

In vitro binding assay and co-immunoprecipitation experiment

We prepared purified S-tagged recombinant LTA and T7-tagged galectin-2 derived from *E. coli* using the pET system (Novagen), and combined them. The co-immunoprecipitation experiments were performed using a monoclonal antibody against LTA (R&D Systems) coupled to HiTrap™ NHS-activated Sepharose HP (Amersham). We visualized the immune complex using T7 tag antibody (Stratagene) and horseradish peroxidase (HRP) conjugated with anti-mouse IgG antibody. For co-immunoprecipitation in mammalian cells, we transfected expression plasmids of Flag or S-tagged LTA, galectin-2 and LacZ (as a negative control) into COS7 cells (HSRRB; JCRB9127) or HeLa cells using Fugene. Immunoprecipitations were done in lysis buffer (20 mM Tris pH 7.5, with 150 mM NaCl, 0.1 % Nonident P-40). Twenty-four hours after transfection, cells were lysed, and immunoprecipitations were performed using anti-Flag tag M2 agarose (Sigma). We visualized the immune complex using HRP-conjugated S-protein (Novagen), anti-Flag M2 peroxidase conjugate (Sigma) or mouse monoclonal antibody against human α -tubulin (Molecular Probes) and HRP-conjugated anti-mouse IgG antibody.

Confocal microscopy

Polyclonal anti-human galectin-2 antisera were raised in rabbits using recombinant protein synthesized in *E. coli*. The antisera showed no cross-reactivity to structurally related molecules galectin-1 and galectin-3, analysed by western blot. Polyclonal anti-galectin-2 antisera and either goat anti-human LTA IgG (R&D Systems) or mouse anti-human α -tubulin monoclonal IgM antibodies were used with Alexa secondary antibodies (Molecular Probes). U937 cells (HSRRB; JCRB9021) were stimulated for 30 min with phorbol myristate acetate (PMA) (20 ng ml^{-1}) and fixed. They were subsequently incubated with the corresponding primary antibodies in phosphate-buffered saline containing 3% bovine serum albumin, and the corresponding Alexa secondary antibodies.

siRNA and over-expression experiments

The target sequences for galectin-2 (5'-AATCCACCATTGCTGCAACT-3') were cloned into pSilencer 2.0-U6 siRNA vector (Ambion). For the over-expression experiment, the galectin-2 was cloned into pFlag-CMV5a vector. After transfection, Jurkat cells were stimulated with PMA (20 ng ml^{-1}) for 24 h, and cells and supernatants were collected separately. LTA concentration was measured using an LTA-specific ELISA system (R&D Systems), and normalized by comparison with total protein concentration. The mRNA quantification procedure has been described previously².

Luciferase assay

A DNA fragment, corresponding to nucleotides 3,188 to 3,404 of intron-1 of *LGALS2*, was cloned into pGL3-enhancer vector (Promega) in the downstream of SV40 enhancer in the 5' to 3' orientation. After 24 h transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Immunohistochemistry

Tissue samples were obtained from 16 patients with MI by elective directional coronary atherectomy. Immunohistochemical protocols were carried out as described previously^{11,12} using goat anti-human LTA IgG (R&D Systems) and rabbit polyclonal anti-human galectin-2 antibody. Staining of adjacent sections was carried out using human-cell-type-specific monoclonal antibodies against SMC 2-actin and CD68 (DAKO). For double-labelled immunohistochemistry, sections were incubated with anti-LTA antibody, then with biotinylated swine anti-goat IgG, and then with avidin-biotin-peroxidase conjugate, followed by visualization with 3,3'-diaminobenzidine tetrahydrochloride (Vector Labs). The section was subsequently incubated with rabbit polyclonal anti-human galectin-2 antibody, followed by incubation with alkaline phosphatase-conjugated swine anti-rabbit IgG and visualized with the 5-bromo-4-chloro-3-indoxyl phosphate and nitro-blue tetrazolium chloride (BCIP/NBT) substrate system.

