LETTERS

Dendritic cell PAR1–S1P3 signalling couples coagulation and inflammation

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Defining critical points of modulation across heterogeneous clinical syndromes may provide insight into new therapeutic approaches. Coagulation initiated by the cytokine-receptor family member known as tissue factor is a hallmark of systemic inflammatory response syndromes in bacterial sepsis and viral haemorrhagic fevers^{1,2}, and anticoagulants can be effective in severe sepsis with disseminated intravascular coagulation³. The precise mechanism coupling coagulation and inflammation remains unresolved^{4–7}. Here we show that protease-activated receptor 1 (PAR1) signalling sustains a lethal inflammatory response that can be interrupted by inhibition of either thrombin or PAR1 signalling. The sphingosine 1-phosphate (S1P) axis is a downstream component of PAR1 signalling, and by combining chemical and genetic probes for S1P receptor 3 (S1P3) we show a critical role for dendritic cell PAR1–S1P3 cross-talk in regulating amplification of inflammation in sepsis syndrome. Conversely, dendritic cells sustain escalated systemic coagulation and are the primary hub at which coagulation and inflammation intersect within the

lymphatic compartment. Loss of dendritic cell PAR1–S1P3 signalling sequesters dendritic cells and inflammation into draining lymph nodes, and attenuates dissemination of interleukin-1 β to the lungs. Thus, activation of dendritic cells by coagulation in the lymphatics emerges as a previously unknown mechanism that promotes systemic inflammation and lethality in decompensated innate immune responses.

Disseminated intravascular coagulation and systemic inflammation are signs of excessive activation of the innate immune system. Both are attenuated by genetic reduction of tissue factor and its protease ligand coagulation factor VIIa, leading to improved survival in endotoxaemia^{6,8}. In a model of severe, but not completely lethal lipopolysaccharide (LPS) challenge⁹, we show that PAR1 deficiency protects mice from lethality (Fig. 1a). PAR1^{−/−} mice initially developed elevated inflammation and coagulation markers indistinguishable from the wild type (Fig. 1b, c). Unlike the wild type, PAR1^{−/−} mice progressively resolved systemic inflammation beginning at 12 h. To address whether coagulation amplifies inflammation

Figure 1 | Coagulation amplifies inflammation and lethality through PAR1 signalling. **a**, Survival advantage of PAR1^{−/−} mice in 90% lethal LPS challenge induced by intraperitoneal injection of 8 mg kg^{−1} LPS (summary of three independent experiments, $n \geq 28$ per genotype, PAR1^{−/−} survival advantage for each individual experiment, $P < 0.05$). **b**, Reduced late-stage inflammation in PAR1^{−/−} mice documented by IL-6 and IL-1 β levels (mean $\pm$ s.d., $n = 18$ per group, asterisks indicate groups that are different from the wild type (WT), $P < 0.05$). **c**, TAT levels in wild-type and PAR1^{−/−} mice, or wild-type mice treated at 10 h with PAR1 antagonist RWJ58259 (P1ant) or the thrombin inhibitor hirudin (Hir). **d**, Intervention with PAR1 antagonist or hirudin improves survival, similarly to PAR1 deficiency ($n = 8$

per group, $P < 0.02$ relative to wild-type control). **e**, Intervention with PAR1 antagonist (5 mg kg^{−1} at 16 and 22 h) improves survival of wild-type mice in the CLP model ($n = 16$ per group, $P < 0.005$, pooled data from two experiments, survival advantage in each experiment for drug-treated group, $P < 0.05$). **f**, Intervention with PAR1 antagonist improves inflammation and coagulation markers. Sham- (surgery without CLP) or CLP-operated mice received PAR1 antagonist or vehicle at 16 h. Pre-drug 16-h retro-orbital and terminal 22-h inferior vena cava blood samples were collected (mean $\pm$ s.d., $n = 3$ for sham, $n = 9$ for CLP drug- or vehicle-treated animals, asterisks indicate groups that are different compared with the control, $P < 0.02$ (TAT), $P = 0.001$ (IL-1 β)).

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through PAR1 signalling at this stage of the model, wild-type mice were treated 10 h after challenge with either the thrombin inhibitor hirudin or a PAR1-selective antagonist. Both pharmacological interventions protected mice from lethality (Fig. 1d). Like PAR1 deficiency, PAR1 antagonism also attenuated late-stage coagulation (Fig. 1c), indicating that continuing activation of coagulation and inflammation are coupled by a common upstream mechanism.

