:7 or 8:

30

"ALK25":	ALKKKKKMPKLRFASRIRKIRKKQF	[SEQ ID NO:7]
"EDC34":	EDCKRACAKALKKKKKMPKLRFASRIRKIRKKQF	[SEQ ID NO:8]

35 or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:7 or 8.

76. A polypeptide according to Claim 75 comprising or consisting of the amino acid sequence of SEQ ID NO:7 or 8.

5 77. A polypeptide according to Claim 58 wherein the naturally occurring protein which modulates blood coagulation is histidine-rich glycoprotein (HRG).

78. A polypeptide according to Claim 77 wherein the HRG is Swiss Port Accession No. P04196.

10

79. A polypeptide according to Claim 77 or 78 comprising or consisting of the amino acid sequence of SEQ ID NO:9:

$(X_1X_2X_3X_4X_5)_n$ [SEQ ID NO:9],

15

wherein

X_1 and X_4 independently represent G, P, L, I, F, T, V, Y or W;

X_2 , X_3 and X_5 independently represent H, R or K; and

20

'n' is an integer from 2 to 6

or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:9.

25

80. A polypeptide according to any one of Claims 77 to 79 comprising or consisting of the amino acid sequence of SEQ ID NO:10:

$(GHHPH)_n$ [SEQ ID NO:10], where 'n' is an integer from 2 to 6

30

or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:10.

35 81. A polypeptide according to any one of Claims 77 to 80 comprising or consisting of the amino acid sequence of SEQ ID NO:11:

"GHH25": GHHPHGHHPHGHHPHGHHPHGHHPH [SEQ ID NO:11]

or a fragment, variant, fusion or derivative thereof, or a fusion of said fragment, variant or derivative thereof, which retains an anti-inflammatory and/or anti-coagulant activity of SEQ ID NO:11.

82. A polypeptide according to any one of Claims 77 to 81 comprising or consisting of the amino acid sequence of SEQ ID NOs: 9, 10 or 11.

83. A polypeptide according to any one of Claims 56 to 82 wherein the polypeptide, or fragment, variant, fusion or derivative thereof, comprises or consists of L-amino acids.

84. A polypeptide according to any one of Claims 56 to 83 wherein the polypeptide, or fragment, variant, fusion or derivative thereof, comprises one or more amino acids that are modified or derivatised.

85. A polypeptide according to Claim 84 wherein the one or more amino acids are modified or derivatised by PEGylation, amidation, esterification, acylation, acetylation and/or alkylation.

86. A polypeptide according to any one of Claims 56 to 85 wherein the polypeptide is between 10 and 200 amino acids in length, for example between 10 and 150, 15 and 100, 15 and 50, 15 and 30, 17 and 30, or 17 and 28 amino acids in length.

87. A polypeptide according to Claim 86 wherein the polypeptide is at least 17 amino acids in length.

88. A polypeptide according to any one of Claims 56 to 87 wherein the polypeptide is linear.

89. A polypeptide according to any one of Claims 56 to 88 wherein the polypeptide is a recombinant polypeptide.

90. A polypeptide according to any one of Claims 56 to 89 wherein the polypeptide is capable of inhibiting the release of one or more pro-inflammatory cytokines from human monocyte-derived macrophages.
- 5 91. A polypeptide according to Claim 90 wherein the pro-inflammatory cytokines are selected from the group consisting of macrophage inhibitory factor, TNF-alpha, HMGB1, C5a, IL-17, IL-8, MCP-1, IFN-gamma, IL-6, IL-1b, IL-12.
- 10 92. A polypeptide according to any one of Claims 56 to 91 wherein the polypeptide is capable of blocking platelet activation.
- 15 93. A polypeptide according to any one of Claims 56 to 92 wherein the polypeptide is capable of interfering with Toll-like receptor (TLR)-signalling in leukocytes, epithelial cells (including keratinocytes) and/or mesenchymal cells (including fibroblasts).
- 20 94. A polypeptide according to any one of Claims 56 to 93 wherein the polypeptide exhibits anti-inflammatory activity in one or more of the following models:
- (a) *in vitro* macrophage models using LPS, LTA, zymosan, flagellin, dust mites, viral or bacterial DNA or RNA, or peptidoglycan as stimulants;
- (b) *in vivo* mouse models of endotoxin shock; and/or
- 25 (c) *in vivo* infection models, either in combination with antimicrobial therapy, or given alone.
- 30 95. A polypeptide according to any one of Claims 56 to 94 wherein the polypeptide exhibits anticoagulant activity.
96. A polypeptide according to any one of Claims 56 to 95 wherein the polypeptide exhibits Toll-like receptor (TLR) blocking activity.
- 35 97. An isolated nucleic acid molecule which encodes a polypeptide according to any one of Claims 56 to 96.

98. A vector comprising a nucleic acid molecule according to Claim 97.
99. A vector according to Claim 98 wherein the vector is an expression vector.
- 5 100. A host cell comprising a nucleic acid molecule according to Claim 97 or a vector according to Claim 98 or 99.
101. A method of making a polypeptide according to any one of Claims 56 to 86 comprising culturing a population of host cells according to Claim 100 under
10 conditions in which said polypeptide is expressed, and isolating the polypeptide therefrom.
102. A method of making a polypeptide according to any one of Claims 56 to 86 comprising liquid-phase or solid-phase synthesis of the polypeptide.
- 15 103. A pharmaceutical composition comprising a polypeptide according to any one of Claims 56 to 86 together with a pharmaceutically acceptable excipient, diluent, carrier, buffer or adjuvant.
- 20 104. A pharmaceutical composition according to Claim 103 suitable for administration via a route selected from the group consisting of topical, ocular, nasal, pulmonar, buccal, parenteral (intravenous, subcutaneous, intratechal and intramuscular), oral, vaginal and rectal.
- 25 105. A pharmaceutical composition according to Claim 103 suitable for administration via an implant.
106. A pharmaceutical composition according to any one of Claims 103 to 105 wherein the pharmaceutical composition is associated with a device or
30 material to be used in medicine.
107. A pharmaceutical composition according to Claim 106 wherein the device used in by-pass surgery, extracorporeal circulation, wound care and/or dialysis.
- 35 108. A pharmaceutical composition according to Claim 106 or 107 wherein the pharmaceutical composition is coated, painted, sprayed or otherwise applied

to a suture, prosthesis, implant, wound dressing, catheter, lens, skin graft, skin substitute, fibrin glue or bandage.

109. A pharmaceutical composition according to any one of Claims 106 to 108
5 wherein the device or material comprise or consists of a polymer, metal, metal oxide and/or ceramic.

110. A polypeptide according to any one of Claims 56 to 86 for use in medicine.

10 111. A polypeptide according to Claim 110 for use in the treatment and/prevention of acute and/or chronic inflammation.

112. A polypeptide according to Claim 110 for use in the treatment and/prevention of microbial infection (e.g. bacterial infection).

15

113. A polypeptide according to Claim 110 for use in the modulation of blood coagulation.

114. A polypeptide according to Claim 110 for use in the treatment of wounds.

20

115. A polypeptide according to any one of Claims 110 or 114 for use in the treatment or prevention of a disease, condition or indication selected from the following:

25

i) Acute systemic inflammatory disease, with or without an infective component, such as systemic inflammatory response syndrome (SIRS), ARDS, sepsis, severe sepsis, and septic shock. Other generalized or localized invasive infective and inflammatory disease, including erysipelas, meningitis, arthritis, toxic shock syndrome, diverticulitis, appendicitis, pancreatitis, cholecystitis, colitis, cellulitis, burn wound infections, pneumonia, urinary tract infections, postoperative infections, and peritonitis.

30

35

ii) Chronic inflammatory and or infective diseases, including cystic fibrosis, COPD and other pulmonary diseases, gastrointestinal disease including chronic skin and stomach ulcerations, other epithelial inflammatory and or infective disease such as atopic

dermatitis, oral ulcerations (aphthous ulcers), genital ulcerations and inflammatory changes, parodontitis, eye inflammations including conjunctivitis and keratitis, external otitis, mediaotitis, genitourinary inflammations.

5

iii) Postoperative inflammation. Inflammatory and coagulative disorders including thrombosis, DIC, postoperative coagulation disorders, and coagulative disorders related to contact with foreign material, including extracorporeal circulation, and use of biomaterials. Furthermore, vasculitis related inflammatory disease, as well as allergy, including allergic rhinitis and asthma..

10

iv) Excessive contact activation and/or coagulation in relation to, but not limited to, stroke.

15

v) Excessive inflammation in combination with antimicrobial treatment. The antimicrobial agents used may be administered by various routes; intravenous (iv), intraarterial, intravitreal, subcutaneous (sc), intramuscular (im), intraperitoneal (ip), intravesical, intratechal, epidural, enteral (including oral, rectal, gastric, and other enteral routes), or topically, (including dermal, nasal application, application in the eye or ear, eg by drops, and pulmonary inhalation). Examples of agents are penicillins, cephalosporins, carbacephems, cephamycins, carbapenems, monobactams, aminoglycosides, glycopeptides, quinolones, tetracyclines, macrolides, and fluoroquinolones. Antiseptic agents include iodine, silver, copper, chlorhexidine, polyhexanide and other biguanides, chitosan, acetic acid, and hydrogen peroxide.

20

25

116. Use of a polypeptide according to any one of Claims 56 to 86 in the preparation of a medicament for the treatment or prevention of inflammation and/or excessive coagulation.

30

117. A use according to Claim 116 wherein the medicament is for use in the treatment or prevention of acute inflammation, sepsis, acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), cystic fibrosis, asthma, allergic and other types of rhinitis, cutaneous and

35

systemic vasculitis, thrombosis and disseminated intravascular coagulation (DIC).

118. A use according to Claim 114 wherein the medicament is for use in the treatment or prevention of sepsis.

119. A use according to Claim 114 wherein the medicament is for use in the treatment or prevention of chronic obstructive pulmonary disease (COPD).

120. A use according to any one of Claims 116 to 119 wherein the medicament is for use in combination with one or more additional therapeutic agent.

121. A use according to Claim 120 wherein the additional therapeutic agent is selected from the group consisting of antibiotic agents, anti-fungal agents, anti-inflammatory agents, immunosuppressive agents, vasoactive agents and antiseptic agents.

122. A use according to Claim 121 wherein the antibiotic agents are selected from the groups consisting of anti-bacterial agents, anti-fungicides, anti-viral agents and anti-parasitic agents.

123. A use according to Claim 121 wherein the additional therapeutic agent is a steroid.

124. A method for treating or preventing inflammation in a patient, the method comprising administering to the patient a therapeutically-effective amount of a polypeptide according to any one of Claims 1 to 86 or a pharmaceutical composition according to any one of Claims 103 to 109

125. A method according to Claim 124 wherein the patient is human.

126. A method according to Claim 124 or 125 for the treatment or prevention of acute inflammation, sepsis, acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), cystic fibrosis, asthma, allergic and other types of rhinitis, cutaneous and systemic vasculitis, thrombosis and disseminated intravascular coagulation (DIC).

127. A method according to Claim 126 for the treatment or prevention of sepsis.
128. A method according to Claim 126 for the treatment or prevention of chronic obstructive pulmonary disease (COPD).
- 5 129. A method according to any one of Claims 124 to 128 wherein the method further administering to the patient one or more additional therapeutic agent.
- 10 130. A method according to Claim 129 wherein the additional therapeutic agent is selected from the group consisting of antibiotic agents, anti-inflammatory agents, immunosuppressive agents, vasoactive agents, receptor blockers, receptor agonists and antiseptic agents.
- 15 131. A method according to Claim 130 wherein the antibiotic agents are selected from the groups consisting of anti-bacterial agents, anti-fungicides, anti-viral agents and anti-parasitic agents.
- 20 132. A method according to Claim 129 wherein the additional therapeutic agent is a steroid.
133. A polypeptide substantially as described herein with reference to the description and figures.
- 25 134. A pharmaceutical composition substantially as described herein with reference to the description and figures.
135. A medical implant or device, or biomaterial for use in the same, substantially as described herein with reference to the description and figures.
- 30 136. Use of a polypeptide substantially as described herein with reference to the description and figures.
137. A method for treating or preventing inflammation substantially as described herein with reference to the description and figures.