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Differential modulation of endotoxin responsiveness by human caspase-12 polymorphisms

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Caspases mediate essential key proteolytic events in inflammatory cascades and the apoptotic cell death pathway. Human caspases functionally segregate into two distinct subfamilies: those involved in cytokine maturation (caspase-1, -4 and -5) and those involved in cellular apoptosis (caspase-2, -3, -6, -7, -8, -9 and -10)^{1,2}. Although caspase-12 is phylogenetically related to the cytokine maturation caspases, in mice it has been proposed as a mediator of apoptosis induced by endoplasmic reticulum stress including amyloid- β cytotoxicity, suggesting that it might contribute to the pathogenesis of Alzheimer's disease³. Here we show that a single nucleotide polymorphism in caspase-12 in humans results in the synthesis of either a truncated protein (Csp12-S) or a full-length caspase proenzyme (Csp12-L). The read-through single nucleotide polymorphism encoding Csp12-L is confined to populations of African descent and confers hypo-responsiveness to lipopolysaccharide-stimulated cytokine production in *ex vivo* whole blood, but has no significant effect on apoptotic sensitivity. In a preliminary study, we find that the frequency of the Csp12-L

allele is increased in African American individuals with severe sepsis. Thus, Csp12-L attenuates the inflammatory and innate immune response to endotoxins and in doing so may constitute a risk factor for developing sepsis.

While cloning human caspase-12 complementary DNAs and sequencing their corresponding genomic DNA, we found that almost all clones contained a TGA (stop) codon at amino acid position 125, as previously described⁴, but a few DNA sources contained a read-through CGA (Arg) codon instead (Fig. 1). This single nucleotide polymorphism (SNP) was contained in exon 4 of the gene encoding human caspase-12 (which was found to be proximal to the caspase-1,-4,-5 gene cluster on 11q23) and resulted in messenger RNAs encoding either a full-length, tripartite caspase

precursor protein (Csp12-L) or a truncated polypeptide (Csp12-S) ending at the junction between the prodomain and the large subunit (Fig. 1a, b). Sequence analysis of more than 1,100 genomic DNA samples from people of distinct ethnic backgrounds showed that most encoded the truncated prodomain-only form of caspase-12 (Csp12-S). The less-frequent CGA (Arg) polymorphism resulting in a full-length caspase polypeptide (Csp12-L) was found only in populations of African descent and was absent in all Caucasian and Asian groups tested (Fig. 1c, d). Although less frequent in humans and confined to about 20% of people of African descent (Fig. 1d and Supplementary Table 1), the full-length form of caspase-12 was found to be encoded in all other species tested, including new world and old world primates and rodents (Supplementary Table 2).

Caspase-12 is naturally polymorphic in ethnic groups of African descent, providing an ideal system in which to examine its *in vivo* role in humans and to circumvent the pitfalls associated with studying caspases in recombinant systems. We chose to examine the apoptotic and inflammatory responsiveness of cells in whole blood obtained from consenting donors of African origin. Owing to the genomic and structural association of caspase-12 with the pro-inflammatory caspase-1,-4,-5 gene cluster, we first examined the effect of the caspase-12 polymorphism on lipopolysaccharide (LPS) and concanavalin A (conA)-stimulated cytokine production in whole blood (Fig. 2). *In vivo* expression of Csp12-L in blood cells was confirmed by western blotting, and the protein was induced by LPS treatment (Fig. 2a). For most cytokines examined, the presence of Csp12-L reduced the magnitude of the LPS-induced response: maximum attenuation occurred in people who were homozygous for the T125C allele (Csp12-L/L) and an intermediate response occurred in heterozygotes (Csp12-S/L) as compared with T125 (Csp12-S/S) homozygotes (Fig. 2b, c).

