Thymosin β4-sulfoxide attenuates inflammatory cell infiltration and promotes cardiac wound healing

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The downstream consequences of inflammation in the adult mammalian heart are formation of a non-functional scar, pathological remodelling and heart failure. In zebrafish, hydrogen peroxide released from a wound is the initial instructive chemotactic cue for the infiltration of inflammatory cells, however, the identity of a subsequent resolution signal(s), to attenuate chronic inflammation, remains unknown. Here we reveal that thymosin β4-sulfoxide lies downstream of hydrogen peroxide in the wounded fish and triggers depletion of inflammatory macrophages at the injury site. This function is conserved in the mouse and observed after cardiac injury, where it promotes wound healing and reduced scarring. In human T-cell/CD14+ monocyte co-cultures, thymosin β4-sulfoxide inhibits interferon-γ, and increases monocyte dispersal and cell death, likely by stimulating superoxide production. Thus, thymosin β4-sulfoxide is a putative target for therapeutic modulation of the immune response, resolution of fibrosis and cardiac repair.
Cardiac injury activates an innate immune mechanism (reviewed in Frangogiannis1) underpinned by sequential recruitment of inflammatory neutrophils and macrophages (leukocytes) to the site of injury. A direct consequence of immune cell infiltration is activation of fibroblasts and their differentiation to myofibroblasts, the principle source of collagen deposition, fibrosis and scarring. Recently, a study in the zebrafish revealed that the earliest ‘damage’ signal that instigates rapid neutrophil and macrophage influx to the wound is hydrogen peroxide ($H_2O_2$). The identity of subsequent downstream signalling molecules or cues that encourage the resolution of immune cells away from the wound, once the acute phase is complete, remains unknown.

Thymosin β4 (Tβ4) is an actin-binding peptide, which exerts both anti-inflammatory and anti-fibrotic effects in the context of tissue wounding; notably following alkali eye injury and bleomycin-induced damage in the lung. Tβ4 is one of the first proteins to be upregulated after wounding and has been shown to modulate oxidative stress, targeting cardiac fibroblasts and cardiomyocyte survival following myocardial infarction. It has been shown to inhibit neutrophil chemotaxis in vitro and attenuate inflammation in carrageenan-injected mice. We initially examined whether mechanical wounding of the tip of the tail fin (Fig. 1b) would lead to the production of pro-inflammatory IFN-γ to reduce the adhesion of activated monocytes and increases cell death downstream of elevated reactive oxygen species (ROS) production.

Results

Tβ4-4SO acts downstream of $H_2O_2$ in the wounded zebrafish.

We initially examined whether mechanical wounding of the zebrafish larval tail fin would lead to production of Tβ4-4SO in vivo. The zebrafish has five β-thymosin isoforms arising from successive rounds of gene duplication, one of which has been described as the orthologue of Tβ4 (Fig. 1a), however, there are no previous reports describing Tβ4-4SO in the zebrafish. Larvae at 72 h post fertilization (h.p.f.) were pre-incubated in the presence or absence of the small molecule inhibitor of H$_2$O$_2$ diphenyleneiodium (DPI), followed by surgical removal of the tip of the tail fin (Fig. 1b). Levels of Tβ4-4SO increased rapidly, 1 h post wounding, by approximately fivefold from $2\% \pm 1.4\%$ s.e.m. to $10.4\% \pm 1.96\%$ s.e.m. ($n = 100$ wounded tail fins per treatment, $n = 3$ experimental replicates; Student’s t-test, $P \leq 0.01$; Fig. 1c). Pre-treatment with DPI resulted in a threefold increase (6.4% ± 0.8% s.e.m.), which was significantly less than that observed in vehicle pre-treated control fish (Student’s t-test, $P \leq 0.05$; Fig. 1d). To investigate whether ectopic administration of Tβ4-4SO might directly affect the presence of leukocytes, latex beads pre-adsorbed with synthetic Tβ4-4SO or vehicle control (PBS), were implanted into Tg[mpoGFP] zebrafish (Fig. 1e,f). Bead implantation acted as an injury stimulus resulting in recruitment of L-plastin+ (MPO−) macrophages in the control setting (Fig. 1g). Imaging of fish at 3 and 6 h, thereafter, indicated a significantly reduced number of macrophages proximal to the Tβ4-4SO bead relative to controls (3 h: co: $20 \pm 4.65$ s.e.m. versus Tβ4-4SO: $8.25 \pm 2.39$ s.e.m. and 6 h: co:$17.25 \pm 4.05$ s.e.m. versus Tβ4-4SO: $8.66 \pm 2.03$ s.e.m. ($n = $mean cell count from five bead-implanted fish per treatment; Student’s t-test, $P \leq 0.01$; Fig. 1h,i), suggesting ectopic Tβ4-4SO either inhibited macrophage recruitment or acted to disperse macrophages at the implantation site. MPO+ neutrophil numbers were not significantly affected in this assay; normal neutrophil function involves stimulation of macrophage recruitment (reviewed in Soehnlein and Lindbom), suggesting Tβ4-4SO was able to alter macrophage incidence despite the presence of neutrophils.