Figure 1

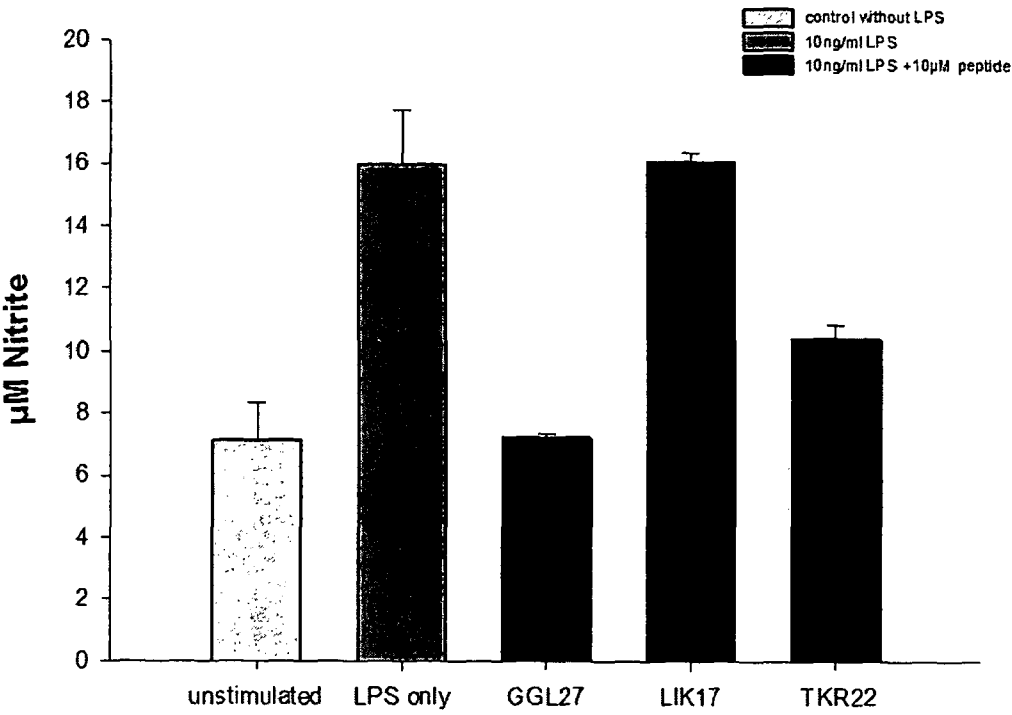


Figure 2

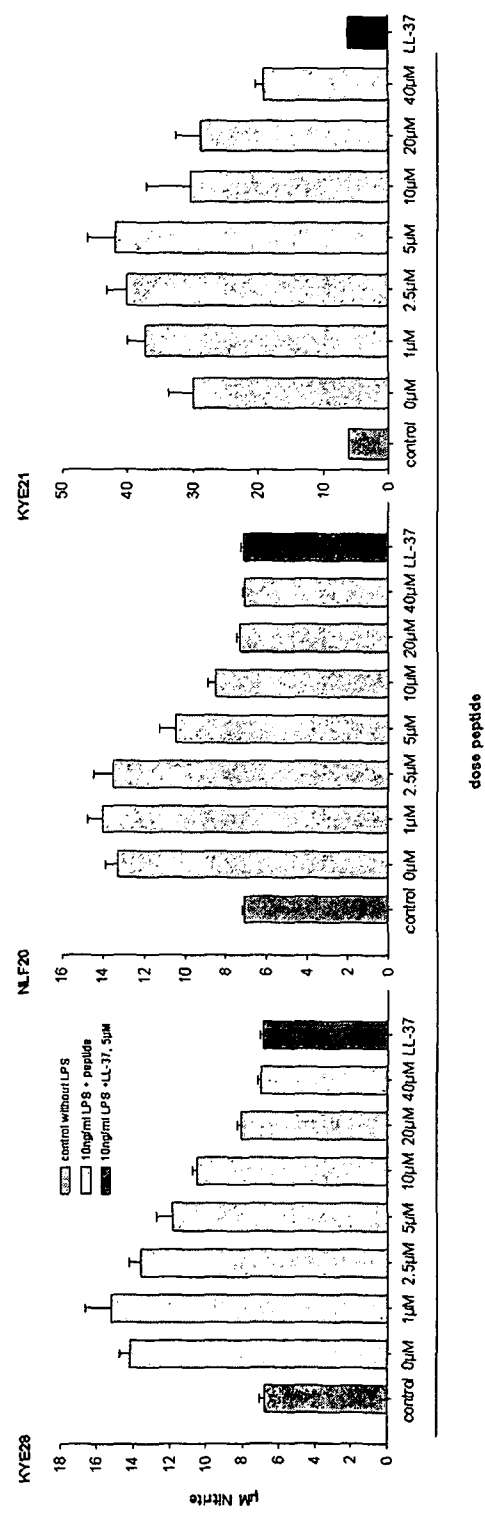


Figure 3

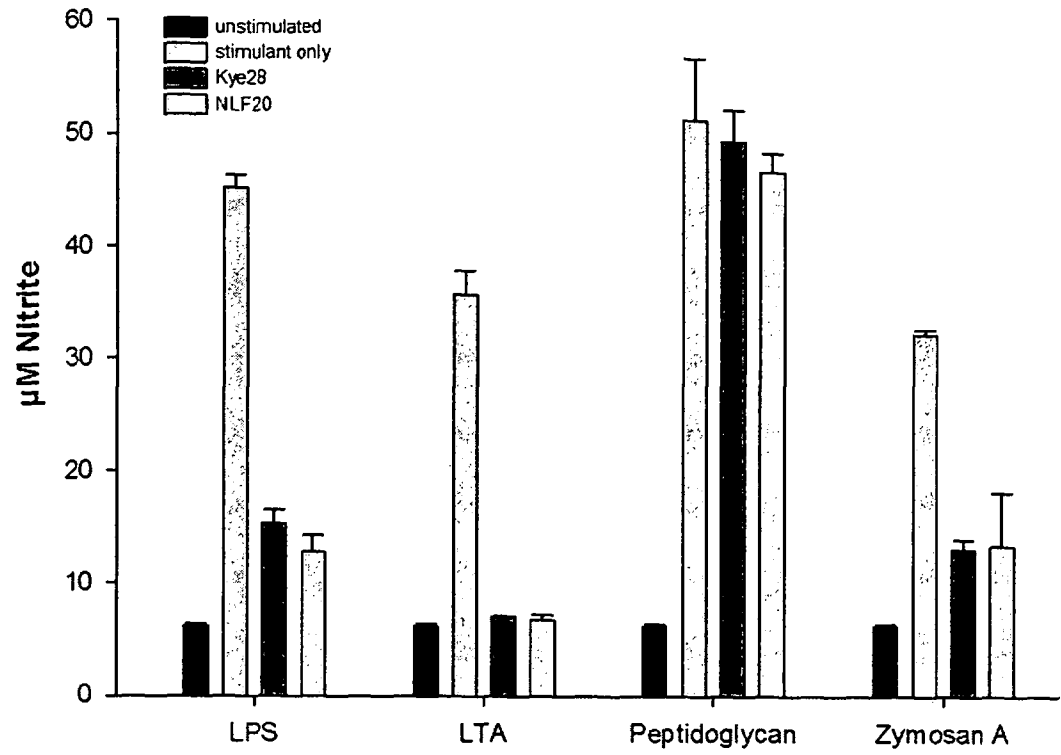


Figure 4

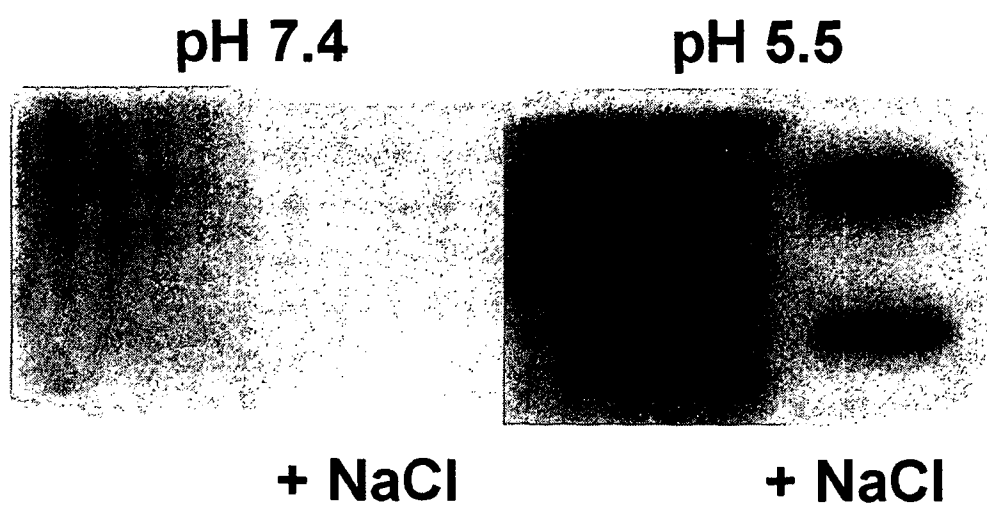


Figure 5

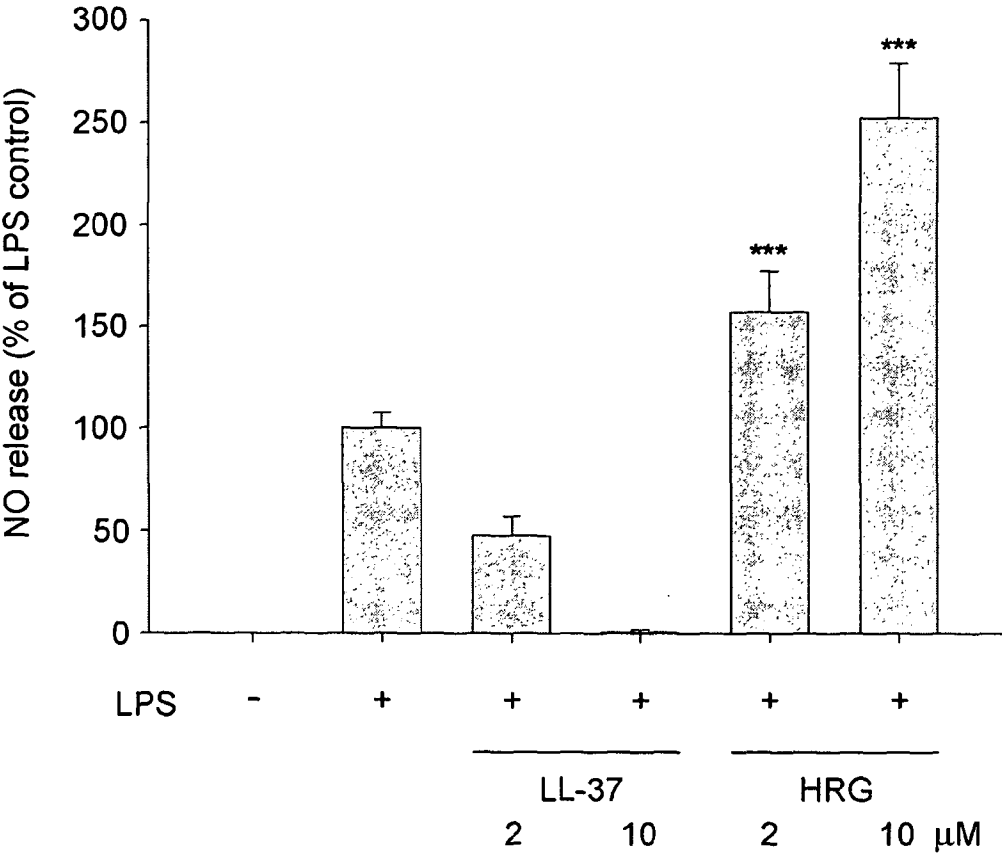


Figure 6

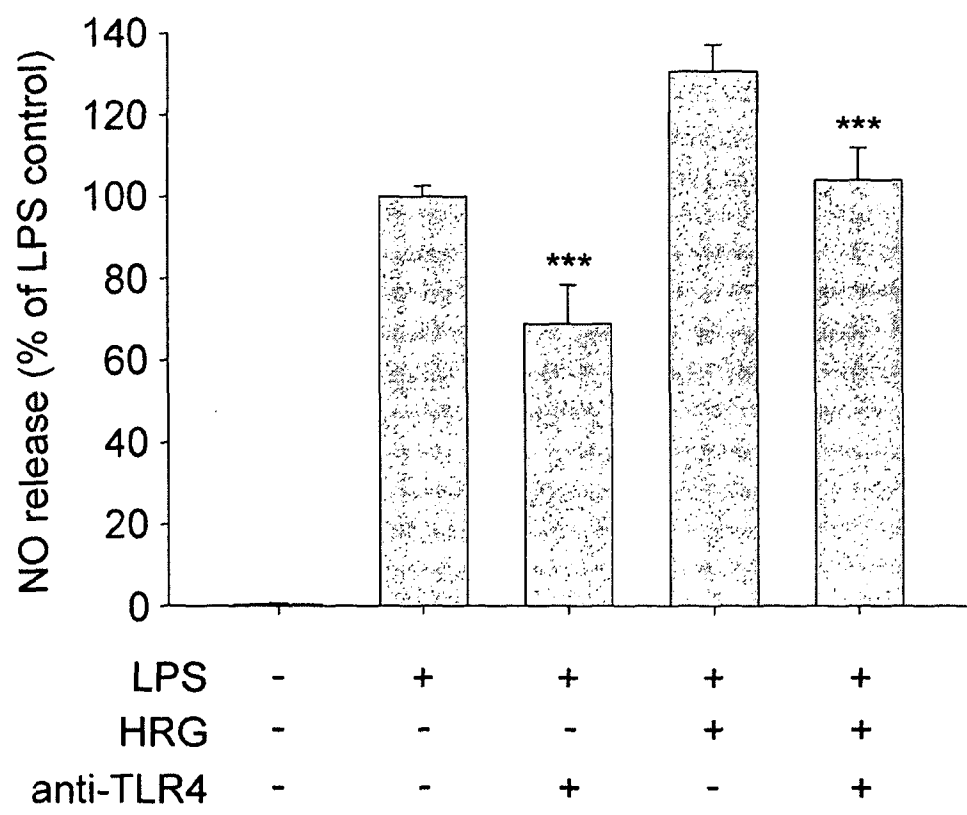


Figure 7

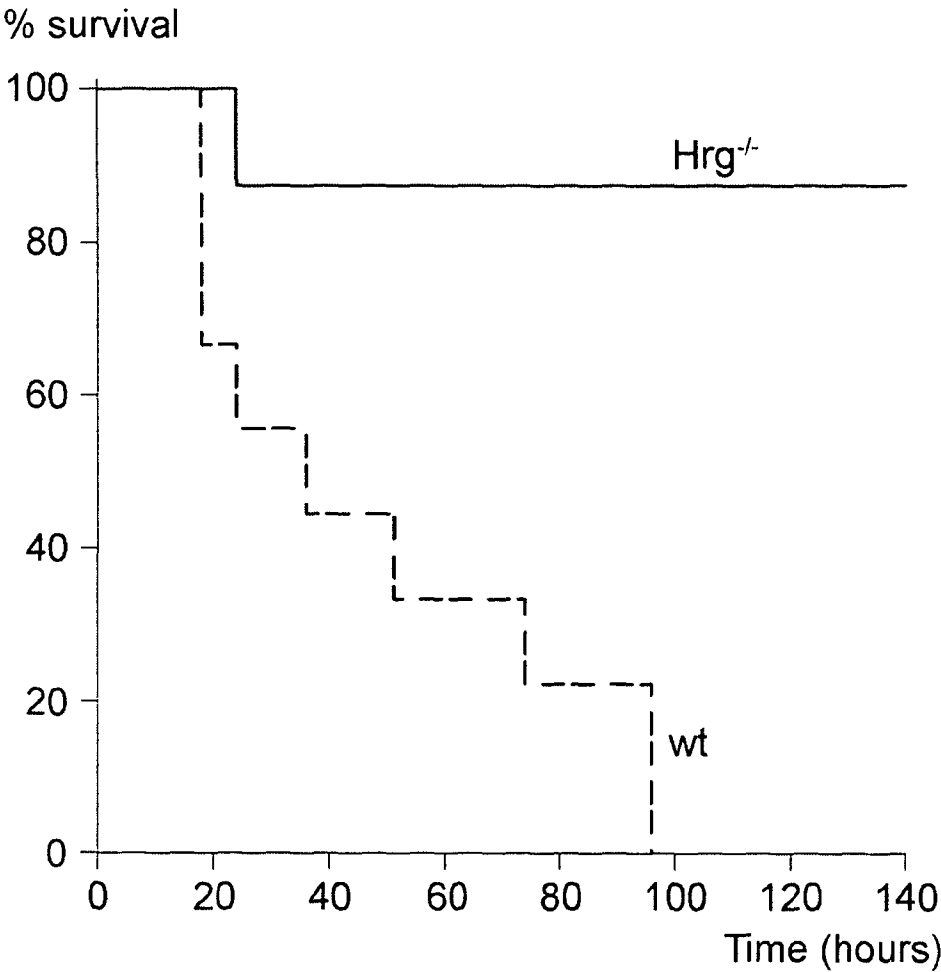


Figure 8

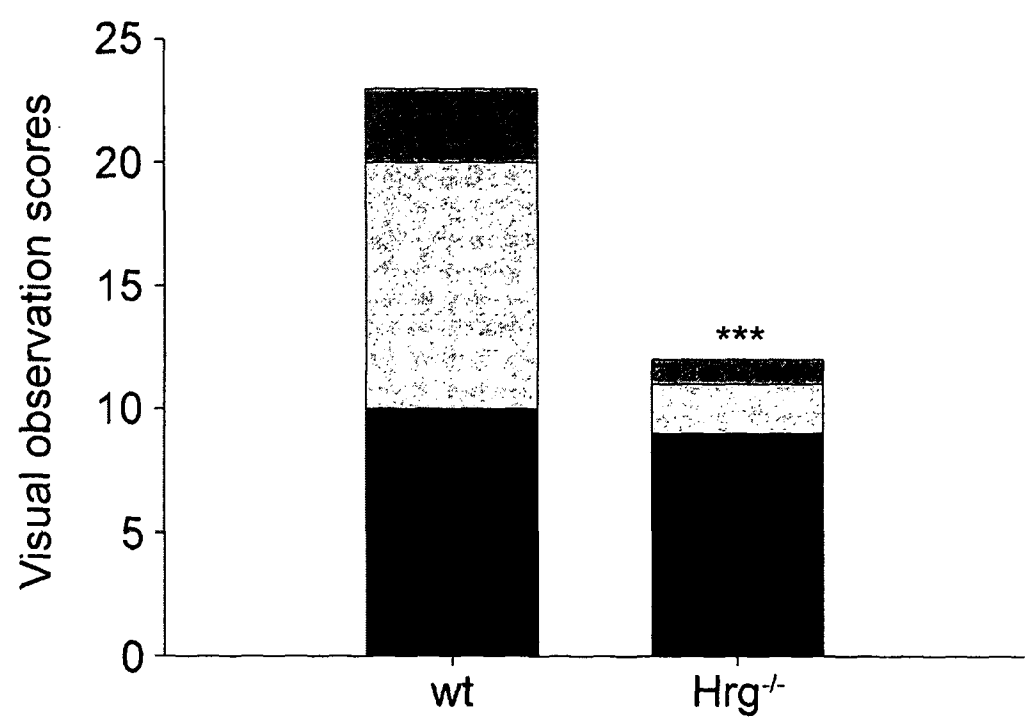