Cytokine production stimulated by conA was unaffected by the two variants of caspase-12 (data not shown), with the exception of interferon- γ , which was substantially increased in response to conA by the presence of one Csp12-L allele and further increased in Csp12-L/L homozygotes as compared with Csp12-S/S controls (Fig. 2d). These results support a role for caspase-12 as a master attenuator of the macrophage-elicited T-helper cell type 1 and type 2 cytokine response, with probable compensatory enabling of T-cell-derived interferon- γ formation. By contrast, the naturally occurring variants of human caspase-12 had no significant effect on apoptotic sensitivity to diverse stimuli, including activators of the extrinsic and intrinsic cell death pathways, as well as agents that provoke apoptosis through endoplasmic reticulum (ER) stress (Fig. 3). These latter findings are contrary to those observed in rodents, where caspase-12 has been proposed to be a key mediator of ER-stress-induced cell death and has been implicated in neurodegenerative disorders including Alzheimer's disease^{3,5}, polyglutamine repeat disorders⁶ and ischaemic brain injury^{7,8}.

These findings suggest that human caspase-12 has a role in modulating endotoxin responsiveness and cytokine release. Because other caspases of this subfamily promote cytokine formation through precursor maturation (for example, caspase-1-mediated cleavage of interleukin-1 β (IL-1 β) and IL-18), an attenuating role of caspase-12 seems counterintuitive. It suggests that full-length caspase-12 (Csp12-L) might act as a dominant-negative regulator of inflammatory caspase activation, potentially by antagonizing the inflammasome complex and associated pro-inflammatory pathways, such as NF- κ B (refs 9–13). In support of this, we found that human caspase-12 was devoid of detectable catalytic activity, in contrast to rodent caspase-12 proteins, which underwent auto-catalytic maturation (data not shown). Furthermore, transfected cell lines expressing Csp12-L showed dampened NF- κ B activation in response to tumour-necrosis factor- α (TNF- α) and reduced

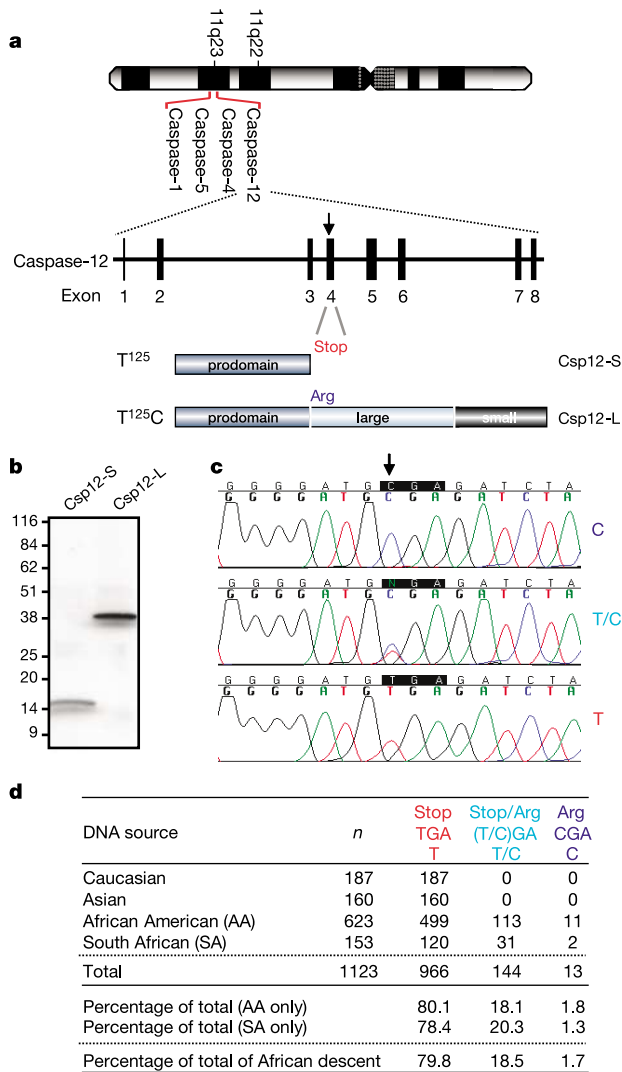


Figure 1 Identification of a caspase-12 point mutation in individuals of African descent. **a**, Map of the region at 11q23. The gene encoding caspase-12 is clustered with those encoding caspase-1, -4 and -5. The exon-intron organization of caspase-12 is shown. Arrow indicates the polymorphism TGA \rightarrow CGA. In wild-type (WT) individuals, a stop codon in exon 4 encodes a prodomain-only protein (Csp12-S). In T125C individuals, an arginine replaces the stop and encodes a full-length protein (Csp12-L). **b**, Western blot. Caspase-12 variants were *in vitro* transcribed and translated, and detected by antibodies specific for the human caspase-12 prodomain. **c**, Electropherograms from control, heterozygous and T125C homozygous individuals. Arrow indicates the position of the polymorphism. **d**, Genotype frequency of T125 and T125C in different ethnic backgrounds.