To address the issue of whether Tβ4-4SO affected leukocyte recruitment versus dispersal, we generated time-lapse movies of the inflammatory cell response in Tg[LysC:dsRed] larval zebrafish, up to 4 h following bead implantation. While similar numbers of LysC+ leukocytes were recruited to the bead wound in the control and Tβ4-4SO-treated setting at 1 and 2 h post injury, those that localized to a PBS bead were retained beyond 3 h post injury, whereas those that encountered a Tβ4-4SO-coated bead dispersed at and beyond this time point, suggesting the latter did not inhibit the initial inflammatory cell recruitment but rather induced their subsequent resolution (Supplementary Fig. S1; Supplementary Movies 1 and 2).

Extrapolating from generic tissue wounding, we next examined the leukocyte response in a model of zebrafish heart injury. Both laser- and needle-induced injuries in the larval heart at 72 h resulted in the release of $H_2O_2$ from the wound site, as determined by free radical spin trap analysis with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO; Fig. 2a). Delivery of Tβ4-4SO or PBS control via microinjection or bathing of the larvae in Tβ4-4SO-containing medium, resulted in significantly reduced incidence of infiltrating leukocytes (L-plastin+) at both 3 and 6 h post injury (3 h: co: $30.4 \pm 2.96$ s.e.m. versus Tβ4-4SO: $22.5 \pm 1.50$ s.e.m.; Student’s t-test, $P \leq 0.05$ and 6 h: co: $35.3 \pm 3.19$ s.e.m. versus Tβ4-4SO: $18.2 \pm 1.90$ s.e.m.; Student’s t-test, $P \leq 0.01$; $n =$ mean cell count from four injured fish per treatment; Fig. 2b–f).

Tβ4 and Tβ4-4SO reduce inflammatory cell infiltration after MI.

To investigate the effect of Tβ4-4SO on leukocyte infiltration during wounding in mammals, we utilized the mouse MI model. It is known that two distinct phases of monocytes participate post MI in the mouse: inflammatory monocytes (Ly-6ch) dominate during the early phase I (days 1–3) and exhibit phagocytic and proteolytic function to digest damaged tissue, whereas monocytes with attenuated inflammatory properties (Ly-6g) dominate later (from days 4–7) during the wound-healing phase II. Hence, we focused on these key time points and on the potential for the parent peptide Tβ4 and derivative Tβ4-4SO to augment the transition from phase I to II.

We initially treated animals with Tβ4 immediately post MI and compared against both vehicle-treated controls and Tβ4 global knockout (equivalent to loss of Tβ4-4SO function) mice. MPO+ neutrophils increased in the border zone of the infarct by day 2 and peaked at day 4 post injury for all mice. Tβ4 treatment attenuated this increase with fewer MPO+ cells present (co: $70 \pm 14.5$ s.e.m. versus Tβ4: $30 \pm 19.1$ s.e.m., $P \leq 0.15$) at the site of injury at both stages (Fig. 3a–d). Global knockout of Tβ4 significantly increased MPO+ cell numbers at the earliest stages...
of infiltration (day 2; wild type: 20.5 ± 4.6 s.e.m. versus Tβ4 KO: 47.7 ± 5.0 s.e.m.; Student’s t-test, \( P < 0.01 \)), thereafter, numbers were equivalent to that observed for control (vehicle-treated) mice (Fig. 3a–d). We next investigated the levels of TNF-α, as the upstream cytokine responsible for initiating the inflammatory cascade.\(^1\) TNF-α was significantly reduced (6.2-fold reduction) after 2 days of Tβ4 treatment post MI as compared with vehicle controls (Fig. 3e,f), as was the downstream pro-inflammatory cytokine IL-6 (4.1-fold reduction), which ordinarily is rapidly induced in the ischaemic myocardium (Fig. 3e,f). In keeping with the role in mitigating inflammatory injury during the early stages post infarct, without interfering with subsequent myocardial healing, Tβ4 stimulated the expression of the inhibitory cytokine IL-10 at 2 days post MI (8.1-fold increase compared with control; Fig. 3e,f). In control mice, the number of F4/80$^{+}$ macrophages significantly increased...
between days 2–7 post MI (Fig. 3g,h,m). Treatment with Tβ4 resulted in an initial increase in F4/80+ cells at day 2 (Fig. 3i,j,m), peaking at day 4 post MI, followed by a significant reduction at day 7 post MI (co: 21 ± 3.4 s.e.m. versus Tβ4: 3 ± 1.4 s.e.m.; Student’s t-test, P ≤ 0.05; Fig. 3m). In Tβ4 knockout mice, we observed the opposite effect, such that F4/80+ cells increased continuously to around fivefold that of control mice by day 7 post MI (21 ± 3.4 s.e.m. versus 135.8 ± 12.4 s.e.m.; Student’s t-test, P ≤ 0.001; Fig. 3k, l, m). The observation that Tβ4 efficiently cleared the macrophage-rich infiltrate by day 7 post MI was consistent with the zebrafish data for Tβ4-SO (Fig. 1h,i).