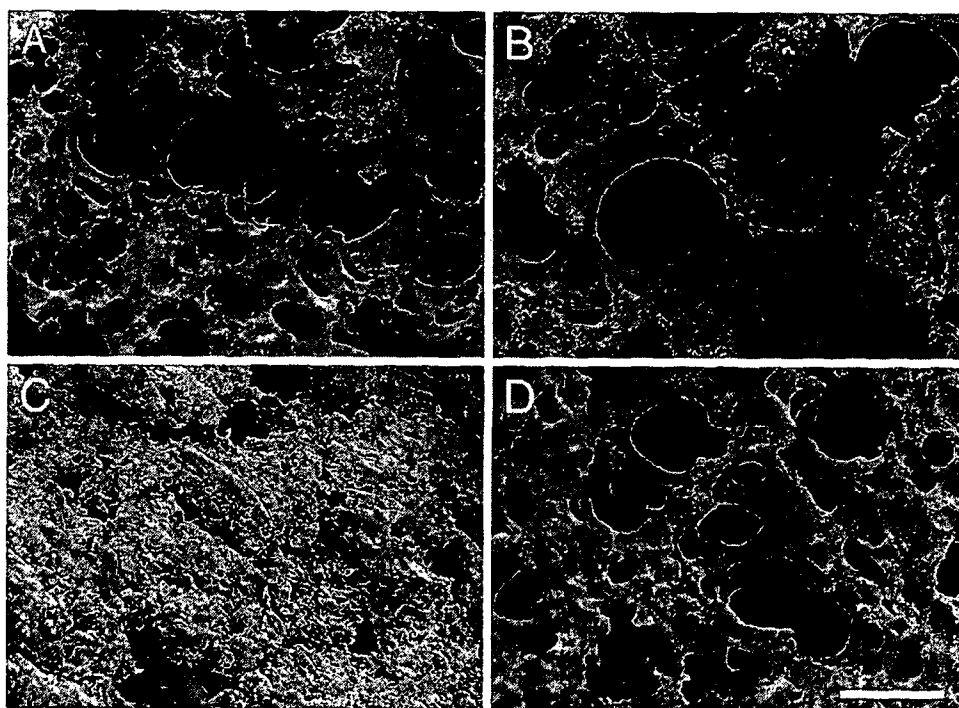
Figure 9

Figure 10

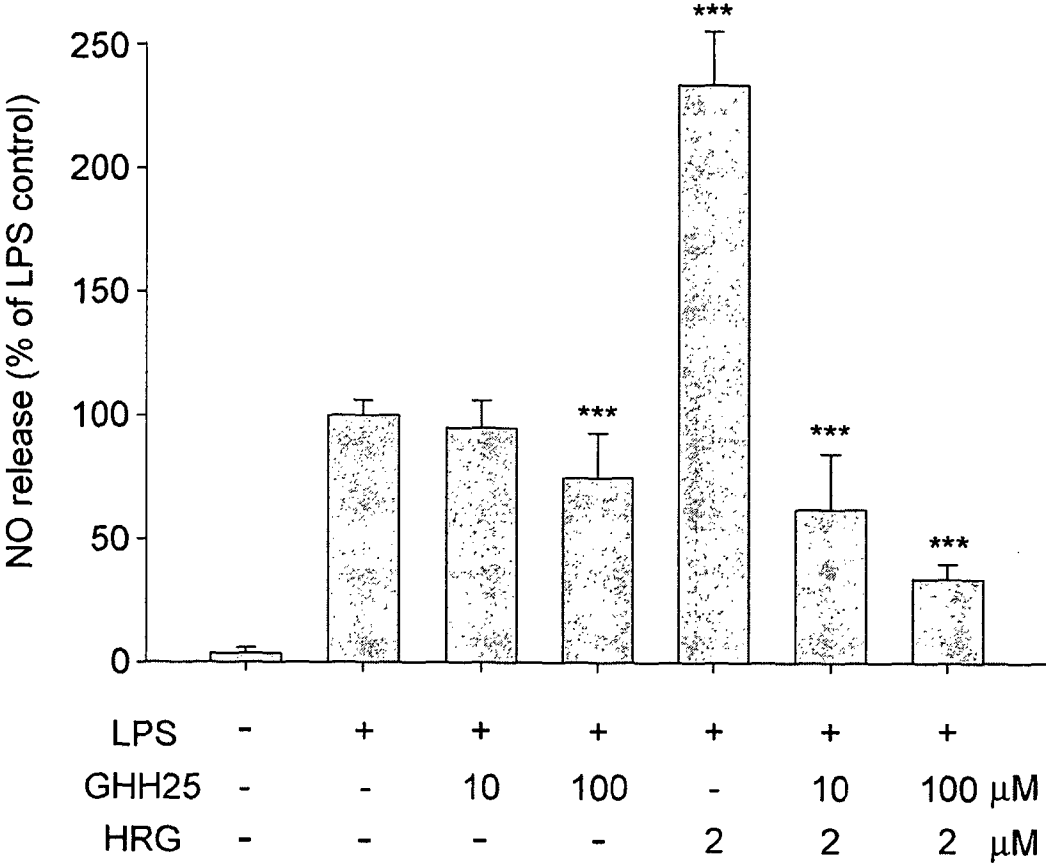


Figure 11

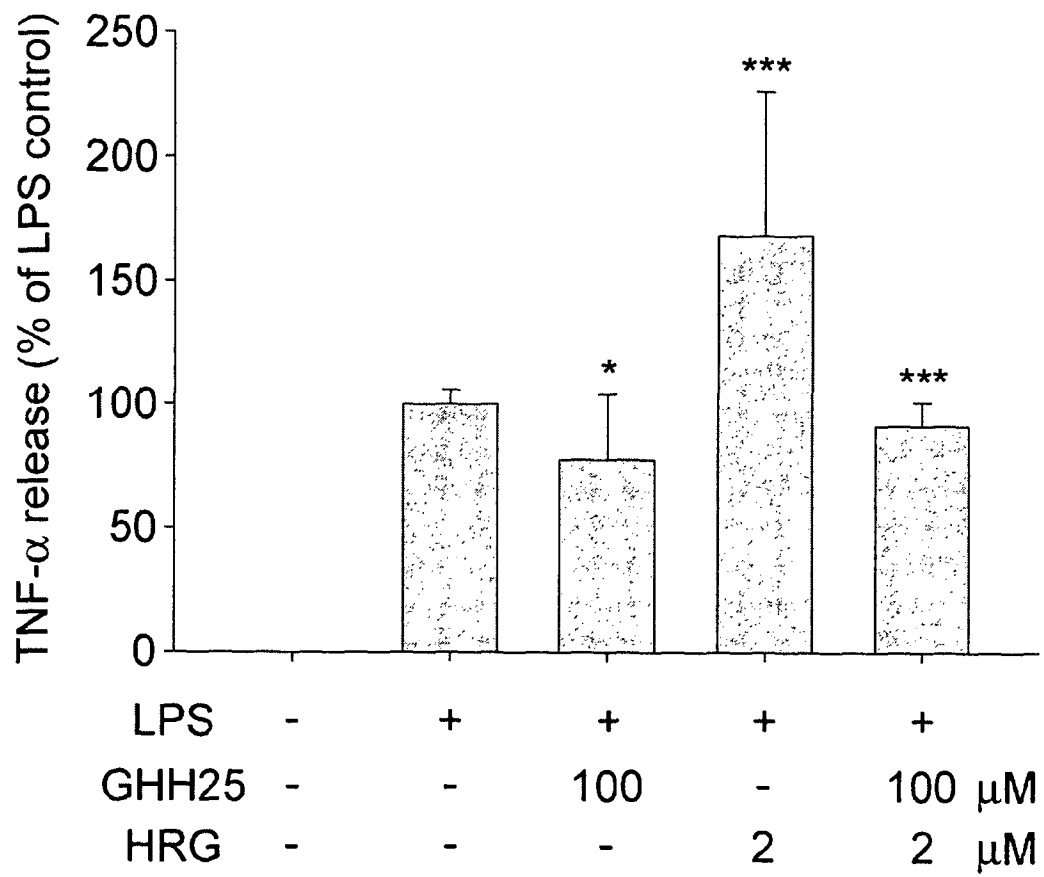


Figure 12

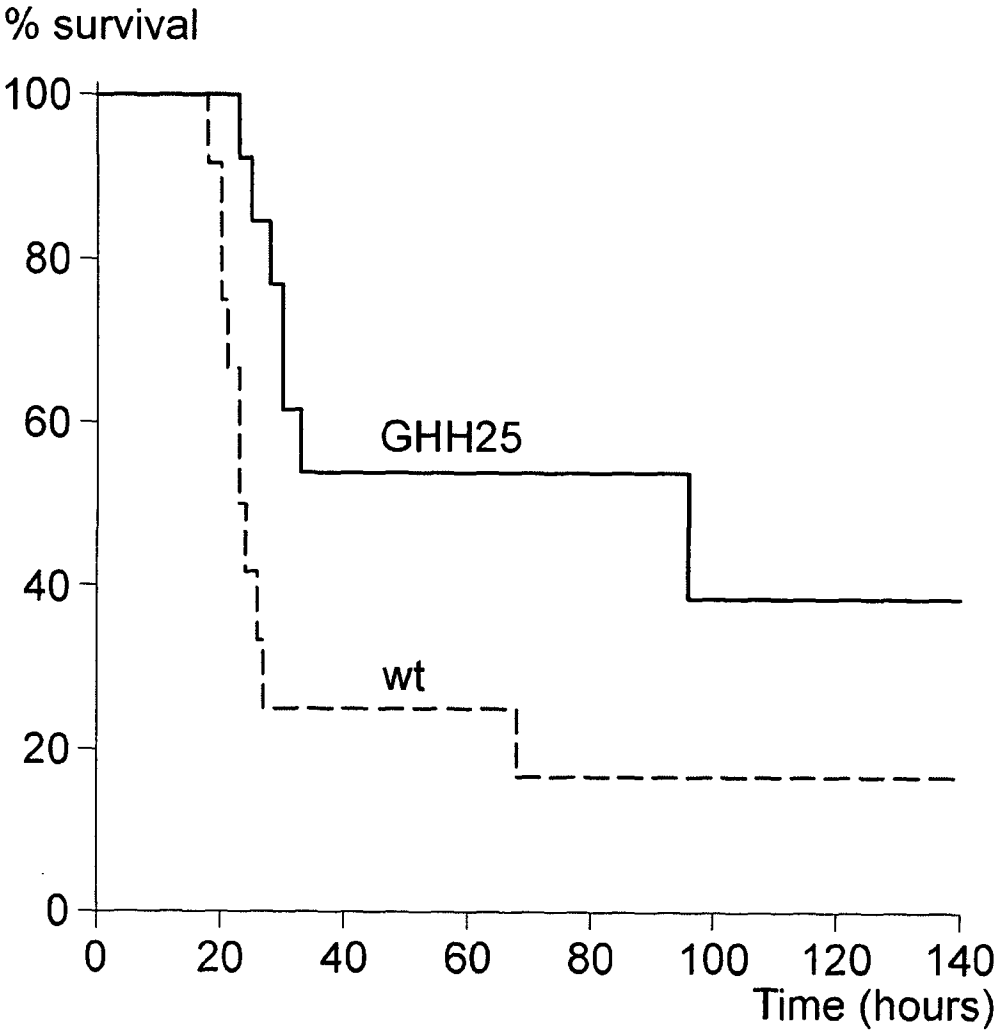


Figure 13

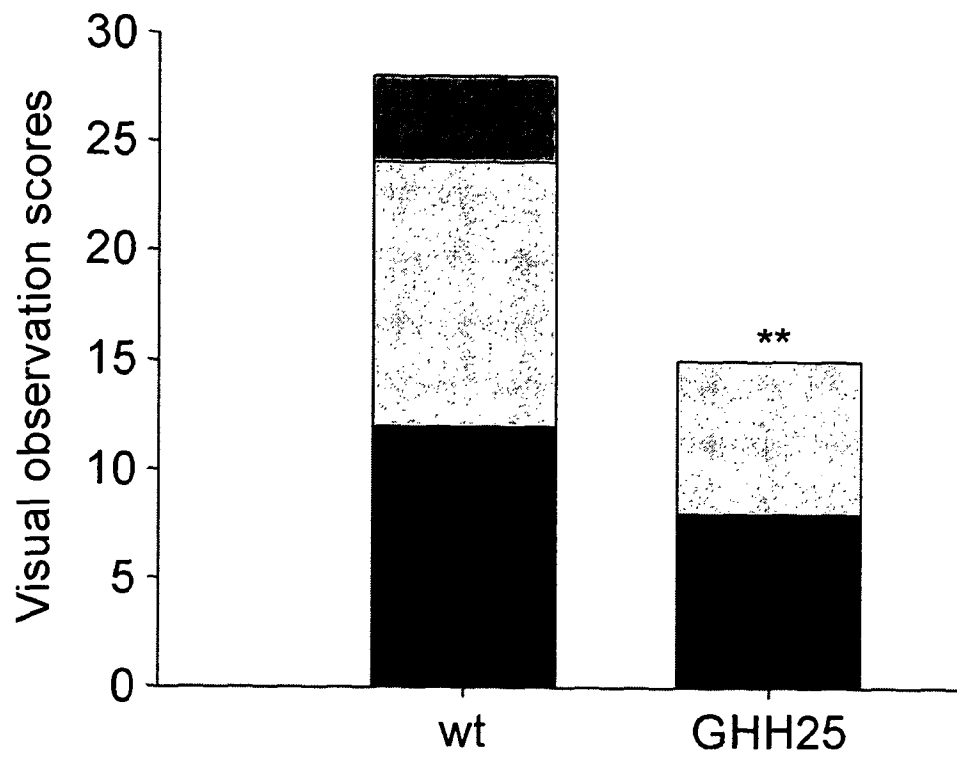


Figure 14

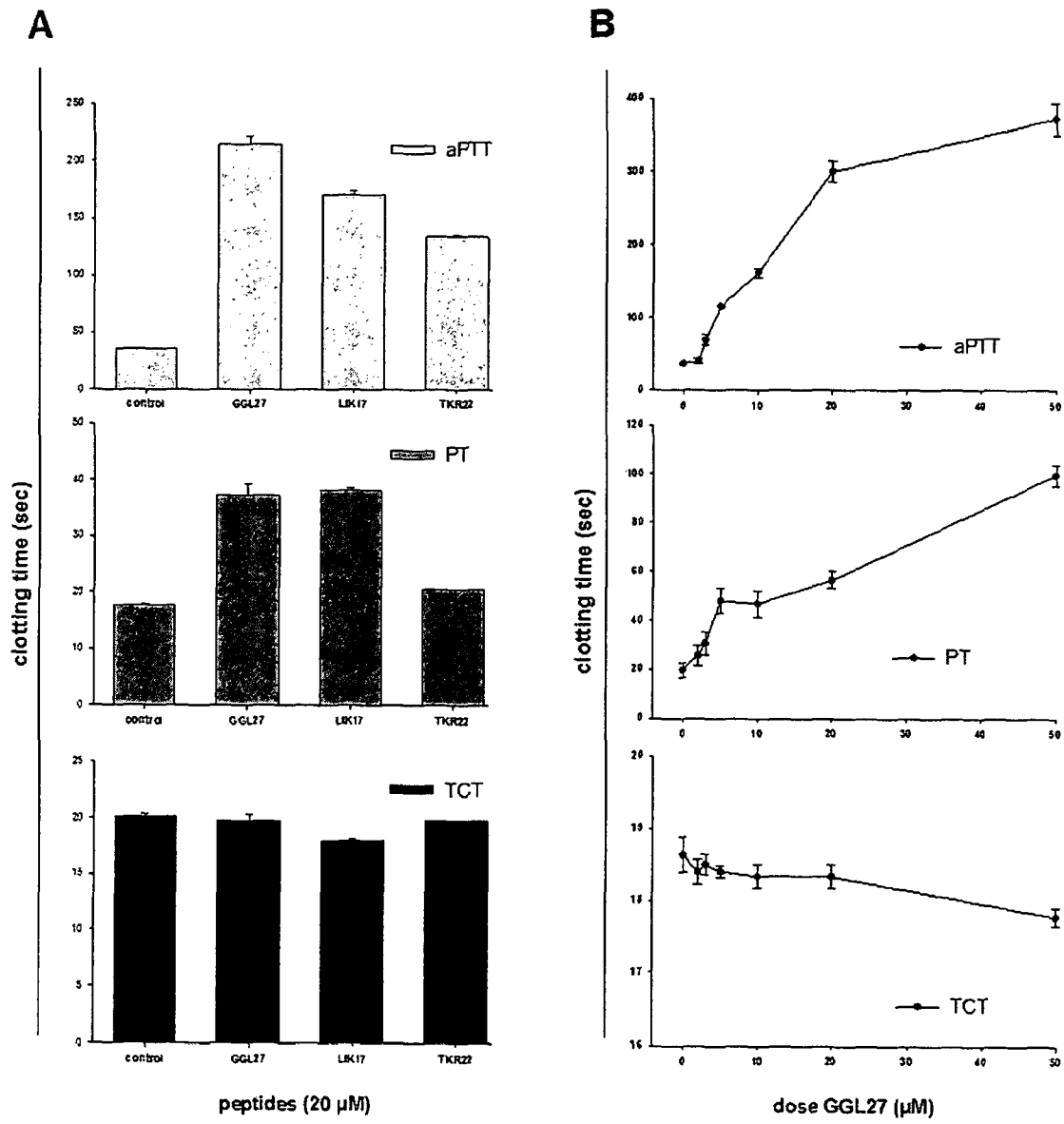


Figure 15

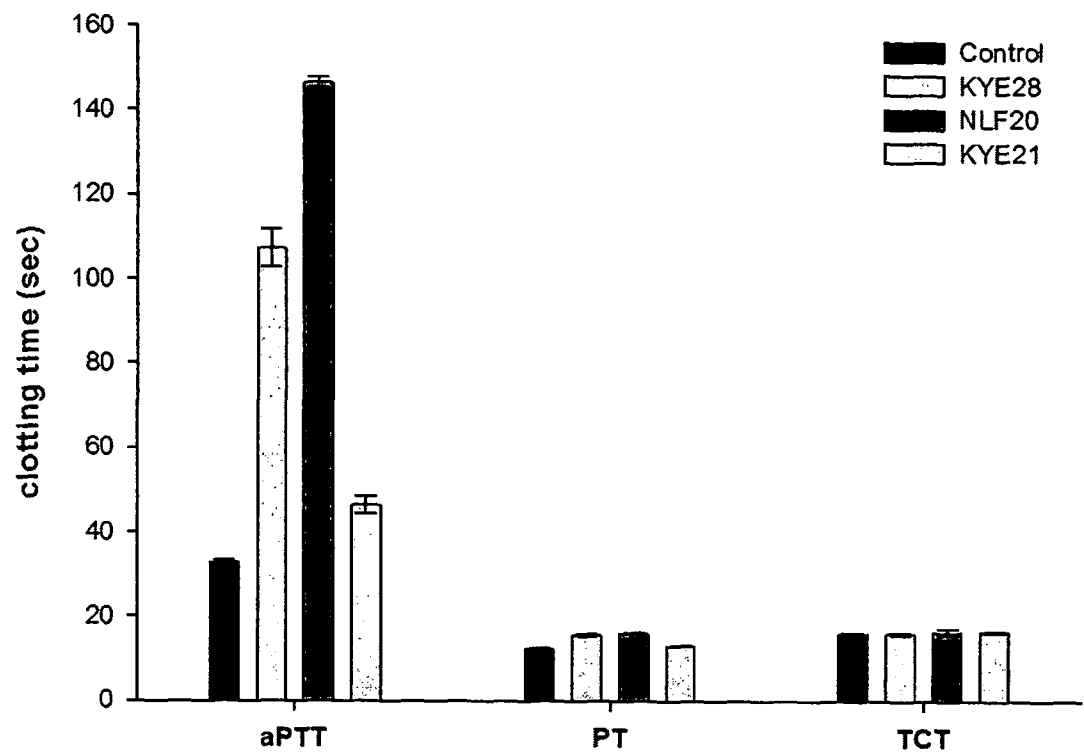


Figure 16