IL-1-stimulated release of IL-8, an NF- κ B-dependent process (Fig. 4). Having only the CARD domain, Csp12-S was a weaker inhibitor of NF- κ B activation. Collectively, these data indicate that human caspase-12 can function as a dominant-negative regulator of inflammatory responses and innate immunity.

Because human caspase-12 modulated endotoxin responsiveness in *ex vivo* human whole blood but had no effect on apoptotic sensitivity, we carried out studies to examine whether there is an association between the caspase-12 polymorphism and either sepsis or Alzheimer's disease in African Americans. We chose sepsis

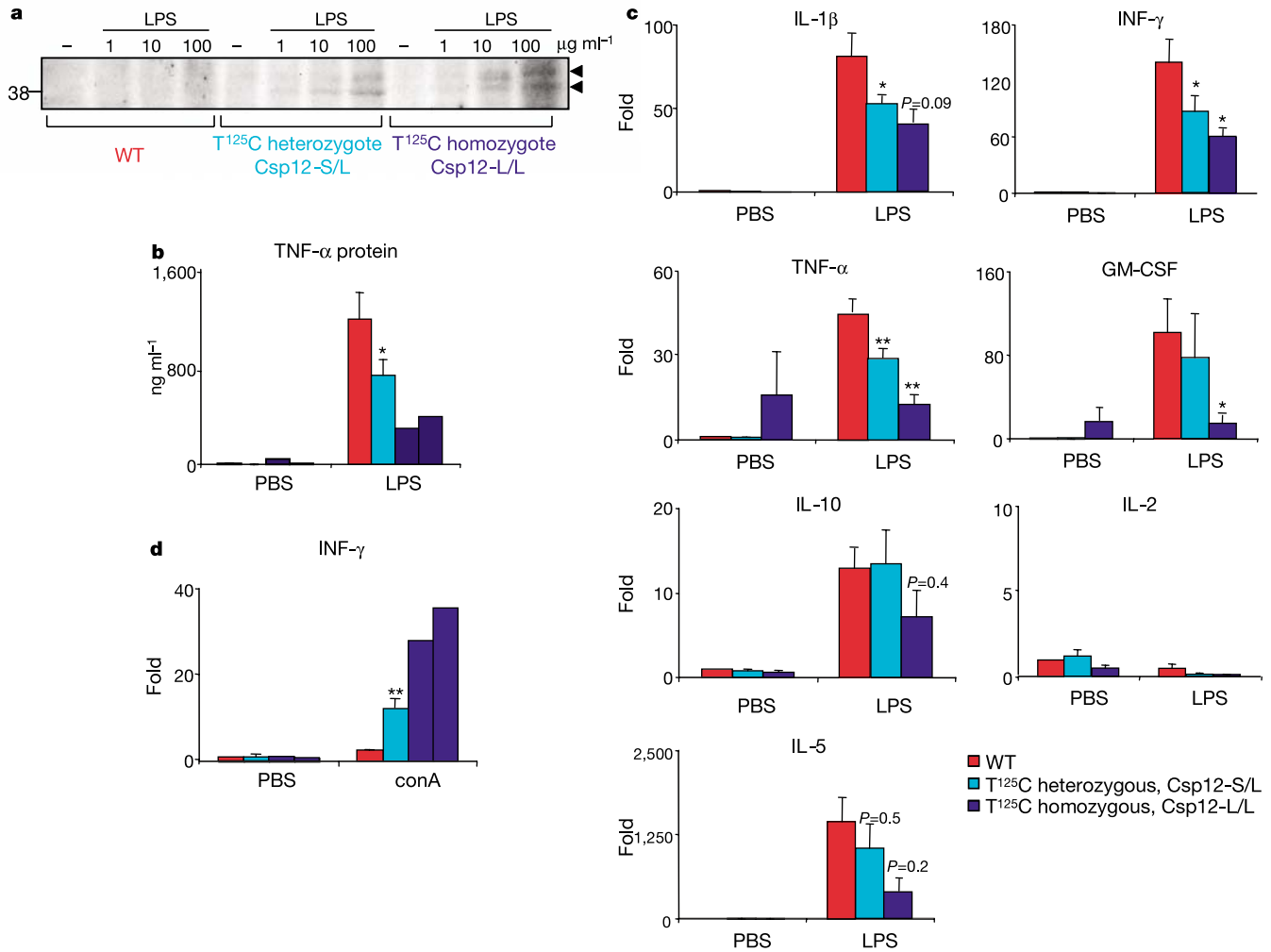


Figure 2 Differential responsiveness to LPS and conA of *ex vivo* whole blood from wild-type, T125C heterozygous and T125C homozygous individuals of African descent. **a**, Csp12-L is expressed in the blood of T125C but not wild-type (WT) individuals. **b–d**, Differential responsiveness to LPS and conA. Whole blood was treated with 1 $\mu\text{g ml}^{-1}$ LPS (**b, c**) or 50 $\mu\text{g ml}^{-1}$ conA (**d**) for 4 h at 37 °C; serum was then collected for TNF- α ELISA (**b**) or total RNA extracted from white blood cells was used for real-time