We next investigated the anti-inflammatory activity of Tβ4-SO by flow cytometry of infarct cell suspensions and observed an early (day 2) significant increase in F4/80+ cells (co: 23.0% ± 2.85% s.e.m. versus Tβ4-SO: 32.0% ± 2% s.e.m.; n = 4; Student’s t-test, P ≤ 0.05; Fig. 4a), which represented an increase in the proportion of macrophages, as the total number of monocytes (CD11bhi, Ly6G, B220, CD49b, CD90.2, NK1.1) in Tβ4-SO: 27% ± 0.4% s.e.m.; n = 4; Student’s t-test, P ≤ 0.05; Fig. 4c). Tβ4-SO treatment of infarct suspensions did not, however, alter the relative proportions of monocyte Ly-6lo versus Ly-6Chi sub-populations within the reduced infiltrate at days 2 and 4 post MI (Supplementary Fig. S2a,b).

These data are consistent with a bi-phasic effect of the influence of Tβ4/Tβ4-SO. During the so-called debris-removal phase 1 (day 2), Tβ4/Tβ4-SO increased macrophage numbers by directing transformation of the stable monocyte pool and subsequently enhanced the transition to the anti-inflammatory

Figure 2 | Tβ4-SO disperses leukocytes after cardiac injury in zebrafish. (a) Laser injury to the heart of a 72-h zebrafish larvae immunostained for DMPO (red) revealed localized H2O2 production at the wound site (b). At 3 h post laser heart injury (h.p.i.), a reduced leukocytic response (revealed by L-plastin staining – magenta; c,d) was seen in Tβ4-SO-treated fish within 500 μm of the focal point of injury (c) compared with controls (b). Larger, needle-induced wounds to the heart region of transgenic fish, resulted in a stronger inflammatory response in PBS-treated control hearts at 6 h.p.i. (d), which was significantly reduced in Tβ4-SO-treated fish (e). Total numbers of L-plastin+ leukocytes was significantly lower at 3 and 6 h.p.i. for Tβ4-SO-treated fish compared with controls; n = 5 fishes per treatment, error bars are s.e.m. (f). Asterisks mark wound site and green outlines delineate the heart as determined by myl7-GFP+. Scale bars, 100 μm (a–e). All statistics Student’s t-test; *P ≤ 0.05, **P ≤ 0.01.