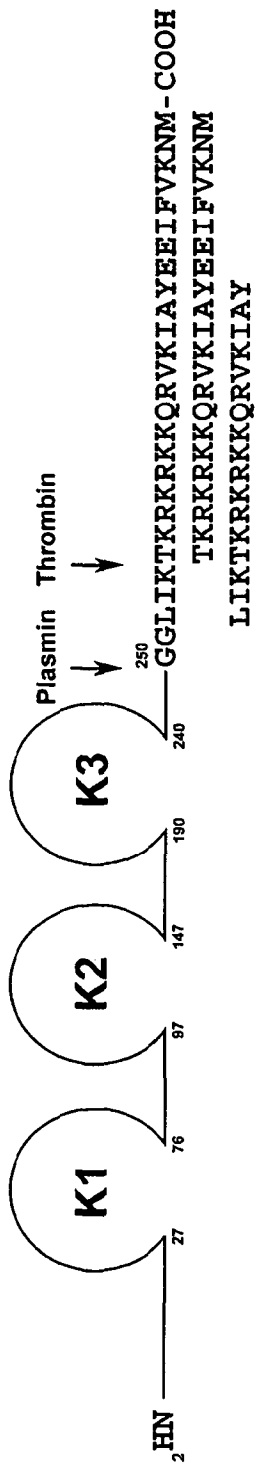


Figure 17

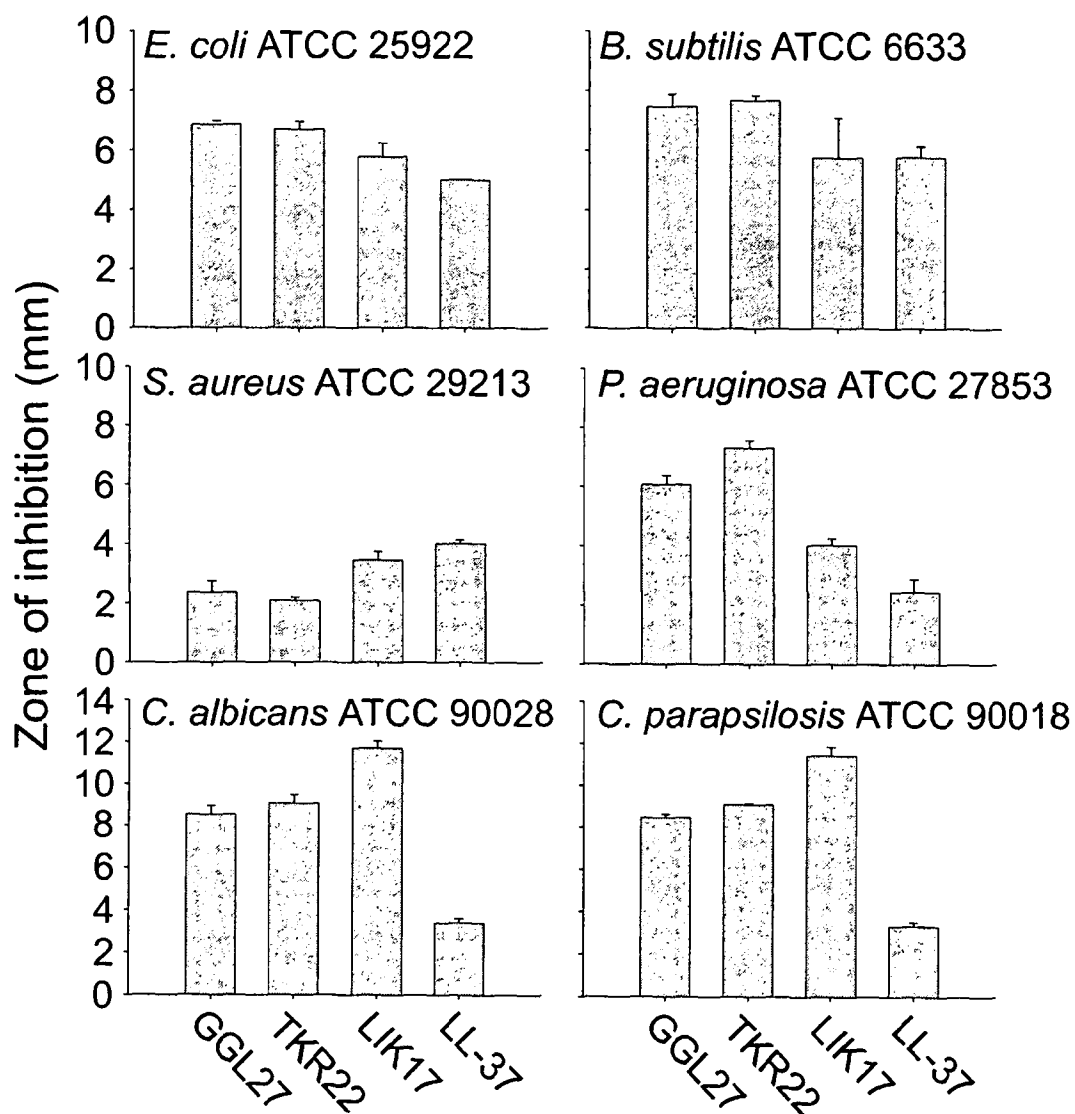


Figure 18

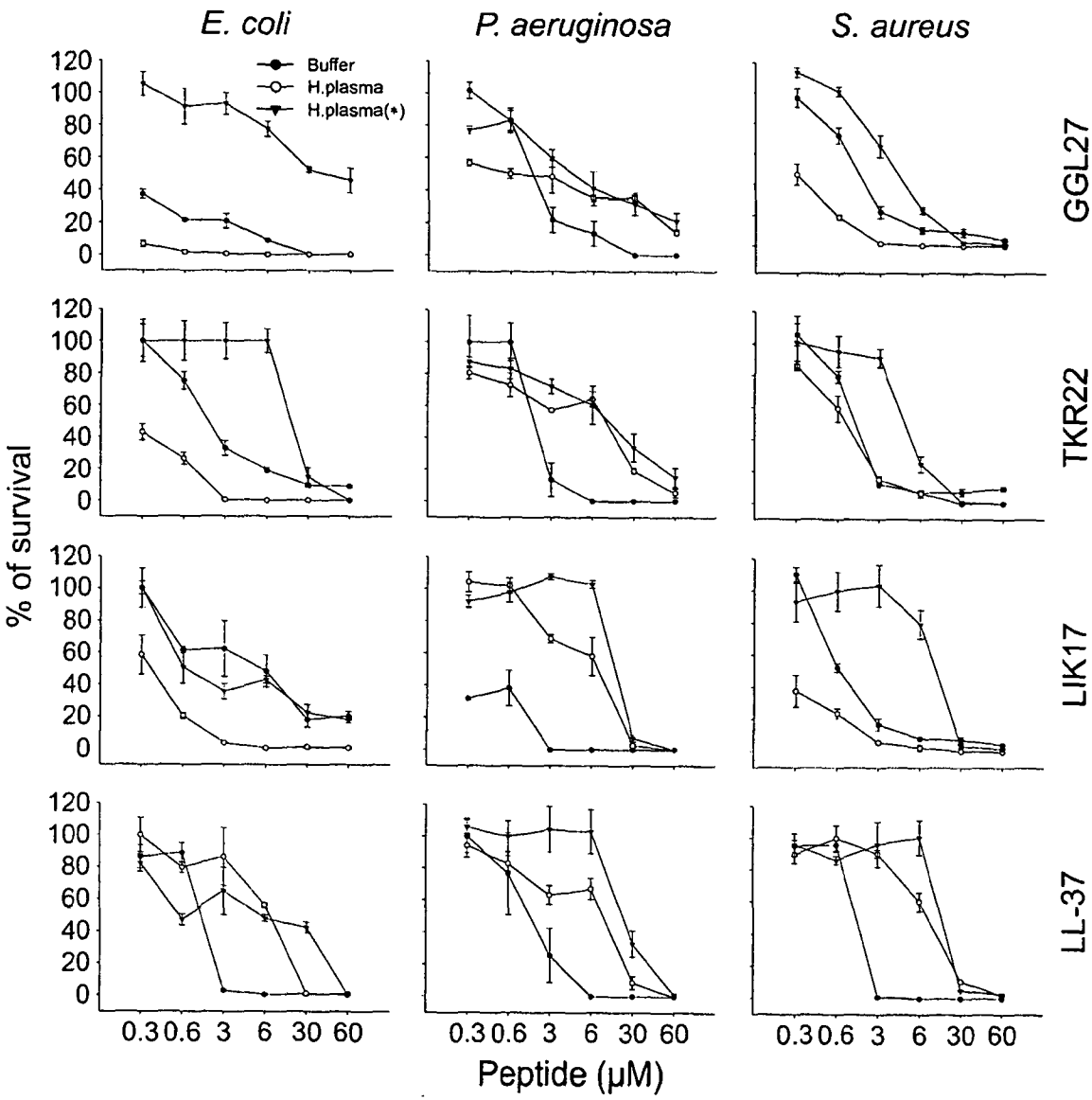


Figure 19

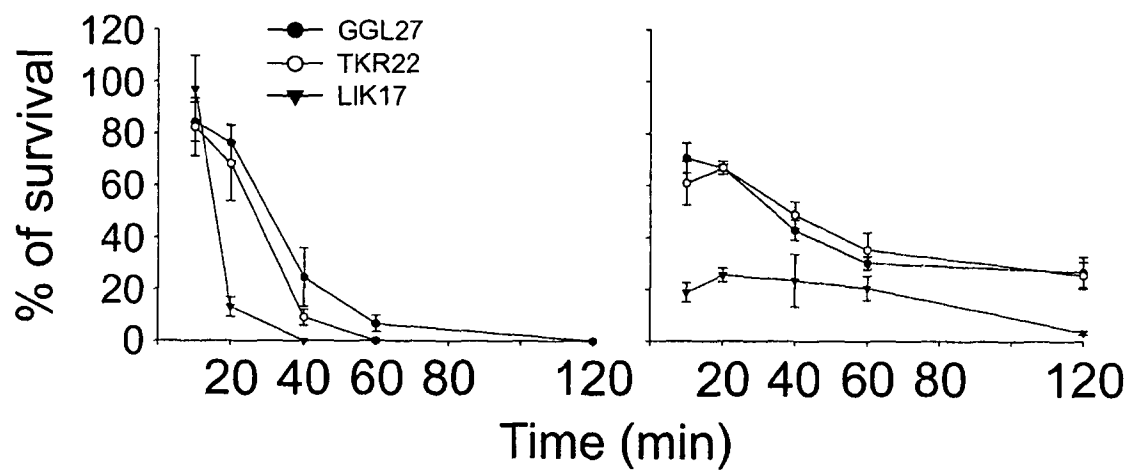


Figure 20

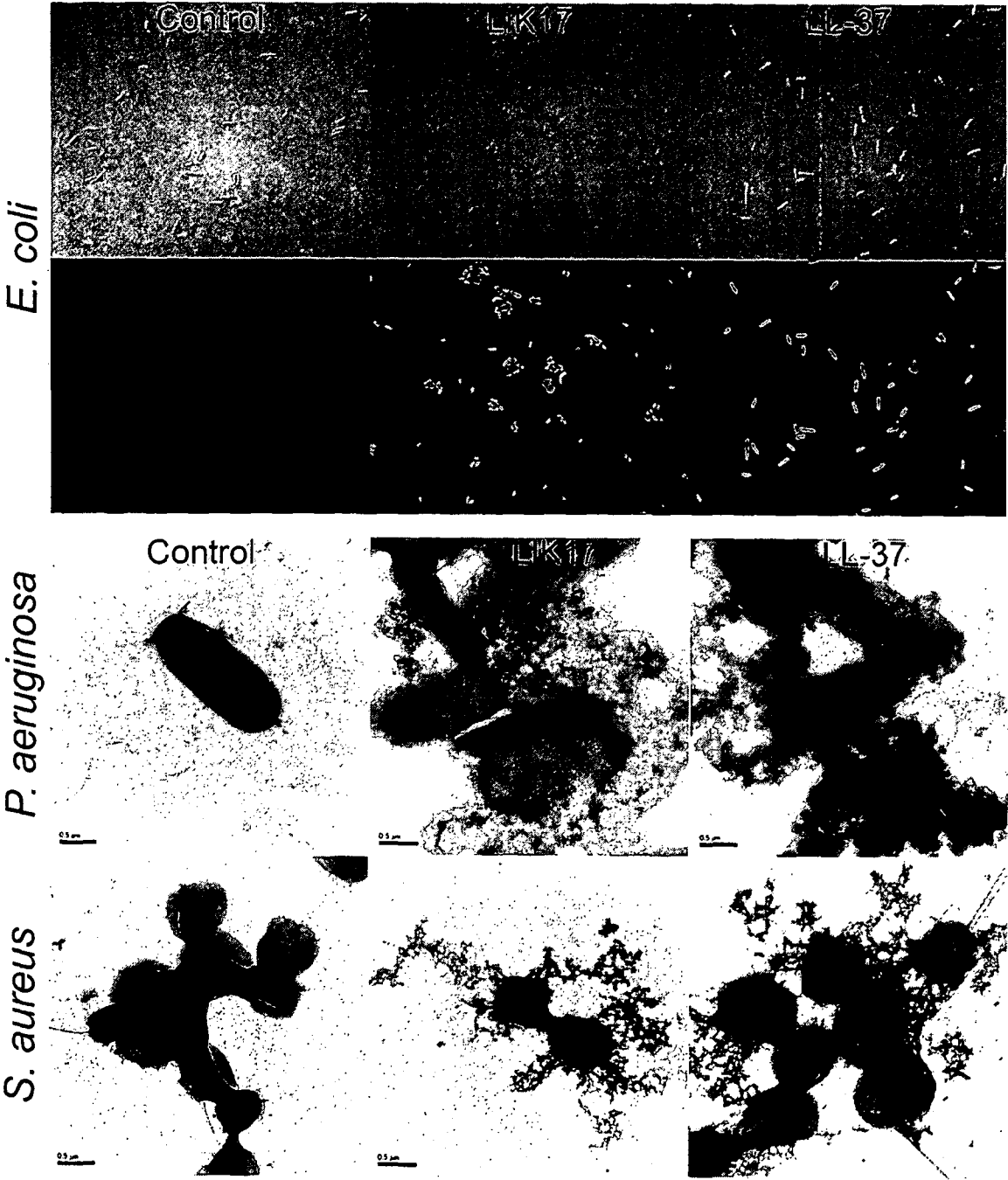


Figure 21

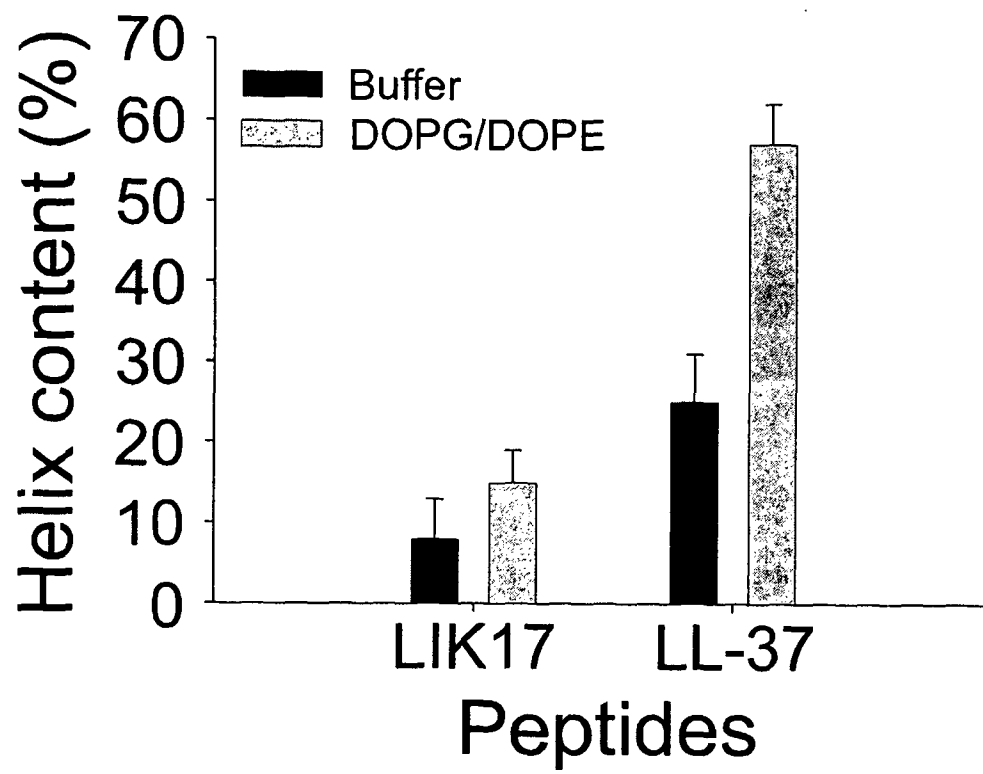


Figure 22

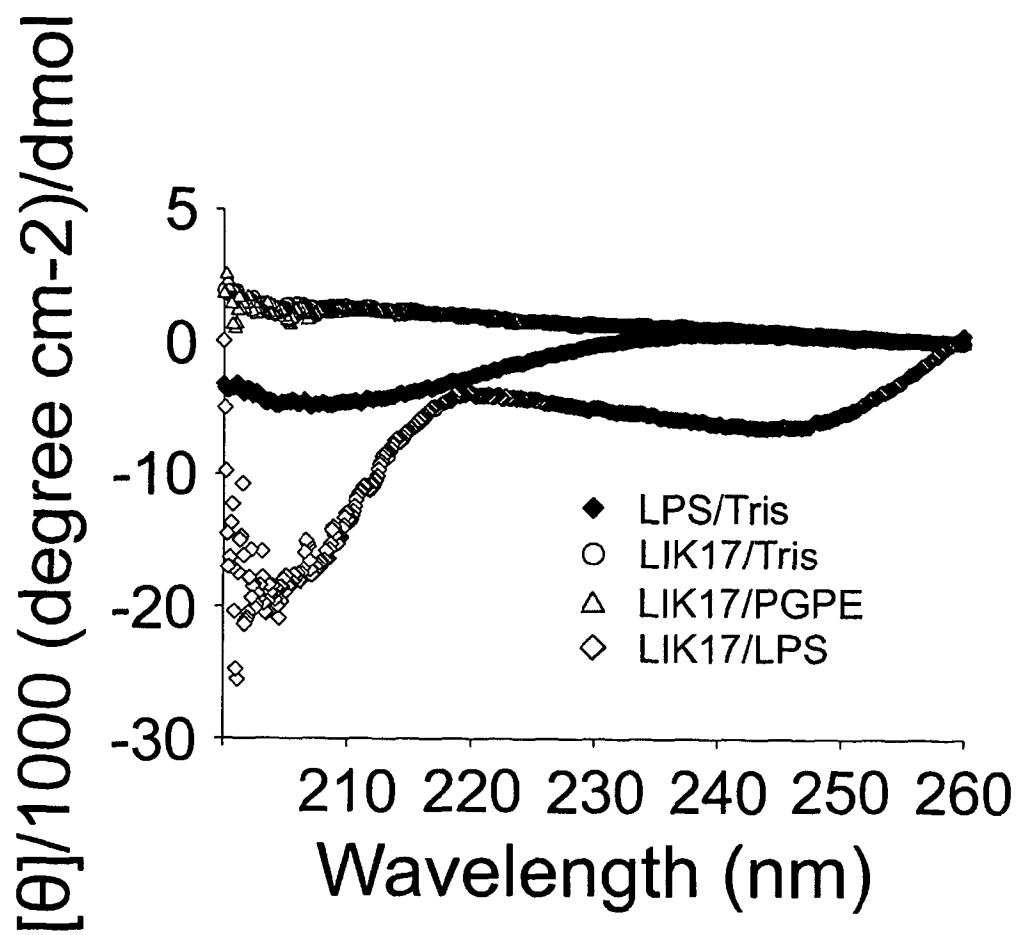


Figure 23

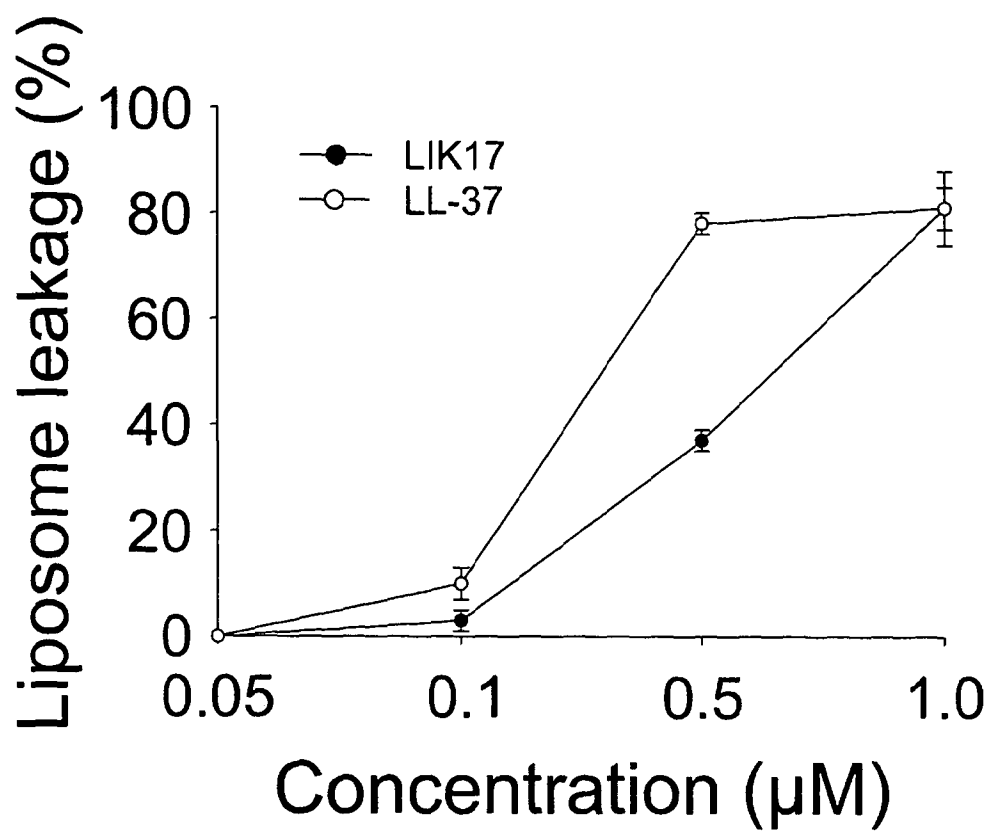


Figure 24