PCR quantification of cytokines (**c, d**). GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN- γ , interferon- γ . Red, light blue and dark blue represent data from the blood of wild-type, T125C heterozygous and T125C homozygous individuals, respectively. Values represent the mean \pm s.e.m. (* $P < 0.05$; ** $P < 0.01$). Wild type: $n = 8$ (**b**), $n = 20$ (**c**), $n = 16$ (**d**); T125C heterozygous: $n = 8$ (**b**), $n = 20$ (**c**), $n = 16$ (**d**); T125C homozygous: $n = 2$ (**b**), $n = 4$ (**c**), $n = 2$ (**d**).

Table 1 Caspase-12 genotype and allele frequency in Alzheimer's or severe sepsis

African American DNA source	Stop TGA (n) T/T	Stop/Arg (T/C)GA (n) T/C	Arg CGA (n) C/C	Total (n)	Genotype frequency (%)			Allele frequency (%)	
					T/T	T/C	C/C	T	C
Reference group	499	113	11	623	80.1	18.1	1.8	89.2	10.8
Alzheimer's disease group	141	40	3	184	76.7	21.7	1.6	87.5	12.5
Sibling controls	80	21	2	103	77.7	20.4	1.9	87.9	12.1
Unrelated controls	56	14	1	71	78.9	19.7	1.4	88.7	11.3
Sepsis group	23	11	4	38	60.5	29	10.5	75	25
Unrelated controls	120	26	2	148	81.1	17.6	1.3	89.9	10.1

The reference group includes all African Americans without disease assessed in this study (from Fig. 1d). Controls for Alzheimer's disease were cognitively normal siblings and unrelated volunteers. Data for the controls for severe sepsis were obtained from intensive-care-unit patients in the same study who were not septic plus other unaffected African Americans from the same region. The Fisher exact test was used for statistical analysis.