Figure 3 | Tβ4/Tβ4-SO modulates the inflammatory response post MI in the mouse heart. Representative images, showing infiltration of the injured myocardium with neutrophils (white arrowheads highlight individual MPO+ cells; a–c) and macrophages (white arrowheads highlight individual F4/80 cells; g–i) in Tβ4-treated, control vehicle-treated and Tβ4-null myocardial infarction (MI) hearts. Significantly fewer cytotoxic myeloperoxidase (MPO)-peroxidase + neutrophils were present in Tβ4-treated, compared with controls and Tβ4-null hearts at days 2 and 4 post MI (a–d). Inset in b illustrates specific MPO staining of a neutrophil at higher magnification. Significantly more neutrophils were present at the infarct site in the Tβ4-null hearts at day 2, consistent with the timing of initial inflammatory cell infiltration (d). Western blots for TNF-α, IL-6, IL-10 and GAPDH at 2, 4 and 7 days post MI in animals treated with Tβ4 as compared with vehicle controls (e) and quantification of protein expression by scanning densitometry (f). Black arrowheads indicate alternate glycosylated isoforms (21 and 24 kDa) of IL-10. The upstream initiator of the acute inflammatory cascade, TNF-α, is downregulated at early stages post MI (2 days) following Tβ4 treatment along with the downstream inflammatory cytokine, IL-6; levels of both TNFα and IL-6 subsequently become elevated with an early reduction in injury and enhanced repair at later stages (e,f). The anti-inflammatory cytokine IL-10 is upregulated after 2 and 4 days of treatment with Tβ4 (e,f). Representative images, F4/80-positive macrophages in the scar region post MI, in co (g,h), Tβ4-treated (i,j) or Tβ4−/− animals (k,l); both at day 2 (g,k) and day 7 (h,j) post MI, with cell counts quantified in m. Tβ4-induced macrophage infiltration peaks at day 4 but is rapidly cleared, to a level significantly reduced relative to vehicle treated by day 7; in Tβ4-null hearts the incidence of macrophages at the wound was significantly elevated relative to the controls as highlighted by cell counts (m). Scale bar, 20 μm (a–c) and 20 μm (g–l); v, vehicle. n = 4–6 MI hearts analysed per time point; error bars are s.e.m. for all bar graphs; all statistics Student’s t-test; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
A Tβ4/Tβ4-SO-depleted monocyte infiltrate reduces scarring. The functional consequences of the Tβ4/Tβ4-SO-depleted monocyte infiltrate were examined by serial MRI of mice treated with Tβ4, to reflect sulfoxide activity, for 7, 14 and 28 days post MI. Tβ4-SO was increased in hearts following the addition of Tβ4 across the time course of the study: percentage oxidized Tβ4 increased from 4.7 ± 1.3% in sham control hearts to 66 ± 5.1% at day 2 post MI, maintained at an equivalent elevated level (68.6 ± 9.3%) through to 28 days post MI (n = 4; repeated measures one-way analysis of variance; P ≤ 0.001). Increase in infarct volume (late gadolinium enhancement; LGE) was significantly higher in PBS compared with Tβ4-treated mice; persisting up to day 28 post MI (co: 43.3 ± 4.9 μl; n = 5 versus Tβ4: 22.0 ± 5.3 μl; n = 4; Student’s t-test; P ≤ 0.05; Fig. 4d) and % infarct relative to left ventricular mass (LVM) followed the same trend (day 28 post MI: co: 27 ± 2%; n = 5 versus Tβ4: 18 ± 4%; n = 4; Student’s t-test,
Figure 4 | Tβ4/Tβ4-SO attenuates inflammatory cell infiltration, scarring and fibrosis. Following treatment with Tβ4-SO, FACS analyses at 2 days post MI revealed a significant increase in macrophages at day 2 post MI (a), with no change in the total number of monocytes recruited (b). In MI samples, 4 days post injury the total number of monocytes was significantly reduced (c). Tβ4 treatment resulted in significantly increased Tβ4-SO levels (refer to main text) and reduced scoring and fibrosis, by 28 days post MI, according to surrogate measures of infarct volume and % infarct relative to LVM (LVM); as determined by longitudinal MRI of LGE; n = 4 hearts per treatment per time point; error bars are s.e.m. (d). Short-axis MRI images of PBS control (e) and Tβ4 (f) treated hearts at day 28 post MI highlighted the extent of infarct and fibrosis (white arrowheads) in the wall of the left ventricle (lv), which is reduced in Tβ4-treated animals (comparing e with f). Infarct volume negatively correlated with ejection fraction at day 28 MI, consistent with the late remodelling phase and reduced LGE in Tβ4-treated hearts (slope: —0.37; P = 0.624) compared with PBS controls (slope: —1.76; P = 0.0234) reflected reduced scar formation (g). Scale bar, 2 mm (f). Statistics repeated measures one-way analysis of variance; *P ≤ 0.05.