The implications of these findings for sepsis therapy were further evaluated in the caecal ligation and puncture (CLP) model. Wild-type mice were treated with PAR1 antagonist after development of severe sepsis symptoms 16 h after surgery. Blocking PAR1 signalling attenuated lethality and interrupted ongoing inflammation as well as coagulation (Fig. 1e, f). These data show that intervention in the PAR1 pathway improves sepsis outcome, and indicate that appropriate LPS challenge adequately models mechanisms by which PAR1 contributes to sepsis lethality.

Multiplex cytokine profiles confirmed that early, LPS-induced inflammatory responses of the innate immune system were unaltered in PAR1^{-/-} mice (Fig. 2a). In contrast, PAR1 deficiency attenuated broadly inflammatory parameters 18 h after LPS challenge (Fig. 2b). Delayed intervention with coagulation inhibitor or PAR1 antagonist in wild-type mice recapitulated the reduction in cytokine levels of PAR1^{-/-} mice. Thus, coagulation amplifies inflammation, and pharmacological blockade of PAR1 is sufficient to rebalance exacerbated systemic inflammation.

S1P signalling has diverse roles in inflammation and immunity¹⁰, and PAR1 and S1P signalling are coupled in endothelial cells^{11,12}. Inflammatory exacerbation was attenuated in mice lacking sphingosine kinase 1 (SphK1), comparable to that seen in PAR1^{-/-} mice (Fig. 2b). To show that PAR1 signalling operated upstream of S1P signalling, S1P receptors of PAR1^{-/-} mice were directly stimulated with an agonist (AAL) for both S1P1 and S1P3, or a selective agonist (AUY) for S1P1. In control experiments, both agonists induced S1P1-dependent sequestration of T cells by > 90% for 12 h, confirming equivalent potency at the dose that was given as a single bolus injection 10 h after LPS challenge. S1P1/3 agonism by AAL, but not selective S1P1 activation by AUY, completely reversed the protection of PAR1^{-/-} mice from exacerbated inflammation (Fig. 2c). S1P3^{-/-} mice showed attenuated cytokine levels similar to PAR1^{-/-} mice, but the S1P1/3 agonist AAL had no pro-inflammatory activity in S1P3^{-/-} mice, confirming that inflammation is specifically amplified by S1P3 signalling.

Although we initially assumed that vascular cells initiated this pro-inflammatory signalling cross-talk, reconstitution of PAR1^{-/-} mice with wild-type bone marrow was sufficient to restore inflammation in PAR1^{-/-} mice (Fig. 3a). The cytokine profile indicated T- and dendritic cell activation, but T-cell counts in late-stage LPS challenge were similar between wild-type and S1P3^{-/-}, PAR1^{-/-} or PAR1-antagonist-treated mice (Supplementary Fig. 2a, b). To address the role of dendritic cells, wild-type bone-marrow-derived dendritic cells (Supplementary Fig. 2c, d) were adoptively transferred 24 h before challenge. This restored late-stage inflammation in PAR1^{-/-}, SphK1^{-/-} or S1P3^{-/-} mice, but did not further amplify inflammation in wild-type mice, showing that adoptive transfer of dendritic cells per se did not exacerbate inflammation (Fig. 3a). In addition, PAR1^{-/-} or S1P3^{-/-} dendritic cells did not increase systemic inflammation after adoptive transfer into S1P3^{-/-} or SphK1^{-/-} mice. Thus, each component of the pathway must be present on the dendritic cell to restore systemic inflammation, indicating that PAR1, SphK1 and S1P3 are coupled in an autocrine, rather than paracrine, pathway.