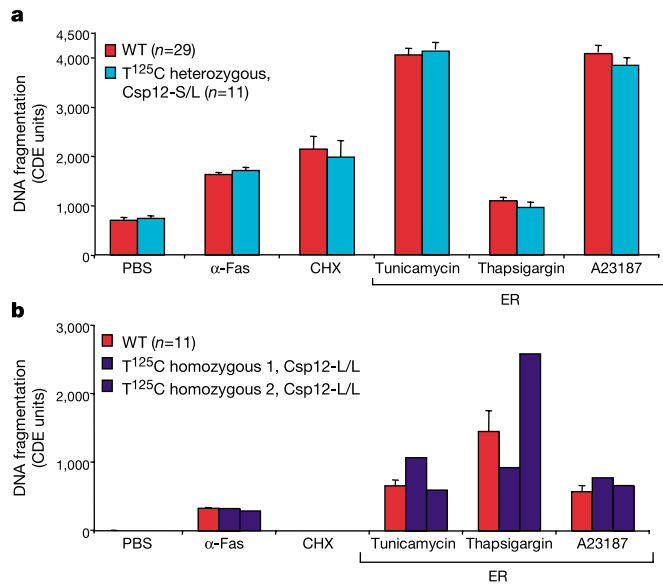


Figure 3 Human caspase-12 is not involved in ER-stress-mediated apoptosis. White blood cells from wild-type (Csp12-S/S), T125C heterozygous (Csp12-S/L) and T125C homozygous (Csp12-L/L) individuals of African descent were treated with the indicated apoptotic stimuli, including three putative ER stressors (ER). After 24 h at 37 °C, cell death by apoptosis was measured by a cell death ELISA. Values represent the mean ± s.e.m. **a**, An experiment in which no homozygous Csp12-L/L individuals were found in the donor group; **b**, an additional experiment in which two homozygous Csp12-L/L individuals (shown separately) were found.

because of the clear link between this disorder and both perturbed cytokine responsiveness and caspases^{14,15}, and Alzheimer's disease because of the reported resistance of cortical neurons derived from caspase-12-deficient mice to amyloid-β cytotoxicity³. In these preliminary studies, the frequencies of the caspase-12 genotypes and alleles in individuals with Alzheimer's disease were indistinguishable from those of non-affected siblings or unrelated African American age-matched controls (Table 1), consistent with our data from whole blood showing that caspase-12 function in humans is not associated with ER stress and is different to that reported in mice. There was, however, a modest but statistically significant increase in the frequency of genotypes encoding Csp12-L in individuals of African descent who were diagnosed with severe clinical sepsis ($P = 0.005$), including a 7.8-fold increase in Csp12-L/L (T125C/T125C) homozygotes. Occurrence of the T125C allele was roughly doubled in individuals of African descent with sepsis, as compared with all other groups (for example, 25% versus 10.1% for control subjects in the same study; $P = 0.002$). Among individuals of African descent with severe sepsis, the mortality rate was 54% in individuals with a T125C allele as compared with 17% in individuals with only T125 (data not shown). Collectively, these results indicate that the presence of the T125C allele (encoding Csp12-L) may increase susceptibility to severe sepsis and also may result in higher mortality rates (up to threefold) once severe sepsis develops.

In summary, caspase-12 seems to modulate inflammation and innate immunity in humans. More specifically, the full-length caspase-12 polymorph (Csp12-L) confers endotoxin hypo-responsiveness, which seems to be manifest in the clinic as an increased susceptibility to severe sepsis and mortality. Mechanistically, Csp12-L functions as a dominant-negative regulator of essential cellular responses, including the IL-1 and NF-κB pathways. These findings indicate that caspase-12 antagonists may have therapeutic use in sepsis and other inflammatory and immune disorders,