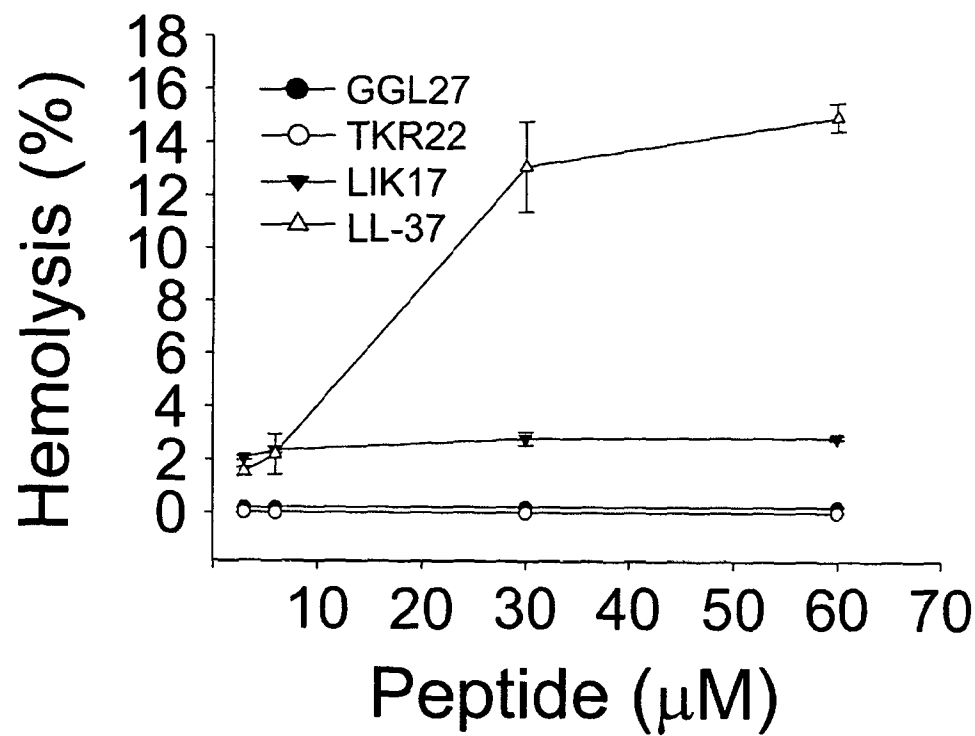


Figure 25

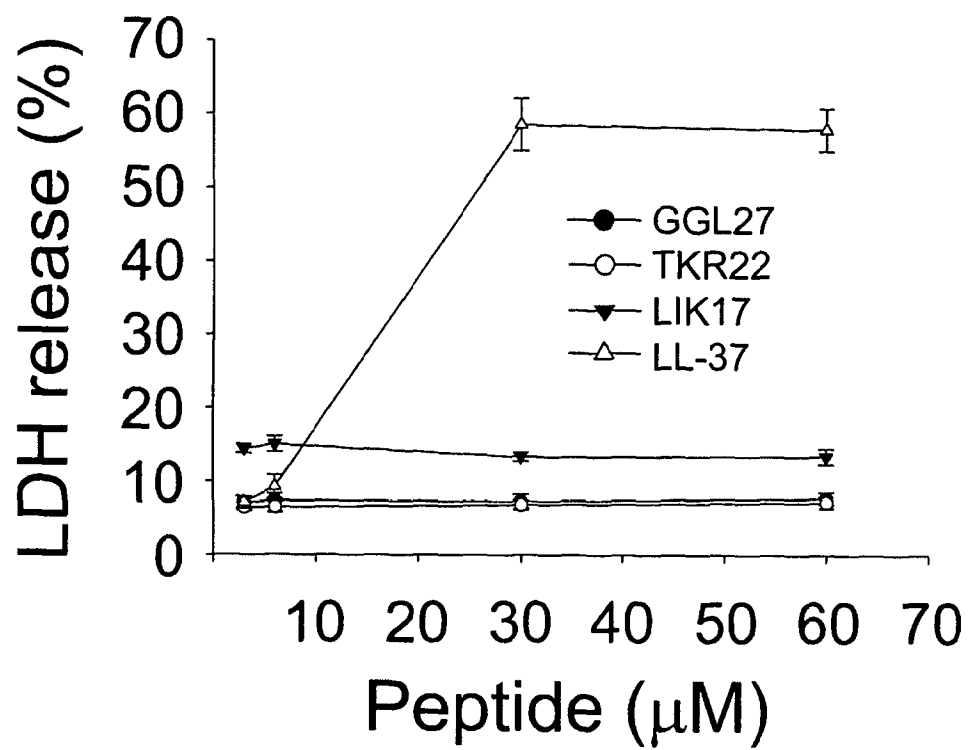


Figure 26

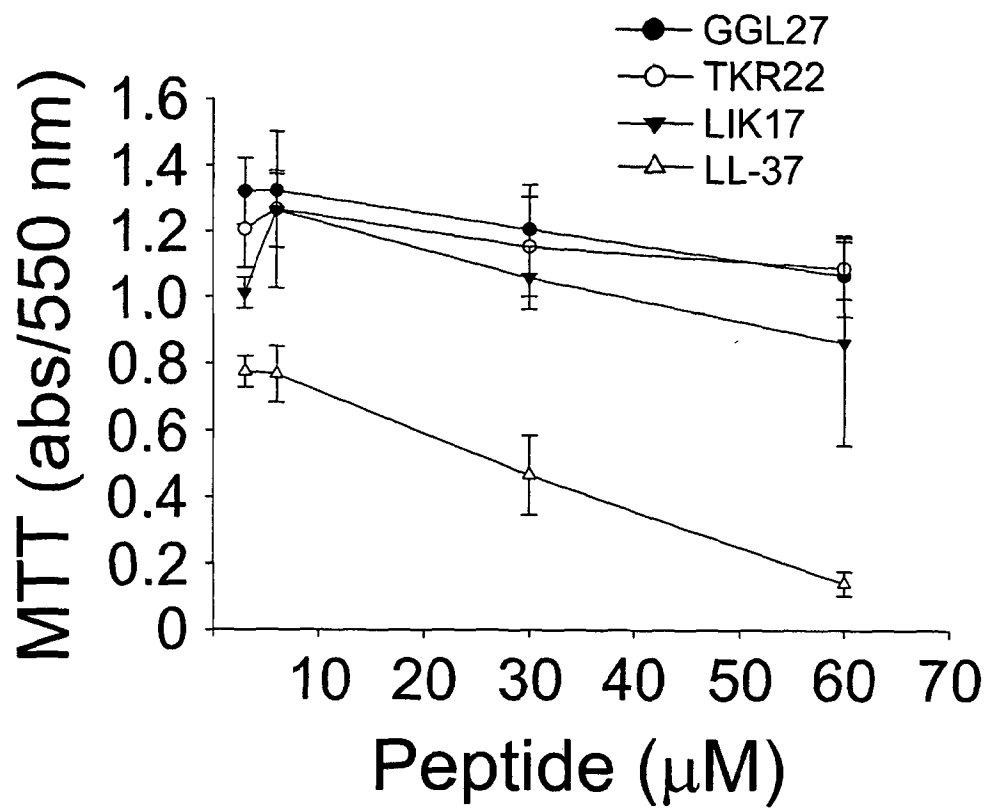


Figure 27

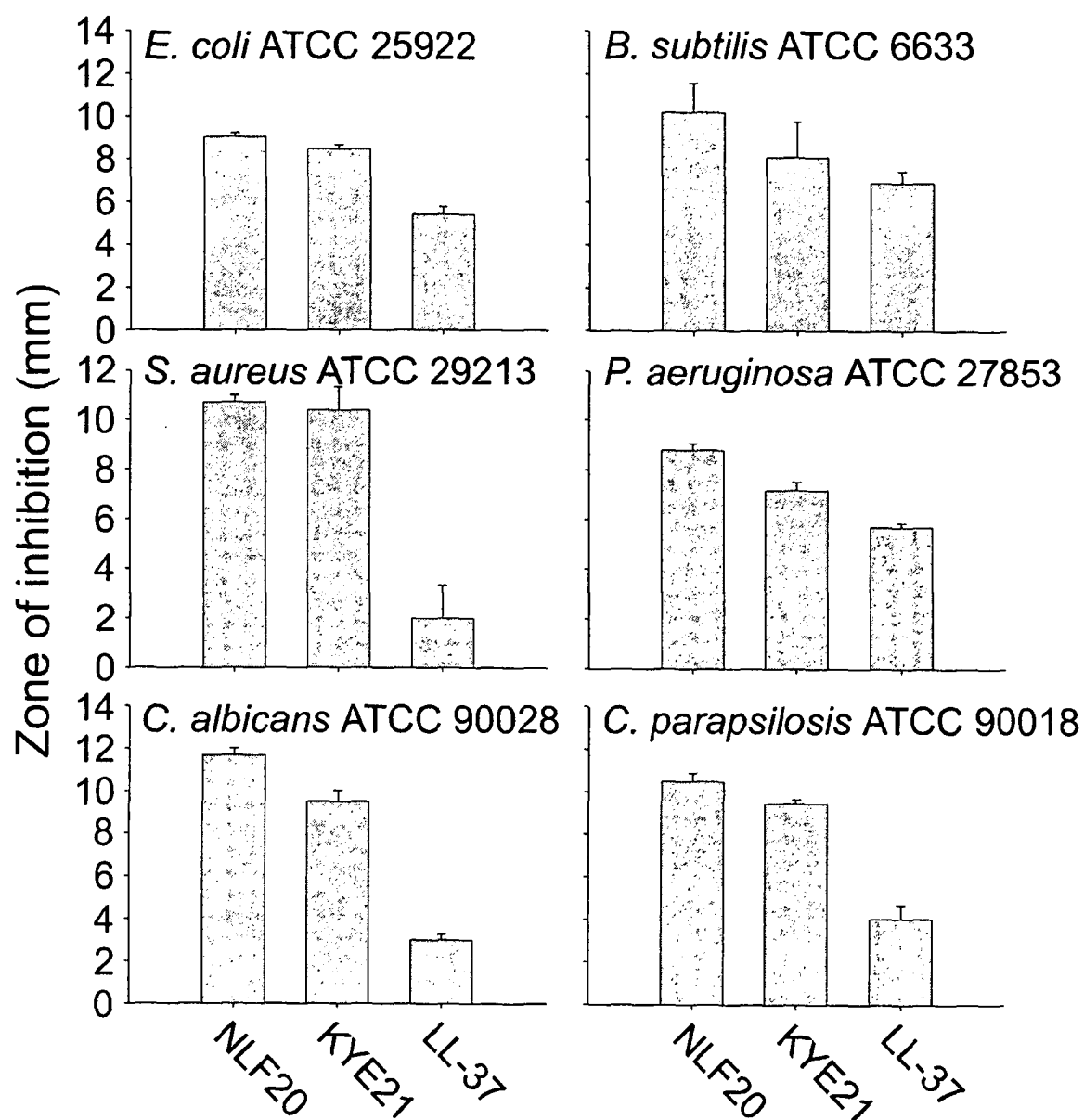


Figure 28

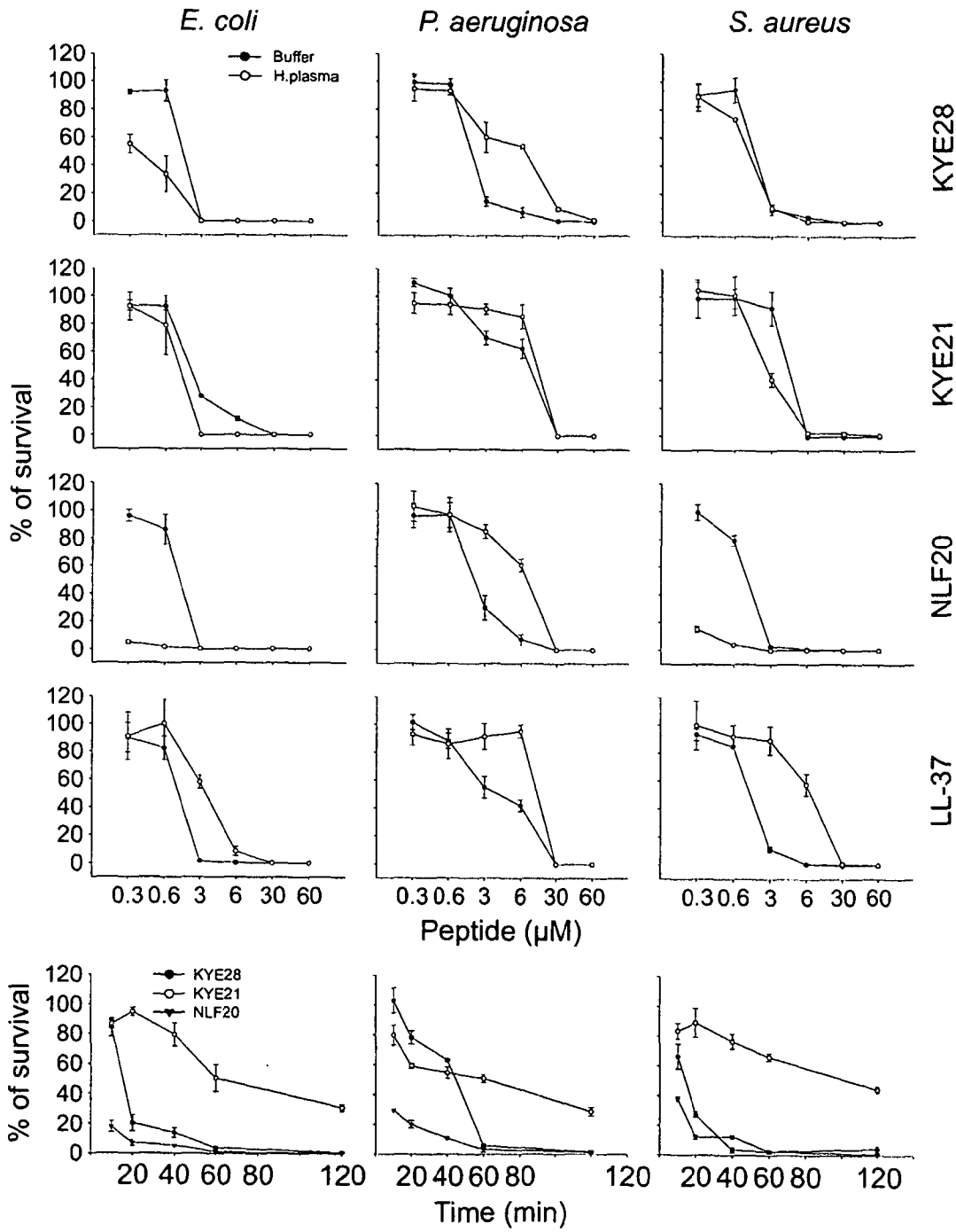


Figure 29

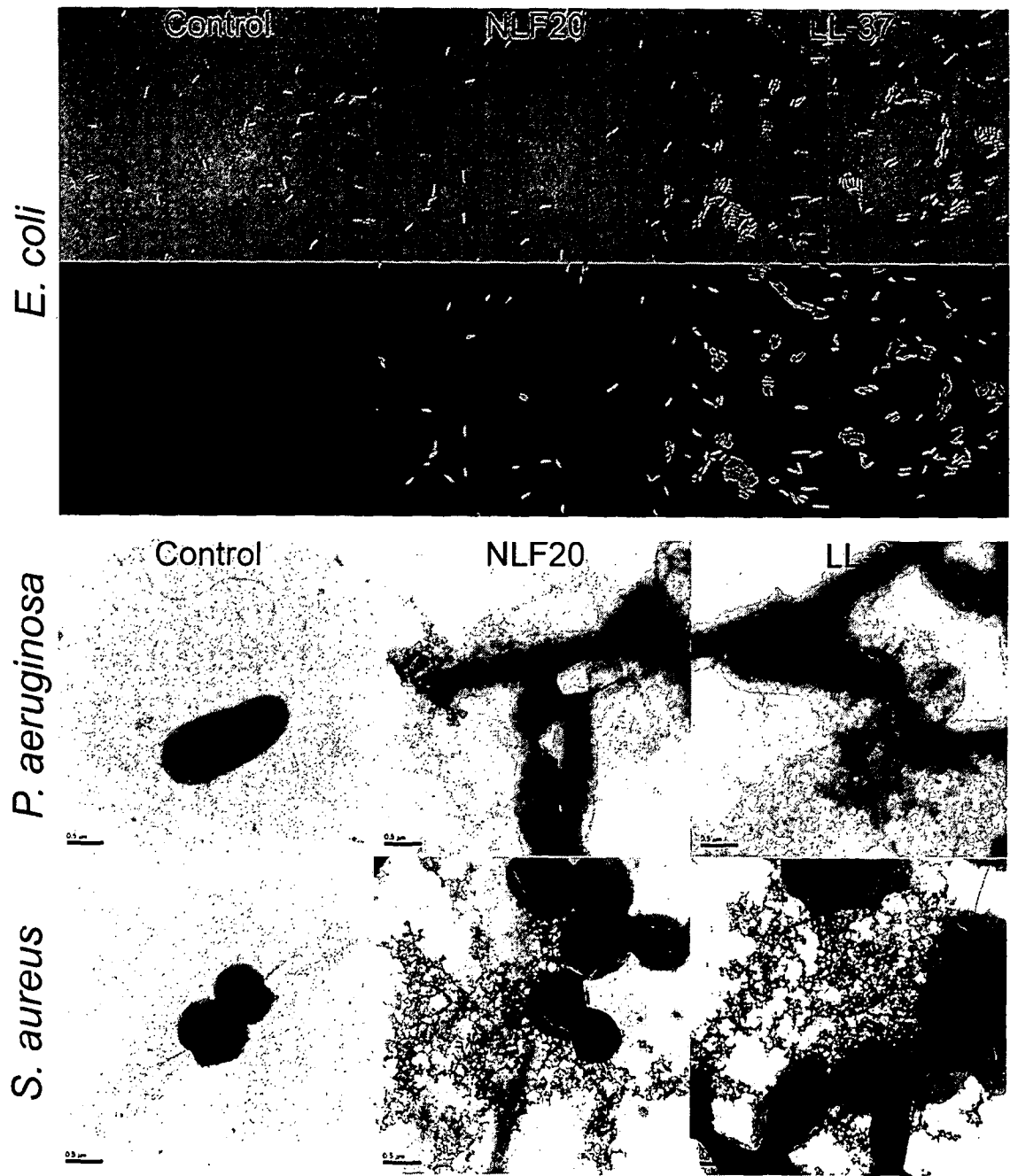


Figure 30

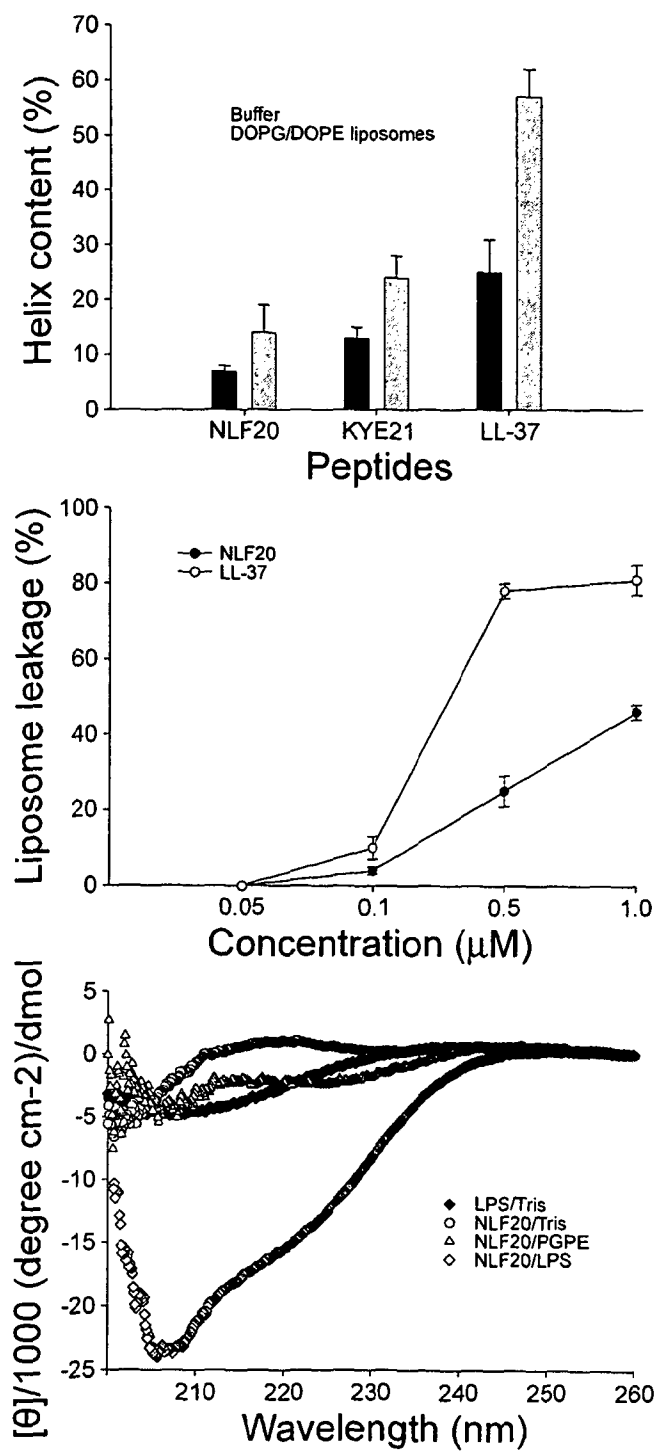


Figure 31

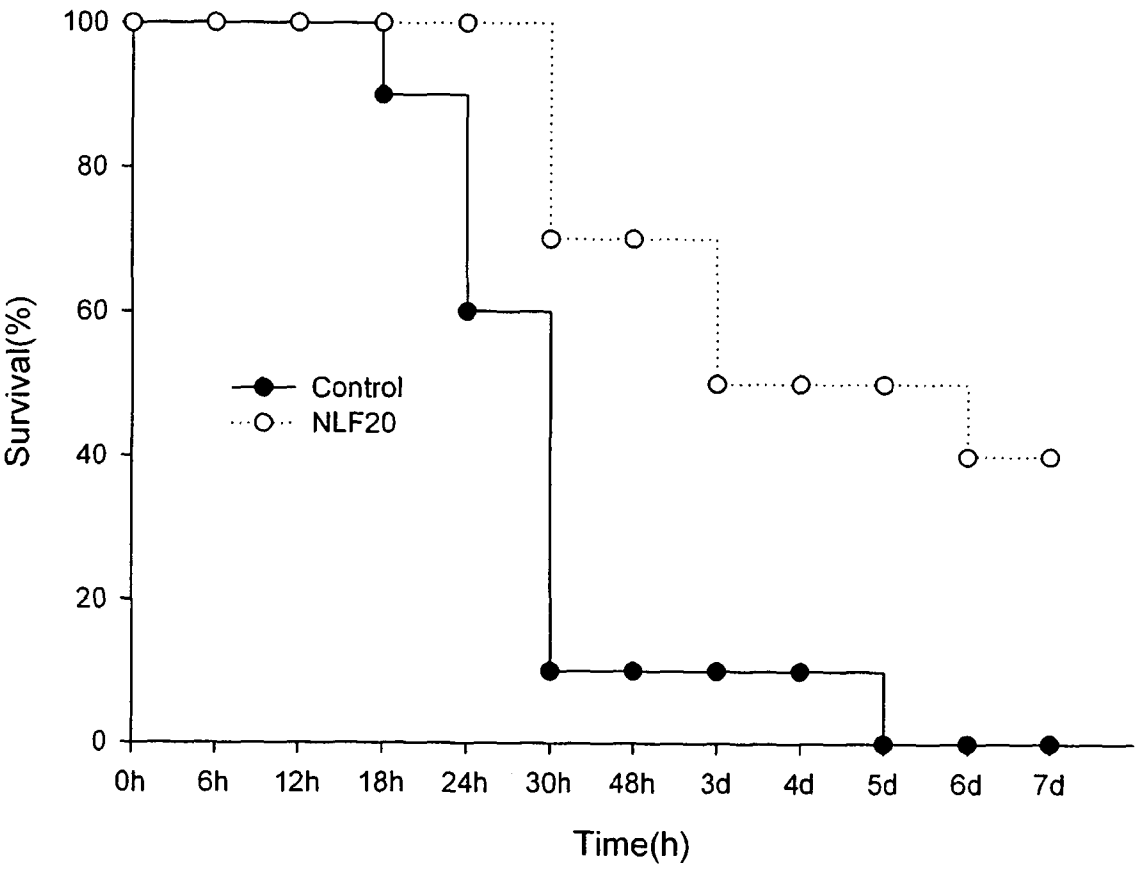