Dendritic cells have important roles in the activation and resolution of innate immune responses^{13,14}, and inflammation mobilizes migratory dendritic cells from the periphery to lymph nodes¹⁵. Because adoptively transferred dendritic cells were recruited to lymph nodes (Supplementary Fig. 3a), we analysed local inflammation in draining mesenteric lymph nodes that are downstream of the severe insult of intraperitoneal LPS administration. Surprisingly, PAR1^{-/-}, SphK1^{-/-} and S1P3^{-/-} mice had increased IL-1 β levels in mesenteric lymph nodes, but IL-1 β levels were conversely reduced in the lungs, which represent the first microvascular bed downstream of lymphatic drainage into the thoracic duct (Fig. 3b). In support of the concept that loss of PAR1–S1P3 signalling specifically contained inflammation in draining lymph nodes, IL-1 β levels in the spleens were unchanged in all knockout strains. Furthermore, wild-type mice receiving S1P3^{-/-} dendritic cells had increased local inflammation in mesenteric lymph nodes, whereas transfer of wild-type dendritic cells into S1P3^{-/-} mice promoted dissemination of inflammation to the lungs. Importantly, PAR1 antagonism in the CLP model induced sequestration of inflammation to draining mesenteric lymph nodes and prevented dissemination of inflammation to the lungs (Fig. 3b). This shows that the PAR1 pathway can be targeted successfully in polymicrobial sepsis syndrome.

Elevated lymph node IL-1 β levels were strictly correlated with increased mesenteric lymph node sizes in the LPS and CLP model

Figure 2 | PAR1 amplifies inflammation through SphK1–S1P3 signalling crosstalk. **a**, Cytokine multiplex analysis⁹ documents that PAR1^{-/-} mice have no alterations in the inflammatory response 6 h after LPS challenge. **b**, Thrombin inhibition, PAR1 antagonism and SphK1 deficiency mimic the broad attenuation of inflammation seen in PAR1^{-/-} mice 18 h after challenge. Note that some cytokines (KC (CXCL1) and IL-2) remain elevated independent of PAR1 signalling. **c**, Intervention at 10 h with the S1P1/3 agonist AAL, but not with the selective S1P1 agonist AUY, reverses attenuated inflammation in PAR1^{-/-}, but not S1P3^{-/-} mice. (mean $\pm$ s.d., $n = 7$ –16, asterisks indicate groups that are different compared with the wild type, hash symbols indicate groups that are different compared with wild type AAL or PAR1^{-/-} AAL, $P < 0.05$ by ANOVA).

(Supplementary Fig. 3b, d), confirming at the macroscopic level that local sequestration of inflammation is controlled by dendritic cells. Because S1P3 signalling enhances dendritic cell motility^{16,17}, we addressed whether loss of PAR1–S1P3 signalling led to accumulation of dendritic cells in lymph nodes. To quantify accurately dendritic cells, without influence from lymph node size variations, wild-type or PAR1^{-/-} dendritic cells tagged with green fluorescent protein (GFP) were adoptively transferred into S1P3^{-/-} mice. Cell density of mesenteric lymph nodes 18 h after LPS challenge was not changed by adoptive transfer, but PAR1^{-/-} dendritic cells were enriched two- to threefold compared with the wild type (Fig. 3c). Thus, loss of PAR1 signalling sequesters dendritic cells into draining lymph nodes.

IL-1 β release from dendritic cells is triggered by ATP-dependent signalling of the P2X7 receptor, which leads to activation of caspase-1 in an adaptor complex, the ‘inflammasome’¹⁸. Adoptive transfer of P2X7^{-/-} dendritic cells into S1P3^{-/-} mice failed to increase lung IL-1 β levels (Fig. 3b). To further corroborate that dendritic cell S1P3 signalling regulates IL-1 β dissemination, S1P3^{-/-} or wild-type mice were adoptively transferred with PAR1^{-/-} dendritic cells. In S1P3^{-/-} mice, PAR1^{-/-} dendritic cells are the only target for S1P3 agonism by AAL delivered 10 h after LPS challenge. Adoptive transfer

of PAR1^{-/-} dendritic cells into S1P3^{-/-} mice did not induce dissemination of IL-1 β to the lungs, unless PAR1^{-/-} dendritic cells were stimulated with the S1P3 agonist. Conversely, PAR1^{-/-} dendritic cells adoptively transferred into wild-type mice increased local inflammation specifically in mesenteric, but not inguinal, lymph nodes, and S1P agonism with AAL reversed increased lymph node IL-1 β levels (Fig. 3b and Supplementary Fig. 3c). Thus, PAR1–S1P3 signalling is coupled to activation of the dendritic cell inflammasome to promote systemic dissemination of inflammation in sepsis.