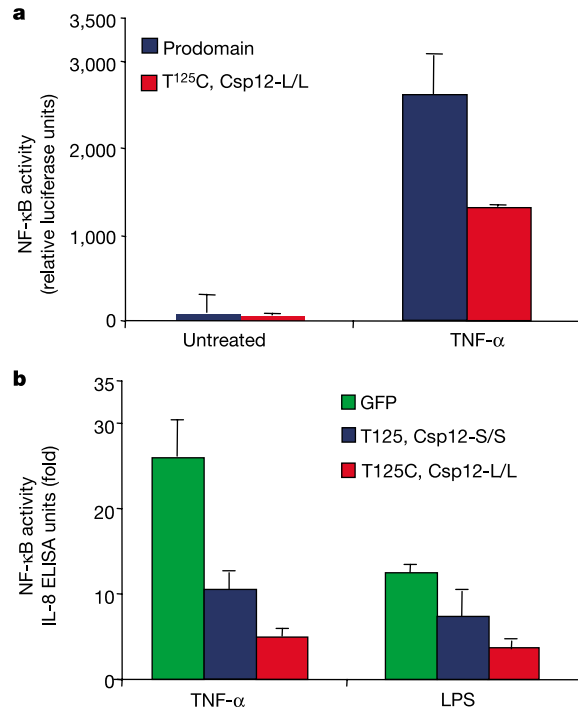


Figure 4 Inhibition of NF-κB activity by human caspase-12. **a**, HEK 293T cells were co-transfected with the pRSV-β-gal and pκB-luc reporter plasmids and pcDNA3.1-caspase12(T125) or pcDNA3.1-caspase12(T125C). **b**, HUVEC cells were co-transfected with pEGFP-N1 and pcDNA3.1, pcDNA3.1-caspase12(T125) or pcDNA3.1-caspase12(T125C). Secretion of IL-8 into the culture medium was measured by ELISA. Values represent the mean ± s.e.m.

where perturbed cytokine responsiveness contributes to disease pathogenesis. □

Methods

Sequencing of caspase-12

Whole blood was collected from humans, squirrel monkeys, capuchins, cynomolgus, rhesus monkeys and Japanese monkeys, and hair was collected from gorillas and chimpanzees. Genomic DNA was extracted from the blood and hair follicles using the QIAamp DNA blood mini kit (Qiagen). We used primers framing a region of 300 base pairs surrounding the T125C polymorphism (sense, 5'-GTCATTCTGTGTGATTAA TTGC-3'; antisense, 5'-CCTATAATATCATACTTTGCTC-3') to amplify the genomic DNA by polymerase chain reaction (PCR). The PCR product was sequenced directly using BigDye Terminators v3.0 (Applied Biosystems).

Blood collection

We collected blood samples from people of African descent through different Black community centres in the Montreal area. For each of five independent experiments, blood donor clinics of roughly 50 donors were organized. We collected 25-ml blood (3 × 8 ml Vacutainer tubes with heparin; Becton Dickinson) from each donor by venous puncture and pooled the blood from each donor before the start of the *ex vivo* treatments. The people of African descent in this study were of different geographical origin: African, Caribbean, African American and South African. For each individual, informed consent for a molecular genetic study was obtained. The blood samples were collected anonymously. In some experiments, blood cells (red blood cells, total white blood cells, neutrophils, basophils, eosinophils, monocytes, lymphocytes) were counted and found to be unaltered among genotypes.

Ex vivo blood treatment

We treated the blood from all donors first and genotyped subsequently. For the inflammation experiments, whole blood (25 ml) was treated with PBS only (12 ml), 1 μg ml⁻¹ LPS (*Escherichia coli* 0111:B4, Sigma; 6 ml) or 50 μg ml⁻¹ conA (*Canavalia ensiformis*, Sigma; 6 ml) for 4 h at 37 °C. After incubation, red blood cells were lysed using erythrocyte lysis buffer (Qiagen) and total RNA was extracted from white blood cells using TRizol reagent (Gibco-BRL). RNA was used for quantitative real-time PCR of cytokine transcripts. For the cell death experiments, blood was collected in Vacutainer CPT tubes (Becton Dickinson). Mononuclear cells were separated from whole blood by centrifugation and were stimulated with PBS only, 1 μg ml⁻¹ α-Fas, 1 mM cyclohexamide, 1 μg ml⁻¹ tunicamycin, 2 μM thapsigargin or 2 μM A23187 for 18 h at 37 °C. Cell death was

measured by quantification of oligonucleosomal DNA fragmentation by using Roche's Cell Death enzyme-linked immunoabsorbent assay (ELISA). In all the blood experiments, 200 μ l of blood was used for genomic DNA extraction and genotyping (see above).

Real-time quantitative PCR and TNF- α ELISA

Total RNA was prepared by an RNeasy mini kit (Qiagen). Reverse transcription of RNA (50 ng) was done with Taqman transcription reagents (PE Biosystems). We purchased the PCR primers and Taqman probes (PE Biosystems) for the target genes and the 18S ribosomal RNA as pre-developed primers and probe sets (see also Supplementary Information). Plasma TNF- α was quantified by ELISA (Abraxis).