Figure 32

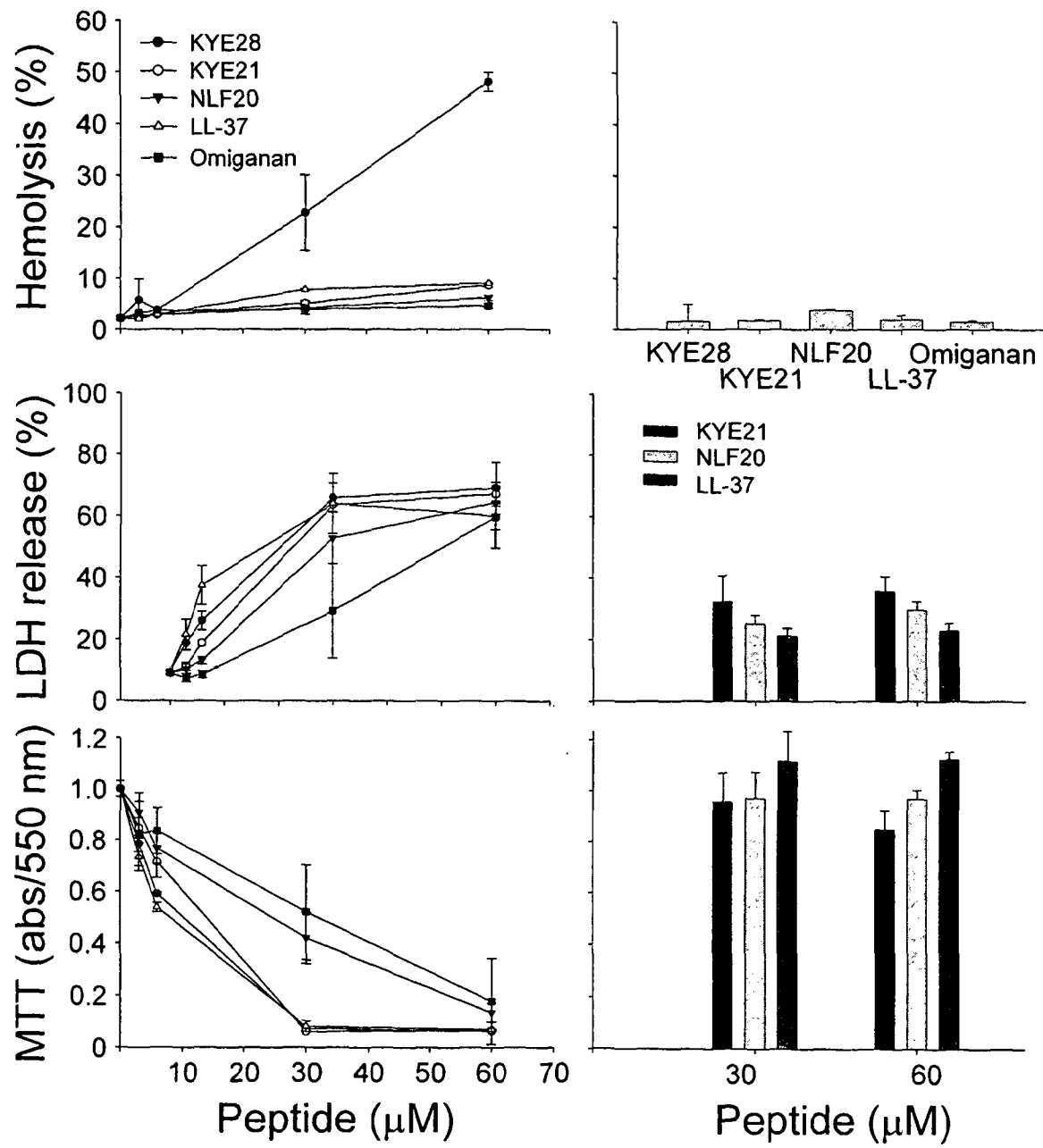


Figure 33

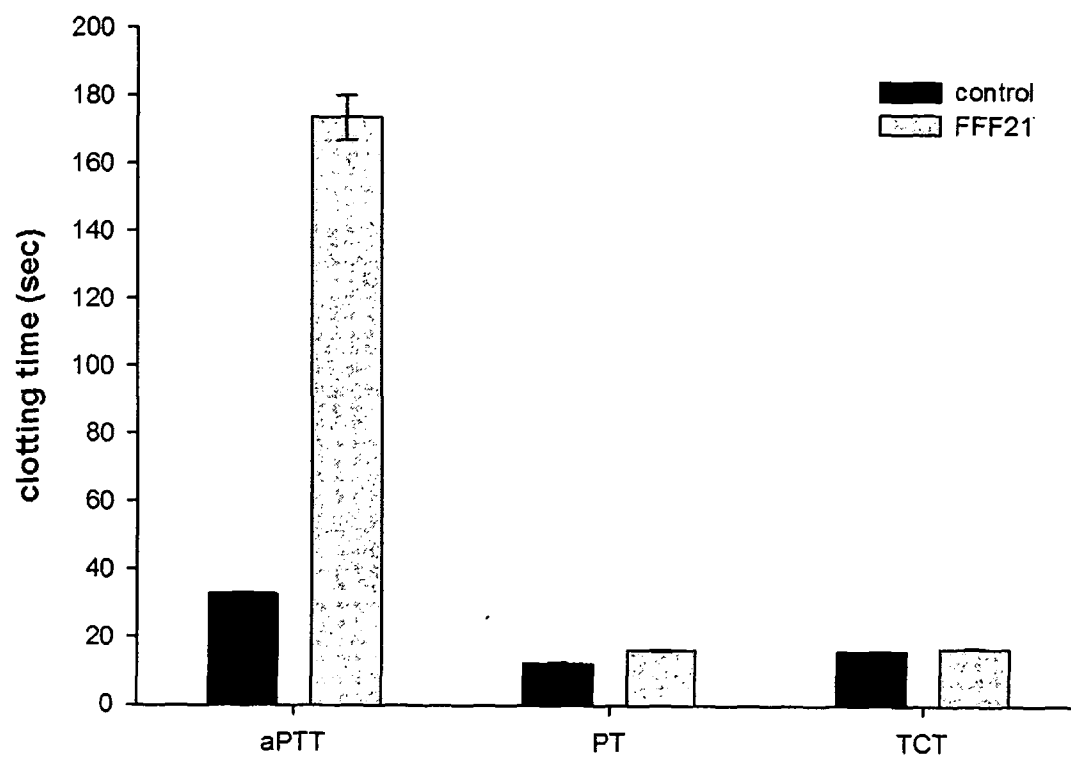


Fig 39

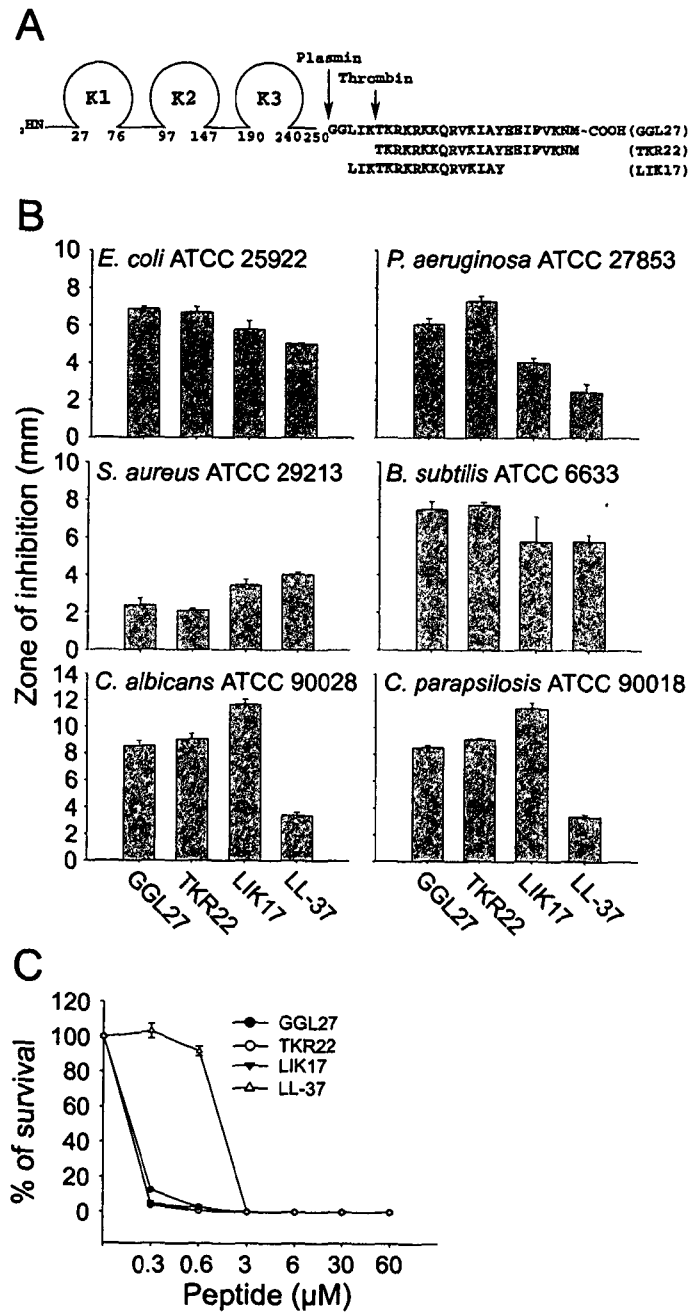


Fig 35

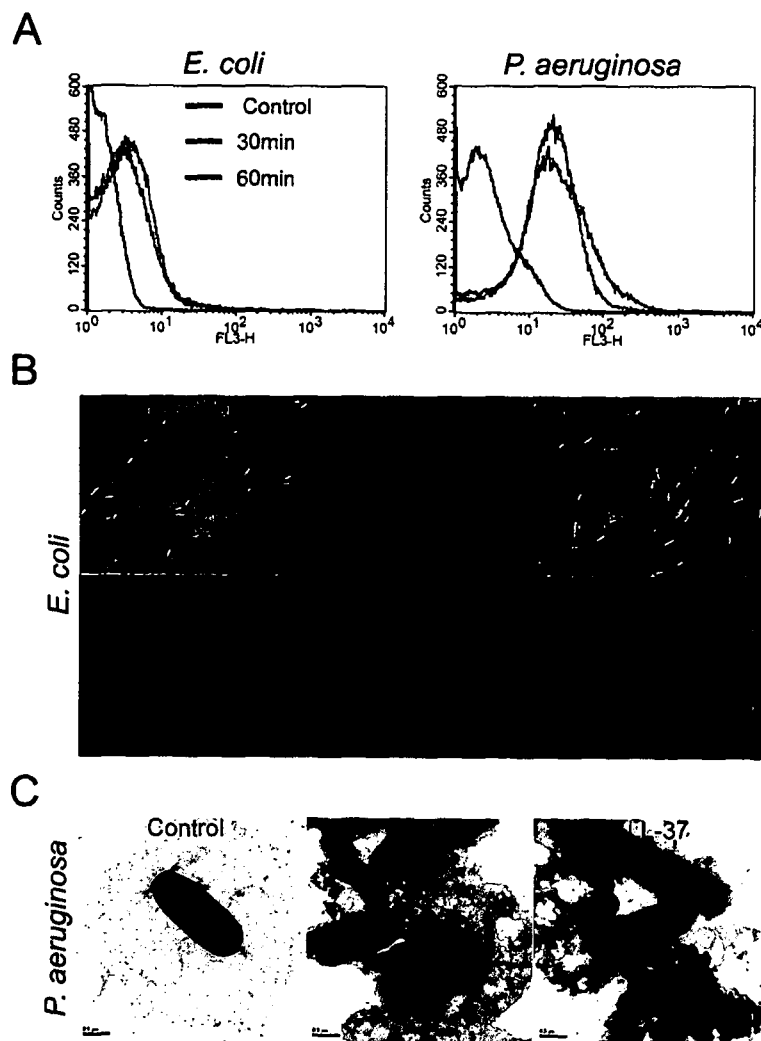


Fig 36

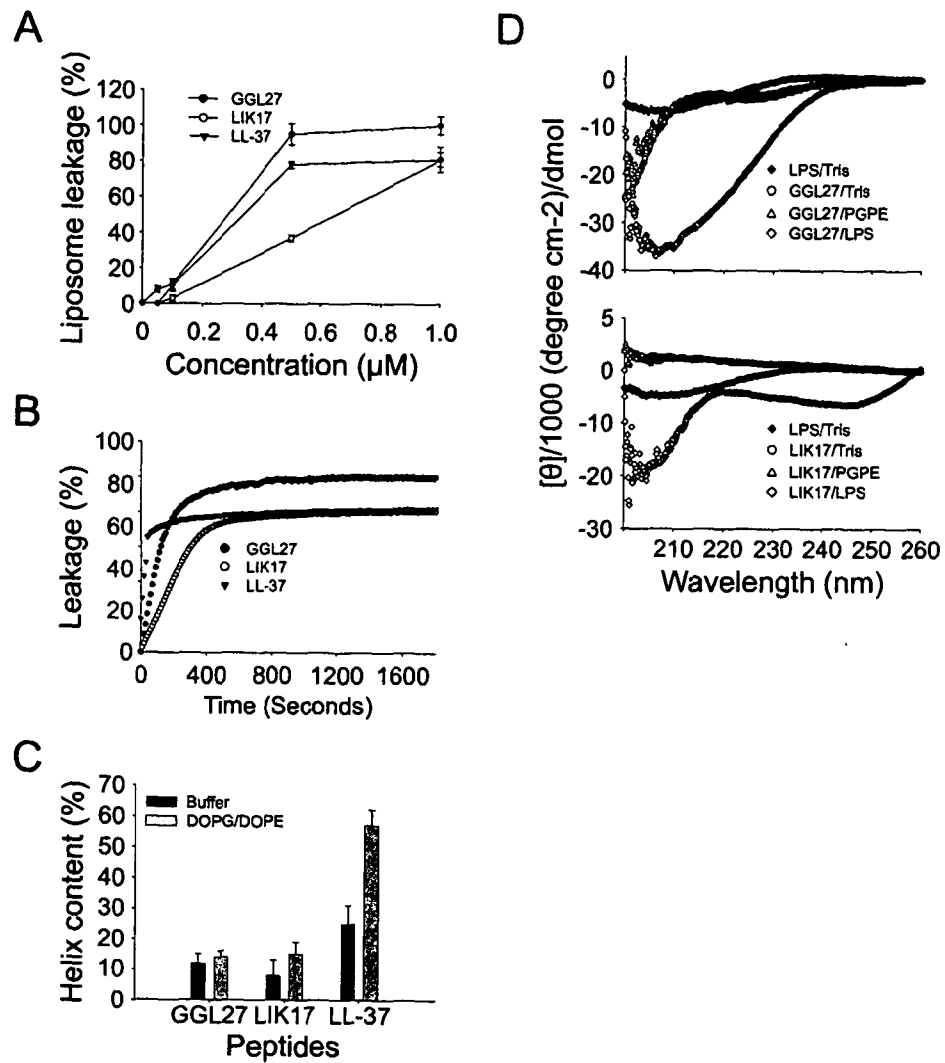


Fig 37

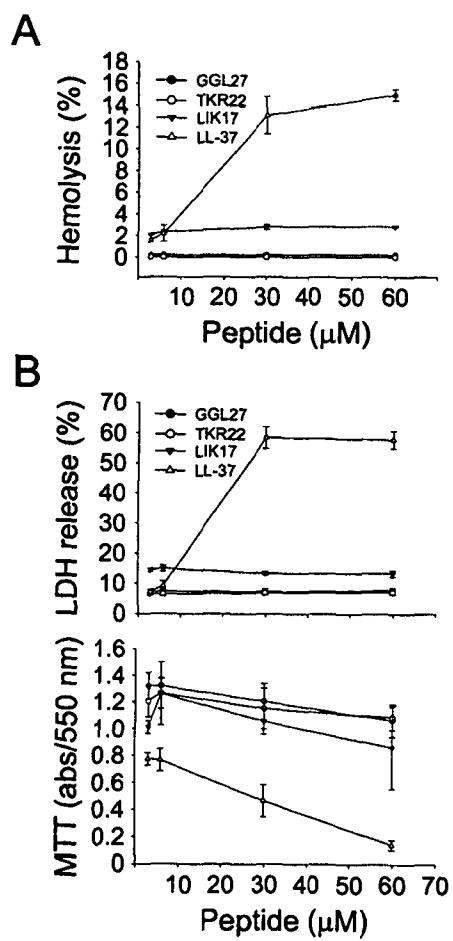


Fig 38

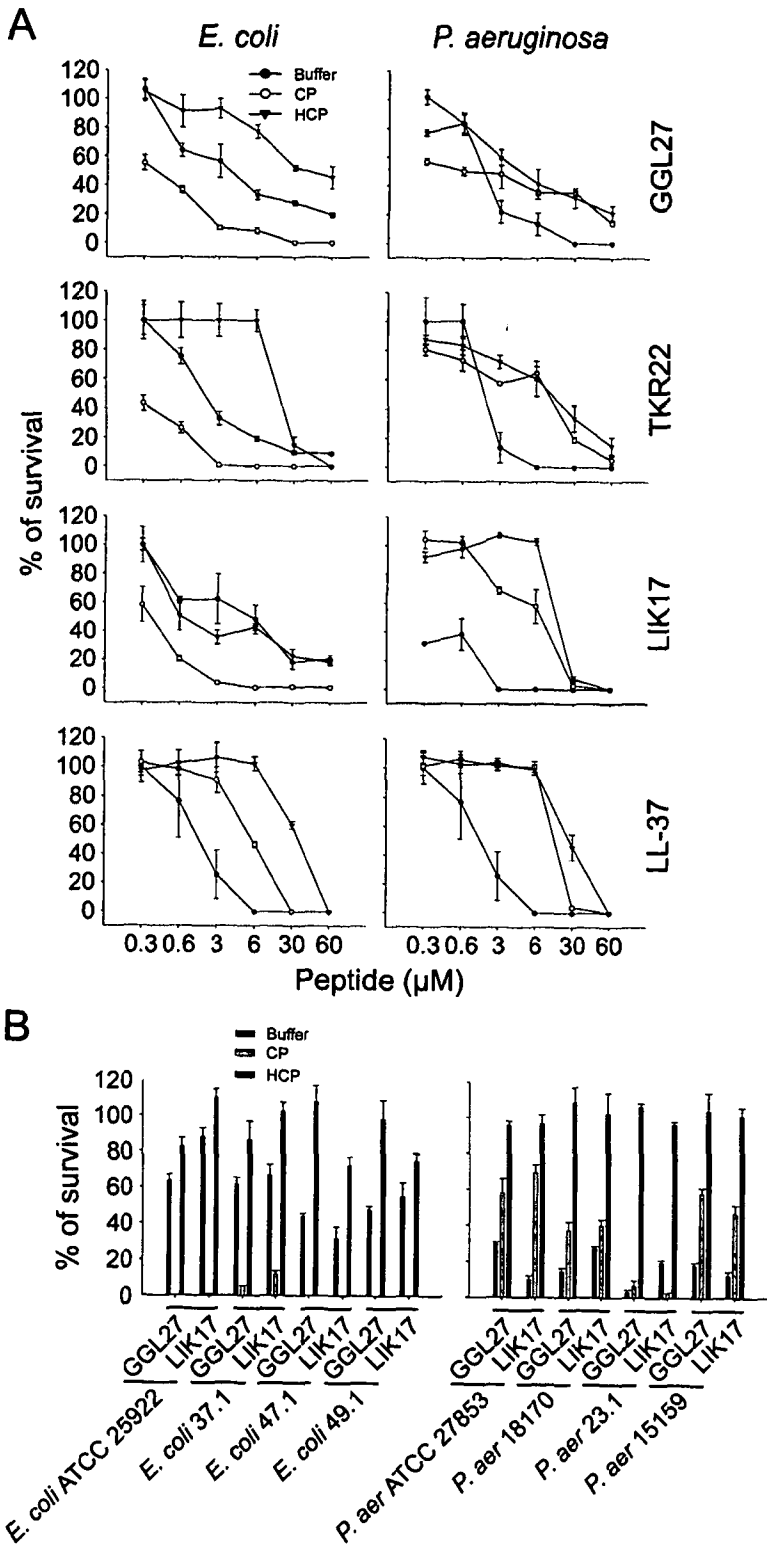


Fig 39

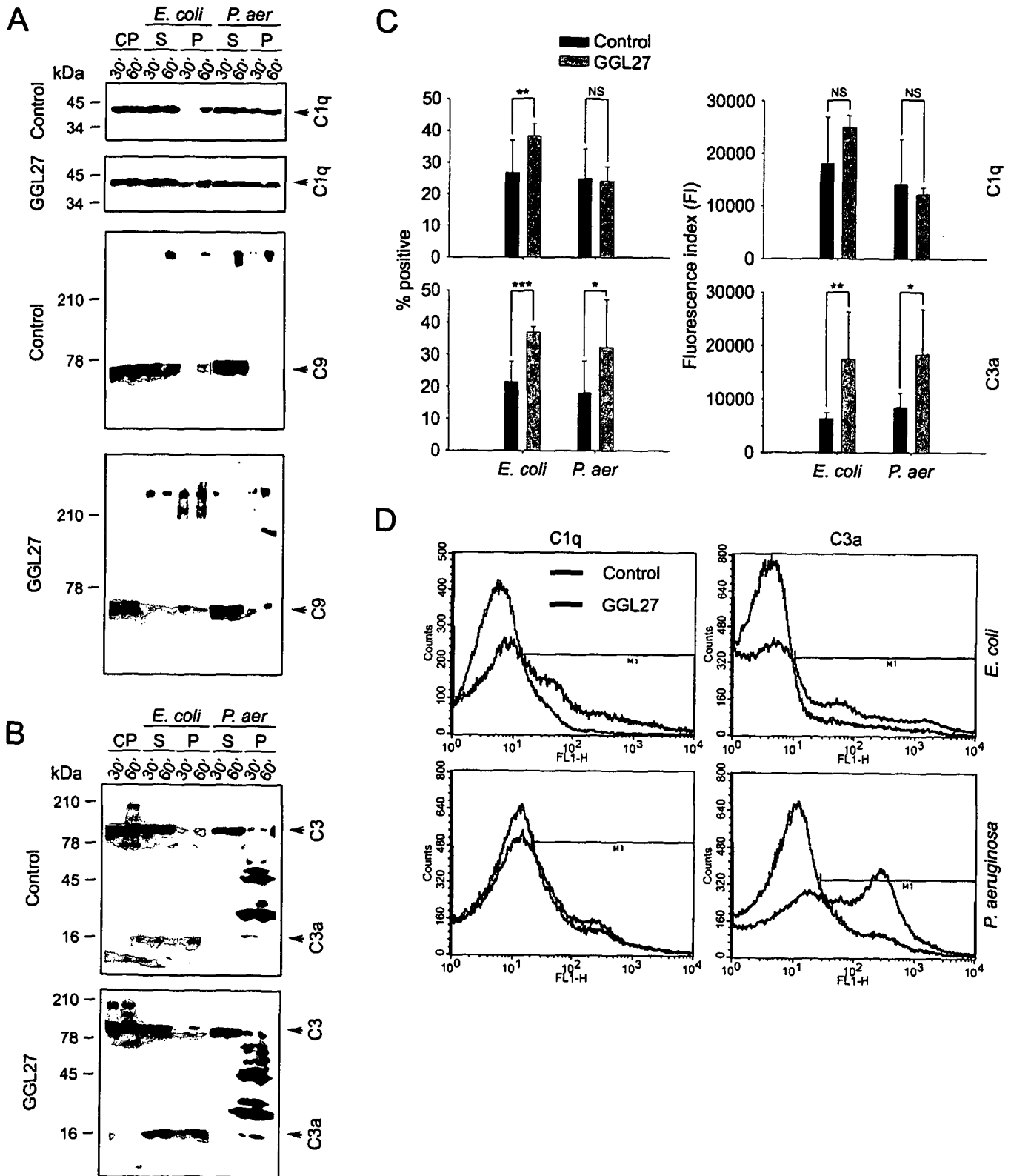