Tβ4-SO has anti-inflammatory effects on human monocytes. Extrapolating the animal model findings to human monocytes/macrophages, we investigated the response to inflammatory stimuli in the presence of Tβ4-SO. Interactions between CD4+ T cells and monocyte/macrophages are known to drive pro-inflammatory and anti-inflammatory responses18. We established co-cultures of CD4+ T cells and CD14+ monocytes from the peripheral blood mononuclear cells of healthy individuals and initially excluded any effect of Tβ4 or Tβ4-SO on the underlying T cells (Supplementary Fig. S3) or the monocyte inflammatory phenotype per se via activation or differentiation (Supplementary Fig. S4). The addition of Tβ4-SO lead to a significant reduction in the release of the pro-inflammatory cytokine IFN-γ, relative to controls (co: 1,830 pg ml⁻¹ ± 365.3 pg ml⁻¹ s.e.m. versus Tβ4-SO: 1,271.5 pg ml⁻¹ ± 483.1 s.e.m., n = 4; Wilcoxon matched-pairs signed rank test, P ≤ 0.05; Fig. 5a). IFN-γ is secreted by T cells and functions to both activate macrophages and establish conditions of adhesion and binding required for their migration (reviewed in Schroder et al.19). CD14+ cells treated with Tβ4-SO were significantly less adherent than their control counterparts, even in the presence of recombinant IFN-γ (co: 219,167 ± 25,834 s.e.m. versus Tβ4-SO: 89,167 ± 13,411 s.e.m.; n = 3; Student’s t-test, P ≤ 0.05; Fig. 5b,c). We next assessed whether effects on CD14+ cell adhesion/displacement might be accompanied by elevated cell death and observed Tβ4-SO significantly increased the number of trypan blue-positive cells compared with IFN-γ and control treated cultures (Tβ4-SO: 135,833 ± 22,928 s.e.m. versus IFN-γ: 55,833 ± 2,203 s.e.m. versus co: 33,333 ± 833 s.e.m.; n = 3; P ≤ 0.05; Fig. 5d). To determine a potential mechanism, we investigated whether Tβ4-SO, in the presence of IFN-γ, might act to increase intracellular ROS and thus provoke oxidative stress-induced apoptosis20. Increased ROS is known to occur downstream of H₂O₂ release and cell survival can be maintained by reversal of H₂O₂-induced oxidation of methionine21. Macrophages from the peritoneal cavity of mice were stimulated with IFN-γ and incubated with either vehicle or Tβ4-SO followed by dihydroethidium, a fluorescent indicator of ROS22. Activating IFN-γ treatment alone resulted in increased ROS, which was significantly augmented by the addition of Tβ4-SO (n = 3; Student’s t-test, P ≤ 0.05; Fig. 5e,f); consistent with Tβ4-SO acting downstream of H₂O₂-methionine oxidation and oxidative stress.

Discussion

Despite a number of previous studies assigning anti-inflammatory and anti-fibrotic properties to Tβ4, there remains no insight as to how this peptide might mediate effects directly on the innate immune-cell response post wounding. Here we sought to test the hypothesis that a naturally occurring oxidized derivative, Tβ4-SO, might act to disperse inflammatory cells from sites of injury in a conserved manner from zebrafish, through rodents, to man. This study was underpinned by prior identification of H₂O₂ as the initial chemo-attractive signal for recruitment of inflammatory cells to a wound2 and the outstanding question as to what might be the downstream factor responsible for resolving these cells to prevent chronic inflammation3. Tβ4-SO is formed by methionine oxidation of the peptide H₂O₂ acting downstream of H₂O₂-methionine oxidation and oxidative stress.
oxidation of TP4 in the presence of H2O2, which alongside the observed upregulation of TP4 established the sulfoxide variant as a potential candidate for immune cell dispersal. We initially generated a simple zebrafish tail fin resection model and revealed by QTOF-MS that TP4-SO is significantly upregulated post wounding in zebrafish in a H2O2-dependent manner. In subsequent zebrafish lateral body wall and heart injury models, ectopic sulfoxide induced the resolution of leukocytes/macrophages from the site of injury downstream of H2O2. Thus, TP4-SO may function as the elusive dispersal signal to prevent chronic inflammation and promote wound healing. Extrapolating to mouse MI, both the parent molecule TP4 and sulfoxide derivative were necessary and sufficient to ensure an elevated presence of macrophages within the stable (Ly-6Chi) monocyte pool, during the debris and necrotic tissue-removal phase I, followed by significantly increased clearance during the transition to the wound-healing phase II following cardiac injury. These findings collectively suggest TP4 through conversion to TP4-SO can function to prevent monocytosis and, therefore, excessive granulation tissue formation as an immediate downstream consequence. Functionally, the altered inflammatory infiltrate likely contributes to the reduced fibrosis and scarring, as determined by MRI, and as previously described. However, TP4 is also known to promote a range of reparative functions post MI, which will have a significant impact here; including the maintenance of