Western blotting

Csp12-L and Csp12-S were *in vitro* transcribed and translated using TNT-coupled reticulocyte lysates (Promega) and were processed for western analysis using rabbit polyclonal antibodies directed against recombinant human caspase-12 prodomain. Alternatively, to detect Csp12-L in blood, isolated white blood cells were lysed in 1 \times SDS-PAGE sample buffer and the protein extracts processed for western analysis using rabbit polyclonal antibodies directed against the large subunit of recombinant rat caspase-12.

NF- κ B activation assays

For the luciferase assays, we co-transfected HEK 293T cells with κ B-luc and β -gal reporter plasmids and a plasmid encoding either Csp12-S (residues 1–125 of the prodomain fused to cMyc) or Csp12-L (T125C caspase-12 fused to green fluorescent protein (GFP)). Twenty-four hours after transfection, cells were treated with 10 ng ml⁻¹ TNF- α for 6 h or were left untreated. Cell extracts were prepared and relative luciferase activity was measured. For the measurement of IL-8 secretion, we transfected HUVEC cells as above, except that the short caspase-12 construct was replaced by the long caspase-12 construct, in which the arginine was mutated to a stop codon at position 125, and pEGFP-N1 was co-transfected as a transfection marker. Twenty-four hours after transfection, the cells were trypsinized and GFP-positive cells were sorted by FACS and allowed to adhere before being treated with PBS, 1 μ g ml⁻¹ LPS or 10 ng ml⁻¹ TNF- α for 18 h. The media from the cultured cells was collected and IL-8 was quantified by ELISA.

Human subjects

Individuals with Alzheimer's disease and matched controls were African American participants of the MIRAGE Study, a multicentre family study of genetic and environmental risk factors for Alzheimer's disease¹⁶. All affected individuals met NINCDS/ADRDA criteria¹⁷ for probable or definite Alzheimer's disease. Controls were cognitively normal siblings and unrelated volunteers (including spouses and age-matched members from the same community as the affected individuals). African American individuals with severe sepsis had both septic bacteraemia accompanied by physiological failure of at least one organ system; matched controls were contributors to the Genetic Predisposition to Severe Sepsis (GenPSS) study of the Project IMPACT. We carried out SNP analysis by TDI-FP¹⁸ and sequencing.

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Nitration of a peptide phytotoxin by bacterial nitric oxide synthase

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Nitric oxide (NO) is a potent intercellular signal in mammals that mediates key aspects of blood pressure, hormone release, nerve transmission and the immune response of higher organisms^{1–4}. Proteins homologous to full-length mammalian nitric oxide synthases (NOSs) are found in lower multicellular organisms⁵. Recently, genome sequencing has shown that some bacteria contain genes coding for truncated NOS proteins; this is consistent with reports of NOS-like activities in bacterial extracts^{6,7}. Biological functions for bacterial NOSs are unknown, but have been presumed to be analogous to their role in mammals. Here we describe a gene in the plant pathogen *Streptomyces turgidiscabies* that encodes a NOS homologue, and we reveal its role in nitrating a dipeptide phytotoxin required for plant pathogenicity⁸. High similarity between bacterial NOSs indicates a general function in biosynthetic nitration; thus, bacterial NOSs constitute a new class of enzymes^{9–11}. Here we show that the primary function of *Streptomyces* NOS is radically different from that of mammalian NOS. Surprisingly, mammalian NO signalling and bacterial biosynthetic nitration share an evolutionary origin.

In mammals, the production of NO is catalysed solely by three highly regulated isoenzymes of NOS. NOSs produce NO from the oxidation of L-arginine (L-Arg) to L-citrulline through the intermediate N-hydroxy-L-Arg^{12–14}. Mammalian NOSs are homodimers that contain an amino-terminal haem oxygenase domain (NOS_{oxy}) and a carboxy-terminal flavoprotein reductase domain (NOS_{red}). The oxygenase domain binds L-Arg, haem and the redox-active cofactor 6R-tetrahydrobiopterin (H₄B), whereas the reductase domain binds FAD, FMN and NADPH. A calmodulin-binding sequence links the oxygenase and the reductase domains and regulates the reduction of NOS_{oxy} by NOS_{red} in those isoforms