Fig 40

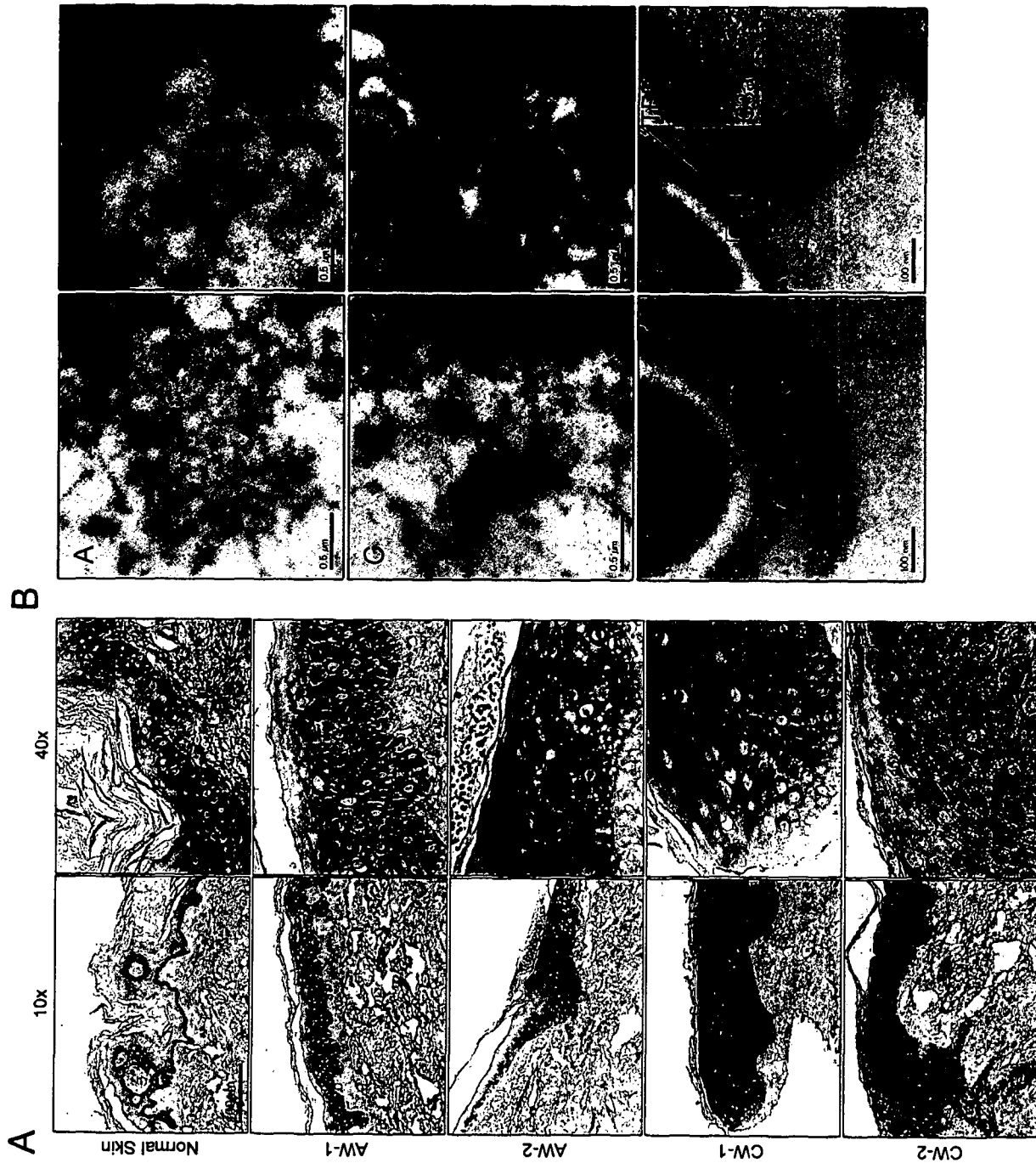


Fig 41

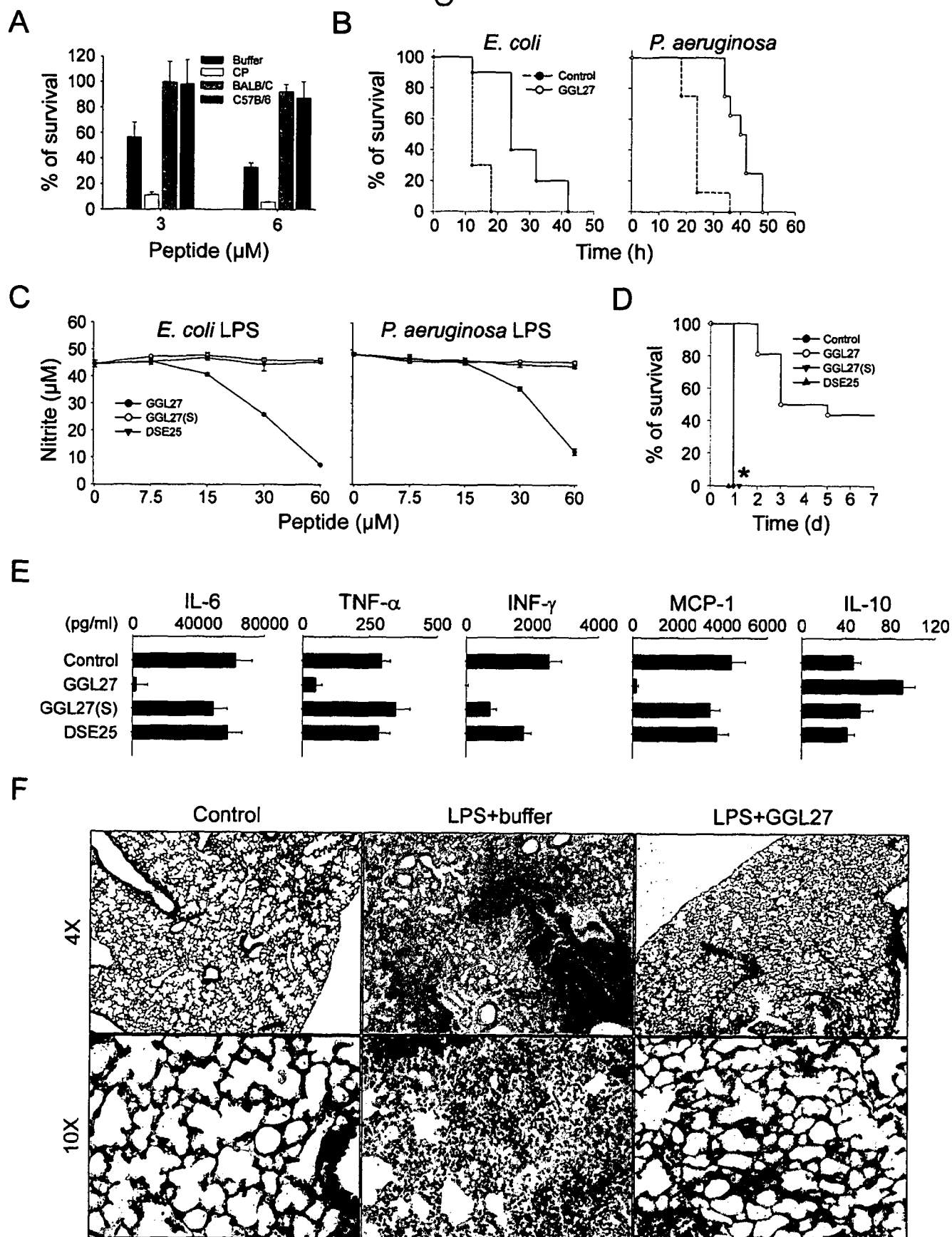


Fig 42

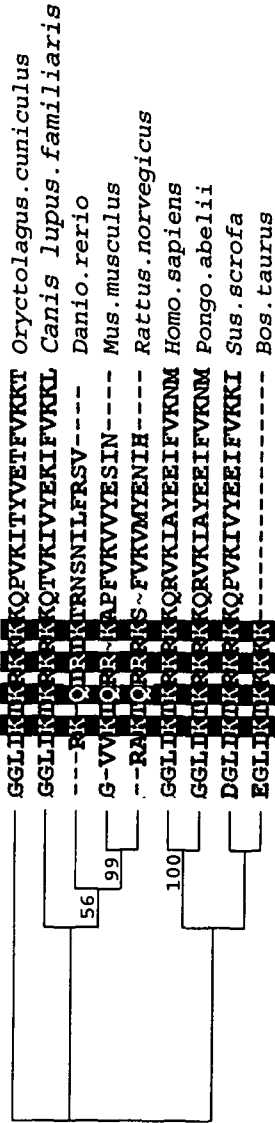


Fig 43

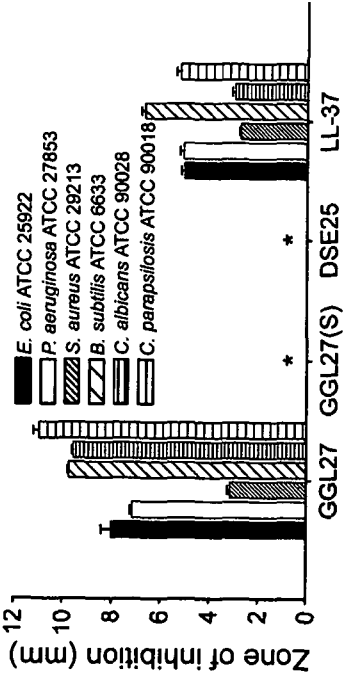


Fig 44



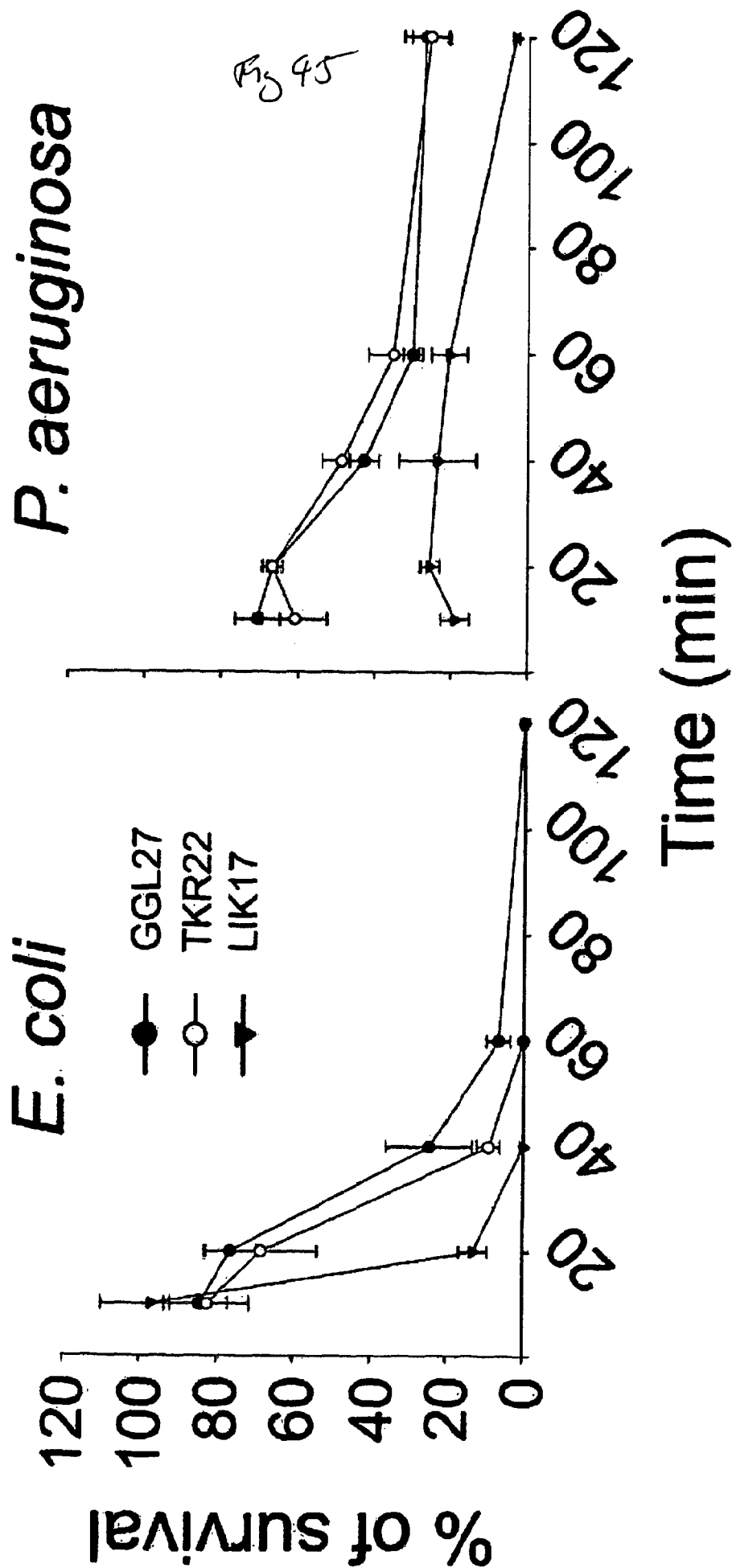


Figure 46

+ NaCl

+ NaCl

pH 7.4

pH 5.5

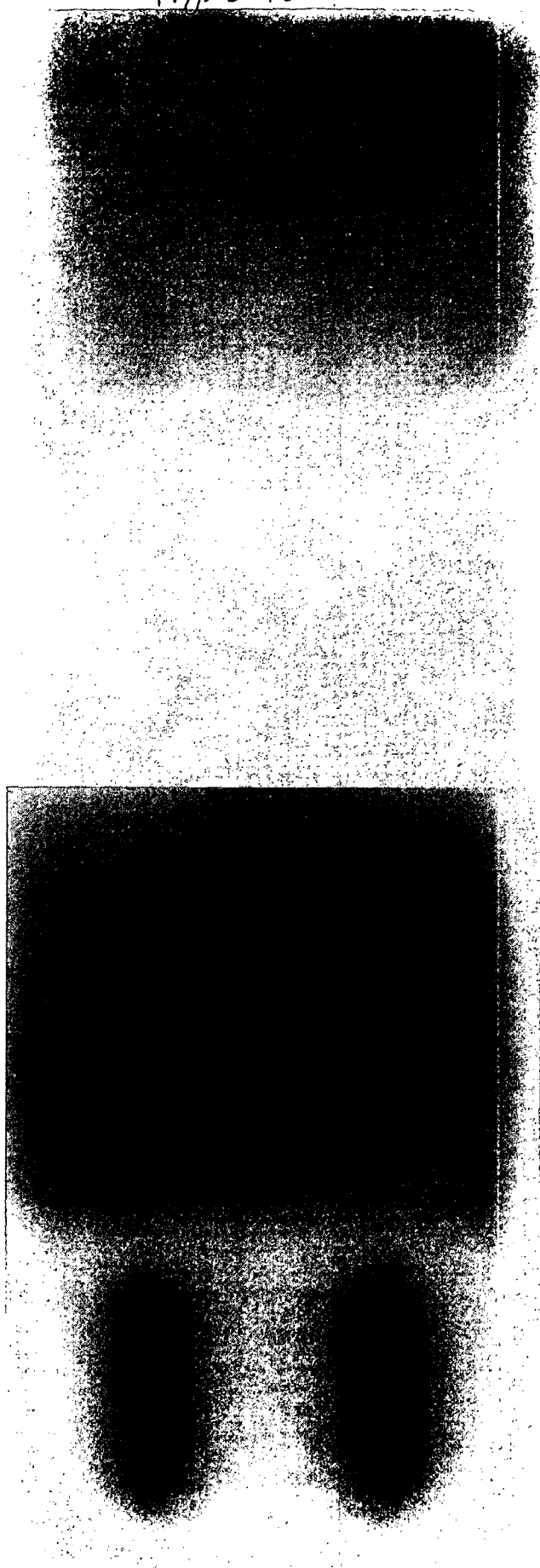


Fig 47

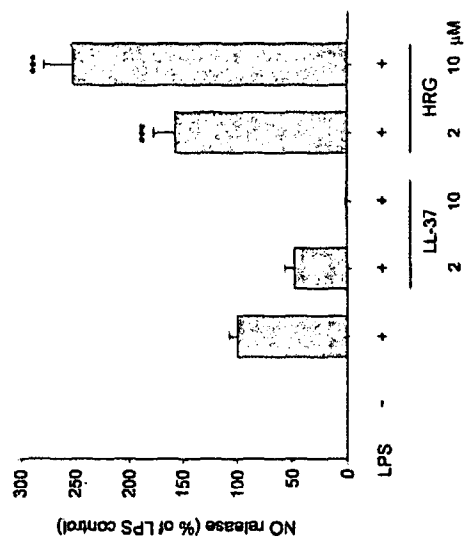