Figure 5 | TP4-SO reduces adhesion and increases cell death of human monocytes via increased ROS. Co-cultures of human CD14+ monocytes and CD4+ T cells treated with either TP4 or TP4-SO resulted in significantly decreased levels of IFNγ; n = 4 co-cultures per treatment; error bars are s.e.m. (a). In both single CD14+ monocyte cultures and co-cultures with CD4+ T cells, TP4-SO significantly reduced the adherence of the cells in the presence of IFNγ; n = 3 cultures per treatment; error bars are s.e.m. (b,c); presence of cells in the right FACS quadrant reflects non-adherent monocytes when treated with TP4-SO (c). Vital dye staining (trypan blue) of CD14+ cultures demonstrated TP4-SO significantly increased monocyte cell death, which was further increased by the additive effect of IFNγ; n = 3 cultures per treatment; error bars are s.e.m. (d). In isolated murine peritoneal macrophages, TP4-SO resulted in a significant fold increase in ROS; n = 3 cultures per treatment; error bars are s.e.m. (e), which was reflected by increased dihydroethidium fluorescence (f). Statistics: Wilcoxon matched-pairs signed rank test (a) and Student’s t-test (b,d,e); *P ≤ 0.05.
cardiomyocyte survival via Akt signalling, the induction of neo-vascularisation and coronary angiogenesis, and reactivation of adult epicardial cells to contribute to myocardial regeneration. Thus, the anti-inflammatory role described for Tβ4-SO is one of a number of contributory factors reflecting the pleiotropy of Tβ4 in the context of cardiac wound healing and repair. Mechanistically, we observed no effect of either Tβ4/Tβ4-SO on monocyte activation or differentiation, as determined by flow sorting against a signature set of markers (CD16, CD40, HLA-DR, CD54, CD86, CD163, CD206). In human co-cultures the clearance and dispersal was dependent upon a combination of modulating IFN-γ-induced cell adhesion, favouring increased human CD14+ monocyte migration, and cell death supported by increased vital dye staining and elevated ROS-mediated apoptosis in murine splenic monocytes. Inflammatory cells are in fact known to release ROS to kill locally invading microbes to prevent infection of wounds; here we introduce the concept of co-opting ROS killing to feedback onto the resolution of the immune cells themselves.

In summary, post-infarction immune cells are required for the transition into a reparative phase, however, their persistence can lead to chronic inflammation, characterized by excessive granulation tissue/scar, attenuated repair and long-term pathological remodelling of the injured heart (reviewed in Frangogiannis et al. and Aoki et al.). Consequently, timely resolution of the inflammatory infiltrate is essential for optimal infarct healing. Our data are consistent with the Tβ4-SO, formed as a by-product of H2O2 production from the wound site, acting to resolve immune cells and modulate cardiac fibrosis and scarring.

Methods

MS analysis of Tβ4-SO. At 72 h.p., zebrafish were pre-treated for 30 min with either 10 nM DPI or DMSO followed by resection of the caudal fin. The fish were recovered for 1 h followed by removal of a small section of tissue immediately proximal to the wound. The tissue was reconstituted in 20 μl of 100 mM Tris, pH 7.8, containing 8 M urea, vortexed for 1 h, and 1.5 μl of 0.2 M dithioerythritol in 100 mM Tris, pH 7.8 was added, vortexed again and the sample incubated at room temperature for 1 h. A volume of 3 μl of 0.2 M iodoacetamide in 100 mM Tris, pH 7.8 was added, vortexed and incubated for 30 min at room temperature. Finally, 165 μl of H2O containing 1 μg of proteomics grade trypsin (Sigma) was added and incubated for ≥16 h at 37°C. Separation of samples and standards was performed using a QuanTof™ performance liquid chromatography (HPLC; Waters, UK). The HPLC protocol: isocratic gradient of 95% dH2O: 5% acetonitrile over a 55-min period; flow rate 0.5 ml min⁻¹, diverted to waste for the first 1.5 min after sample injection, to minimize accumulation of endogenous compounds on the ionization source. The HPLC was coupled to a triple quadrupole tandem mass spectrometer (MicroMass Quattro; Waters) operating in negative-ion mode using the following conditions: C, cone voltage 30 V, cone gas flow rate 90 l h⁻¹, C₁₂, desolvation gas flow rate 600 l h⁻¹, desolvation temperature 350°C, cone voltage 30 V, cone gas flow rate 900 l h⁻¹ and desolvation gas flow rate 900 l h⁻¹. The MRM mode was used for quantitation and data were acquired and processed using MassLynx software (Waters).