Dendritic cells are known to express tissue factor¹⁹, and therapeutic efficacy of hirudin implicated thrombin in a feedback loop that regulates dendritic cell function. Fibrin was detected in lymphatic sinuses that were identified by LYVE-1 staining, confirming thrombin generation in this unexpected location of mesenteric lymph nodes (Fig. 4a). In addition, a protective thrombin inhibitor dose reduced lymphatic fibrin deposition. Although fibrin deposition was sparse in S1P3^{-/-} lymph nodes, PAR1^{-/-} lymph nodes showed abundant fibrin throughout the parenchyma that contrasted with the predominant wild-type staining within lymphatic ducts. These data indicated that wild-type mice lost the ability to sequester coagulation activation to draining lymph nodes.

Figure 3 | Dendritic cell PAR1–SphK1–S1P3 signalling controls dissemination of inflammation from the lymphatics. **a**, Wild-type bone marrow (BM) chimaeras of PAR1^{-/-} mice lose protection from systemic inflammation. PAR1^{-/-} or wild-type GFP⁺ bone marrow resulted in similar reconstitution of PAR1^{-/-} mice (peripheral blood GFP⁺ cells: CD4⁺, 44 ± 15 versus 51 ± 15%; CD8⁺, 31 ± 19 versus 37 ± 19%; CD11c⁺, 62 ± 21 versus 72 ± 5%; B220⁺, 93 ± 2 versus 92 ± 2%). Wild-type dendritic cells (DC) adoptively transferred by tail vein injection 24 h before challenge reverse attenuated inflammation in protected strains (mean ± s.d., $n = 4–6$ mice per group, asterisks indicate groups that are different compared with the wild-type bone marrow, hash symbols indicate groups that are different from the wild-type dendritic cell group, $P < 0.05$ by ANOVA). **b**, Blood and tissue IL-1 β levels of spleens, mesenteric lymph nodes and lungs measured 18 h after LPS challenge with AAL administration at 10 h, or 22 h after CLP as described in Fig. 1f (mean ± s.d., $n = 4–6$ mice per group, asterisks indicate groups that are different from challenged wild type without drug, hash symbols indicate groups that are different compared with animals receiving wild-type dendritic cells, $P < 0.05$ by ANOVA). **c**, Retention of PAR1^{-/-} dendritic cells in S1P3^{-/-} lymph nodes during late-stage endotoxaemia. Typical experiment of quantitative image analysis (two independent experiments with $n = 3$, $*P < 0.05$) and views of three-dimensional reconstructions of 30- μ m sections from mesenteric lymph nodes. The grid lines are 20 μ m apart; HPF, high power field.

Thrombin-antithrombin (TAT) levels in the periphery further support the concept that dendritic cells initiate dissemination of intravascular coagulation from the lymphatics. TAT levels in peripheral blood samples were close to baseline in $S1P3^{-/-}$ mice (Fig. 4b), although several independent parameters indicated normal LPS-induced tissue-factor expression in $S1P3^{-/-}$ mice (Supplementary Fig. 4). $PAR1^{-/-}$ or $SphK1^{-/-}$ mice had higher peripheral TAT levels compared with $S1P3^{-/-}$ mice. The precise mechanism for the complete loss of late-stage coagulation activation in $S1P3^{-/-}$ mice requires further study. However, adoptive transfer of wild-type dendritic cells restored peripheral TAT levels in $S1P3^{-/-}$, $PAR1^{-/-}$ and $SphK1^{-/-}$ mice to levels of wild-type mice. In addition, $PAR1^{-/-}$ dendritic cells increased coagulation in $S1P3^{-/-}$ mice to levels observed in $SphK1^{-/-}$ or $PAR1^{-/-}$ mice. Thus, dendritic cells are the primary source and determine the extent of disseminated coagulation activation during inflammatory exacerbation.

Attenuation of coagulation is not required for survival in the LPS model⁹ and improved survival of $S1P3^{-/-}$ and $SphK1^{-/-}$ mice indicated that dissemination of inflammation caused lethality (Fig. 4c).