Fig 48

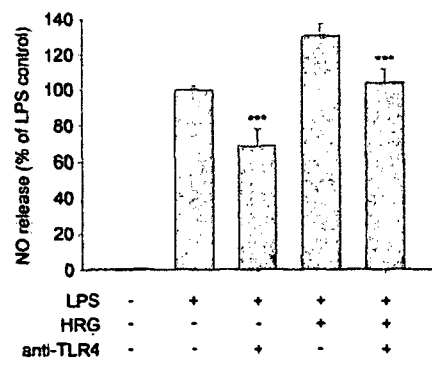


Fig 49

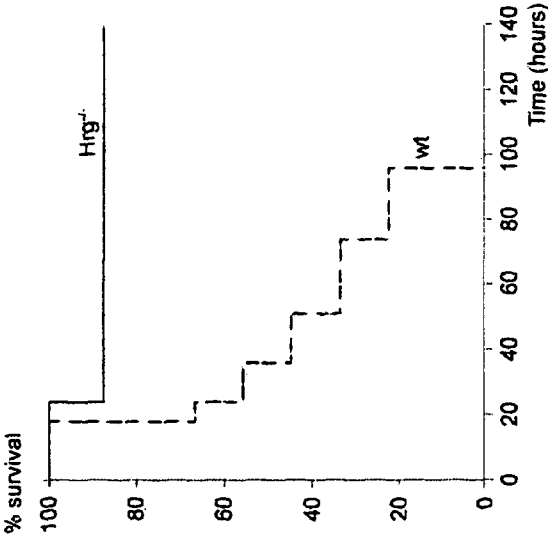


Fig 50

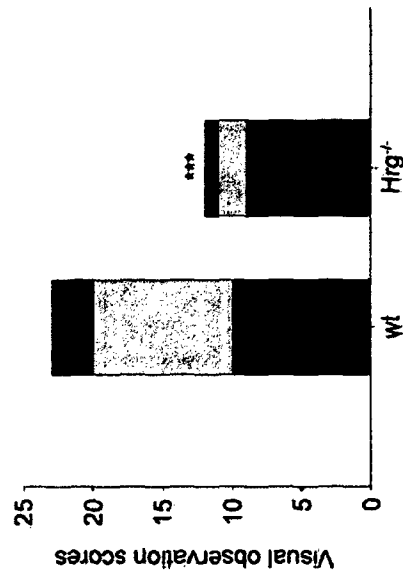


Fig 51

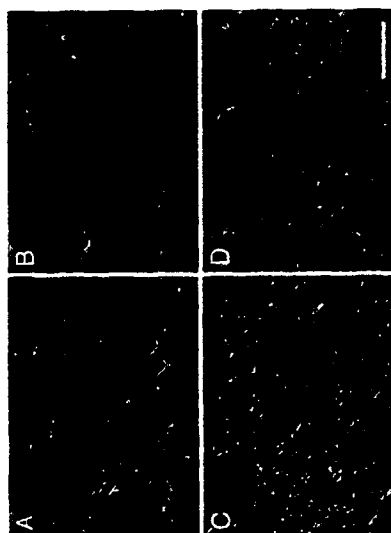


Fig 52

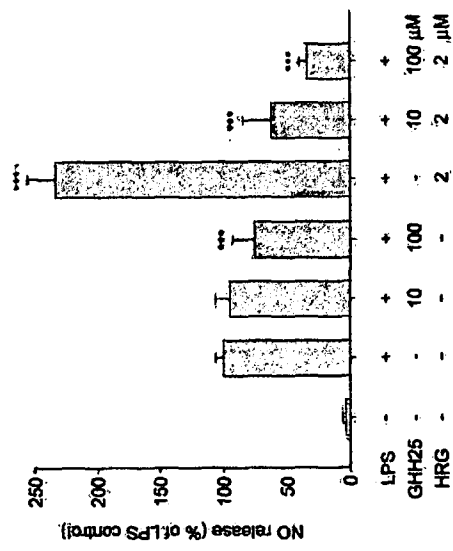


Fig 53

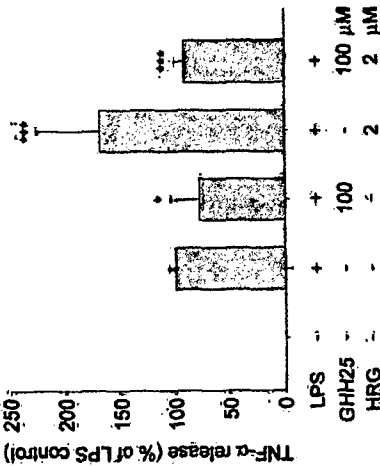


Fig 54

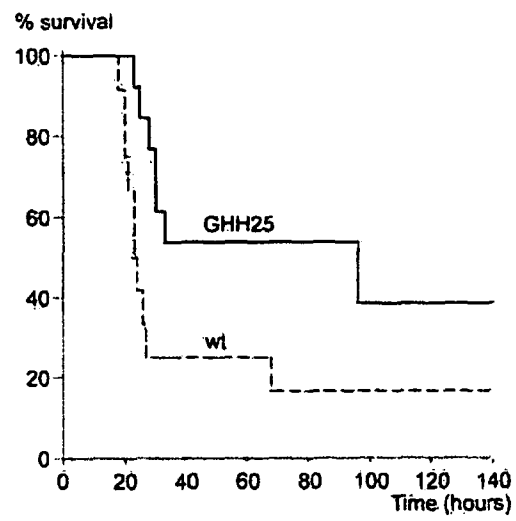


Fig 55

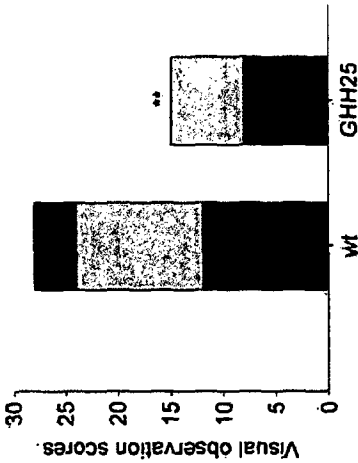


Fig 86