In vivo analysis of leukocyte recruitment in zebrafish. Zebrafish were housed and maintained in environmentally controlled aquaria. All surgical and pharmacological procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 (ASP; Home Office, UK). Wild-type, Tg(mpo:GFP), Tg(LysCsRed) or Tg(mpg72GFP) zebrafish larvae (72 h.p.) were anesthetized in MS-222 and placed in 0.3% methyl cellulose in 0.3% Danieau’s solution on a glass slide. Heparin-Ceramic Hydrogel Composite beads (Sigma-Aldrich) were rinsed for 5–10 min in 0.3% Danieau’s solution five times and pre-warmed at 38°C. Heparin-Ceramic Hydrogel Composite beads (Sigma-Aldrich, A9414) and wound a nitrogen ablation laser (Spectra-Physics) attached to a Zeiss Axioplan 2 wide-field imaging system. For imaging of H2O2 production, 72 h.p. Tg(mpg72GFP) larvae were incubated in Danieu’s medium containing 200 mM DMO (Enzo Life Sciences) for 5 h, wounded and then killed at 30 m.p.i. Larvae were fixed in 4% paraformaldehyde overnight at 4°C and subjected to whole-mount immunostaining using rabbit anti-L-plastin (1:500), rabbit anti-DNAP (1:200; ALX-210-530-R050, Enzo Life Sciences) or chicken anti-GFP antibodies (1:200; Invitrogen, A10262). Secondary antibodies: Alexa Fluor 546 or 647 goat anti-rabbit and Alexa Fluor 647 goat anti-chicken (all 1:200; Invitrogen, A11035, A21245, A21449, respectively). Images were acquired on a Leica SP5 confocal microscope. For live imaging of LysCsRed+ positive leukocyte recruitment to a wound, fish were anesthetized and a bead inserted as described above. Fish were then embedded in low-gelling point agarose and imaged using the Leica confocal system. Immune cell numbers within a 500-μm radius of the wound site were counted manually using Fiji software.

Myocardial infarction. Mice were housed and maintained in a controlled environment. All surgical and pharmacological procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 (ASP; Home Office, UK). Myocardial infarction was induced in isoflurane-anesthetized mice by permanent ligation of the left anterior descending artery. On recovery, animals received interperitoneal (i.p.) injection of either Tβ4 (6 mg kg⁻¹), Tβ4-SO (6 mg kg⁻¹) or vehicle (PBS). Further injections were given every second day unless otherwise stated. Hearts were harvested at 2, 4 and 7 days after ligation and either prepared for flow cytometry or immunofluorescence.

Immunofluorescent quantification of cells in MI hearts. Immunofluorescence was performed on hearts post MI. Hearts were first fixed in 4% paraformaldehyde, mounted in OCT and serially sectioned. Representative sections from the scar region were then stained with the following antibodies: anti-F4/80, anti-MPO (Abcam). Images were acquired using a Zeiss Axiosmager and cells were counted using the ‘cell counter’ plugin in ImageJ; at least four fields of view per section were quantified from ≥3 sections per heart.

Western blotting. Post-MI hearts were lysed in Lammi buffer and proteins resolved on a 2D SDS–PAGE gel. Western blotting was performed using standard methods with antibodies against TNF-α, IL-6, IL-10 (Abcam) and GAPDH (Chemicon). Scanning densitometry was performed and signal quantified using ImageJ.

Flow cytometry. To measure murine macrophages post MI, hearts isolated post MI were digested into a single cell suspension using 0.1 M collagenase type II (Worthington Biochemical) and cells were stained using a standard protocol with the following antibodies (all from BD Biosciences unless stated otherwise): anti-CD90-APC-Cy7, 53-2.1, anti-B220-PE, RA3-62B, anti-CD49b-PE, DX5, anti-NK1.1-PE, PK136, anti-Ly-6C-PE, I-Ag4, anti-Ly-6G-APC, I-MJ70, anti-F4/80-biotin, C57/6. (4D1). Anti-CD11c-biotin, I-L202, anti-ly-6G-FTC, AL-21, Strep-PE-Cy7 was used to label biotinylated antibodies. Monocytes were identified as CD11b⁺ CD90⁺/B220⁻/CD49b⁻/NK1.1⁻/Ly-6G⁻ (F4/80⁻/I-A⁻/CD11c⁻) Ly-6C⁺/Ly-6G⁻. Macrophages/dendritic cells were identified as CD11b⁺ CD90/220⁻/CD49b⁻/NK1.1⁻/Ly-6G⁻ (F4/80⁻/I-A⁻/CD11c⁺) Ly-6C⁺/Ly-6G⁻. The sub-population percent aggregation was expressed relative to the total number of cells detected by the cytometer unless otherwise stated.