To specifically show that $PAR1$ signalling on dendritic cells drives lethality, wild-type or $PAR1^{-/-}$ dendritic cells were adoptively transferred into $SphK1^{-/-}$ mice. Consistent with changes in inflammatory cytokines, wild-type, but not $PAR1^{-/-}$ dendritic cells reversed the survival benefit of $SphK1^{-/-}$ mice (Fig. 4d). In contrast, adoptive transfer of the same number of wild-type, bone-marrow-derived macrophages⁹ into $SphK1^{-/-}$ mice had no adverse effect on survival, demonstrating specificity. Furthermore, administration of the direct $S1P1/3$ agonist (AAL) reversed the therapeutic benefit of $PAR1$ antagonism in wild-type mice (Fig. 4e), despite potential vascular protective $S1P1$ agonistic activity¹¹.

To show directly that dendritic cell $S1P$ signalling initiates lethality, $S1P3^{-/-}$ mice were adoptively transferred with $PAR1^{-/-}$ dendritic cells. This had no adverse effect on survival of $S1P3^{-/-}$ mice, but stimulation of $S1P3$ with AAL on adoptively transferred $PAR1^{-/-}$ dendritic cells was sufficient to induce lethality (Fig. 4f). Consistent with detrimental effects of $S1P3$ signalling on both vascular and dendritic cells, $S1P3^{-/-}$ mice showed delayed lethality in the CLP model (see Supplementary Figs 5, 6 for further discussion of vascular

Figure 4 | Dendritic cell $PAR1$ - $S1P3$ signalling promotes disseminated intravascular coagulation and lethality. **a**, Fibrin staining (green) of mesenteric lymph nodes from wild-type, $S1P3^{-/-}$ or $PAR1^{-/-}$ mice 18 h after LPS challenge with hirudin administration at 10 h. Sections were counterstained for LYVE-1 (red) to visualize lymphatic ducts. **b**, Dendritic cells are responsible for exacerbated disseminated intravascular coagulation. The graph shows TAT levels at 18 h in peripheral venous blood samples of LPS-challenged mice that were adoptively transferred with dendritic cells from the indicated knockouts or the indicated bone-marrow chimaeras in $PAR1^{-/-}$ mice (mean $\pm$ s.d., $n = 4-10$, $P < 0.05$ versus respective wild-type dendritic cell or wild-type bone marrow by ANOVA). **c-f**, Dendritic cell $S1P$ signalling promoted lethality. **c**, Protection of $S1P3^{-/-}$ and $SphK1^{-/-}$ mice from LPS challenge ($n = 8$ per group, $P < 0.01$ relative to wild type). **d**, Adoptive transfer of wild-type, but not $PAR1^{-/-}$ dendritic cells reverses improved survival of $SphK1^{-/-}$ mice ($P = 0.008$, $n = 9$ per group) and lethal

effects are specific for transfer of dendritic cells, but not macrophages (Mac) ($P = 0.003$, $n = 8$ per group). **e**, $S1P3$ agonism with AAL reverses survival of $PAR1$ antagonist-treated mice ($P = 0.004$ for $PAR1$ antagonist versus wild-type control, $P = 0.016$ for $PAR1$ antagonist $\pm$ AAL, $n = 8$ per group). **f**, Dendritic cell $S1P$ agonism with AAL at 10 h promotes LPS-induced lethality in $S1P3^{-/-}$ mice adoptively transferred with $PAR1^{-/-}$ dendritic cells ($P = 0.0008$, $n = 8$ per group). **g**, Adoptively transferred wild-type, but not $PAR1^{-/-}$ dendritic cells promote lethality in $S1P3^{-/-}$ mice in the CLP model ($n \geq 20$ per group, pooled data from two independent experiments; $n = 12$ for $S1P3^{-/-}$ + $PAR1^{-/-}$ dendritic cells. Survival advantage: $S1P3^{-/-}$ versus wild type, $P = 0.0001$; $S1P3^{-/-}$ + wild-type dendritic cells versus wild type, $P = 0.0001$; $S1P3^{-/-}$ + $PAR1^{-/-}$ dendritic cells versus wild type, $P = 0.0001$; $S1P3^{-/-}$ versus $S1P3^{-/-}$ + wild-type dendritic cells, $P = 0.0001$; $S1P3^{-/-}$ + $PAR1^{-/-}$ dendritic cells versus $S1P3^{-/-}$ + wild-type dendritic cells, $P = 0.002$; $S1P3^{-/-}$ versus $S1P3^{-/-}$ + $PAR1^{-/-}$ dendritic cells, $P = 0.5$).

roles of S1P and PAR signalling). Importantly, adoptive transfer of wild-type, but not PAR1^{-/-} dendritic cells impaired survival of S1P3^{-/-} mice in the CLP model (Fig. 4g), confirming the crucial role of dendritic cell PAR1 signalling in inducing sepsis lethality.

These experiments uncover a new link between protease and sphingolipid signalling in the immune system, and position coagulation signalling in the lymphatics upstream of continuing disseminated intravascular coagulation, lung injury and peripheral vascular dysfunction (Supplementary Fig. 1). Dendritic cells emerge as both the target for pro-inflammatory protease signalling and the origin of coagulation activation. This provides a surprisingly simple mechanism for the intimate coupling of exacerbated inflammation and coagulation in deregulated innate immune responses. Although dendritic cells are known to regulate sepsis outcome^{20–22}, the results presented emphasize the role of dendritic cells as promoters of systemic inflammation and sepsis lethality²³. Pharmacological blockade of PAR1 is sufficient to interrupt exacerbated systemic dissemination of inflammation and coagulation, and to improve survival in sepsis. The requirement to block thrombin in the lymphatics for anti-inflammatory benefit may explain the limited clinical efficacy of intravenously administered plasma protease inhibitors, such as antithrombin³. Importantly, blockade of dendritic cell PAR1–S1P3 signalling in the lymphatics attenuates systemic inflammation by containing inflammation to draining lymph nodes downstream of severe inflammation or infection. We propose that intervention in the coagulation–PAR1–S1P3 axis may avoid immune paralysis and compromised host defence that has hampered therapeutic development of direct anti-inflammatory strategies in decompensated infectious diseases.

METHODS SUMMARY

Under approved protocols, pathogen-free C57BL/6-wild-type, PAR1^{-/-} (ref. 24), S1P3^{-/-} (ref. 25), SphK1^{-/-} (ref. 26), or P2X7 (ref. 27) mice were challenged with LD90, ~8 mg kg⁻¹ LPS⁹. PAR1 antagonist RWJ58259 (5 mg kg⁻¹), S1P1/S1P3 agonist AAL(R) (2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol) (0.5 mg kg⁻¹)²⁸, S1P1 agonist AUY (AUY954) (1 mg kg⁻¹)²⁹ or thrombin inhibitor hirudin (120 mg kg⁻¹ lepirudin, 1 mg kg⁻¹ PEG hirudin) were bolus-injected intravenously. For CLP, mice were anaesthetized (ketamine/xylazine, 100/8 mg kg⁻¹) to ligate 1 cm of caecum for two punctures (21G needles), confirmed to be patent by extrusion of stool. After repositioning of the caecum and wound closure, mice received subcutaneously 0.5 ml of warm saline for recovery. Bone marrow chimaeras were generated by reconstituting lethally irradiated PAR1^{-/-} mice. Bone-marrow-derived dendritic cells were expanded for 7 days³⁰, positively selected for CD11c⁺ with paramagnetic beads, and 1 × 10⁶ cells per mouse were injected intravenously 24 h before challenge. Cytokines and TAT were determined in vena cava inferior plasma. Tissue IL-1β levels were measured in cleared extracts (30 mM Tris pH 7.4, 150 mM NaCl, 1% Triton-X100, 2 mM CaCl₂ and 2 mM MgCl₂, protease inhibitor cocktail) from homogenized lymph nodes, spleens and lungs with normalization for total protein concentration. Immunohistochemistry used frozen 5-μm methanol-fixed sections permeabilized with 0.1% Triton for staining with antifibrin antibody (Nordic), anti-LYVE-1 (Reliatech) and counterstaining with DAPI (Vector). Z-stacks of 30-μm frozen sections were taken with the BioRad Radiance 2100 Rainbow laser scanning confocal microscope (Nikon TE 2000-U). Reconstruction of a three-dimensional z-stack and quantitative analysis was performed with the IMARIS software (Bitplane). Means ± s.d. are given and normally distributed data were evaluated by analysis of variance (ANOVA) followed by Fisher's PLSD, or by Kruskal–Wallis followed by the Mann–Whitney U-test. Survival advantages were analysed by Kaplan–Meier curves and the log rank test with Bonferroni correction as needed.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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