To measure murine peritoneal macrophages, cells were incubated with IFN-γ (10 ng ml⁻¹) and Tβ4-SO (100 ng ml⁻¹) for 16 h and then isolated on ice for 30 min, followed by centrifugation. Cells were then washed once in PBS and stained using a standard protocol for flow cytometry with the following antibodies: anti-Ly-6G-PE, I-Ag4 (BD Biosciences), anti-CD11b-APC, I-MJ70 (BD Biosciences), DAPI (Sigma), dihydroythidium (1 μm).

For FACS experiments with human cells, cells were isolated as above, washed once in PBS, fixed using 2% paraformaldehyde and stained for flow cytometry with the following antibodies: anti-CD4 PerCP-Cy5.5 (BD Biosciences), anti-CD14-APC (Serotec), anti-CD163-FTC (Santa Cruz Biotechnology), anti-CD206-PE (Immuneotech), anti-CD25-PE (Millenyi Biotech), anti-CD28-FTC (Serotec), anti-CD69-FTC (Serotec) and anti-OX40-PE (Biolegend). Anti-CD16 AF488 (Biolegend), anti-CD14-APC-cy7 (Biolgend), anti-CD40 PE (Serotech), anti-HLA-DR-PE-Cy7 (BD), anti-CD86 Pacific Blue (Biolegend) and anti-CD54 AF647 (Biolegend).

Mure cells and human samples were acquired on a BD LSRII equipped with four lasers (350, 405, 488 and 633 nm) and BD Canto II analyser, respectively, using FACSDiva and FlowJo software.

MRI analysis. Wild-type C57BL/6J mice treated with Tβ4 or vehicle, were subjected to MRI 7 days after left anterior descending artery ligation. In cases where infarct size was within the range of 15–40%, longitudinal studies were performed at 14 and 28 days post MI. Mice were anaesthetized with isoflurane (4%) and maintained at 37 ± 0.5°C with oxygen and anaesthetics (1–2% isoflurane), supplied via a nose cone (11 min⁻¹). Cardiorespiratory monitoring and gating were performed using an MR-compatible system (SA Instruments) with needle electrodes placed on the anterior chest wall and a respiratory pillow placed on the chest. Imaging was performed using a 9.4T VNRMS horizontal bore scanner (Varian) with a shielded gradient system (1,000 mT m⁻¹) using a 39-mm diameter volume...
Mouse peritoneal macrophage isolation and assessment of ROS. Adult wild-type C57BL/6 mice were humanely euthanized in accordance with AUP (AUP) A 1986, injected i.p. with 5 ml at 4 °C PBS and then agitated to release macrophages from the peritoneal cavity. The PBS containing the cells was re-isolated and the cells centrifuged at 600 g for 10 min and washed once with PBS. Cells were then resuspended in RPMI medium 1640 supplemented with 1% penicillin/streptomycin, 1% glucose, and 10% heat-inactivated FCS and allowed to adhere to the surface of a 24-well plate for 3 h. Non-adherent (non-macrophage) cells were removed from the well with vigorous washing and macrophages were stimulated with IFN-γ (100 ng ml⁻¹) for 4 h at 37 °C.

Peripheral blood cell isolation. Ethical approval for the use of peripheral blood from healthy volunteers was obtained from the Bromley Research Ethics Committee and all subjects provided informed consent. Peripheral blood mononuclear cells were isolated by density-gradient centrifugation (lymphocyte separation media; PAA, Pasching, Austria). Purification of cell subsets was performed by magnetic particle isolation using anti-CD14 microbeads and T cells were isolated by positive selection using anti-CD14 microbeads and T cells were isolated by positive selection using anti-CD14 microbeads (Miltenyi Biotec).

Human cell culture. Peripheral blood was collected from healthy volunteers, following written informed consent. Ethics approval for this study was obtained from the Bromley Research Ethics Committee and all subjects provided informed consent. Peripheral blood mononuclear cells were isolated by density-gradient centrifugation (lymphocyte separation media; PAA, Pasching, Austria). Purification of cell subsets was performed by magnetic particle isolation using anti-CD14 microbeads and T cells were isolated by positive selection using anti-CD14 microbeads (Miltenyi Biotec).

Quantification of adherence and death of human monocytes. Following 3 days in culture, non-adherent and adherent cells were removed separately taking first the media for non-adherent cells and then adding ice-cold PBS before detaching the adherent cells incubated on ice. The number of non-adherent and dead monocytes was then quantified using a trypan blue exclusion assay.

ELISA. Supernatants were collected and analysed for IFN-γ (R&D Systems) and analysed as per the manufacturer’s instructions.

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M.F.L. performed MRI to assess scarring and cardiac function in Tβ4-treated mouse hearts. P.R.R. devised the initial hypothesis, supervised experiments and collaborations and wrote manuscript.

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