

Published in final edited form as:

Nat Commun. 2013 ; 4: 2081. doi:10.1038/ncomms3081.

Thymosin β 4-sulfoxide attenuates inflammatory cell infiltration and promotes cardiac wound healing

Mark A. Evans¹, Nicola Smart¹, Karina N. Dubé¹, Sveva Bollini¹, James E. Clark², Hayley G. Evans³, Leonie S. Taams³, Rebecca Richardson⁴, Mathieu Lévesque⁴, Paul Martin⁴, Kevin Mills⁵, Johannes Riegler^{6,7}, Anthony N. Price⁸, Mark F. Lythgoe⁶, and Paul R. Riley^{1,*}

¹Department of Physiology, Anatomy and Genetics, Sherrington Building, University of Oxford, South Parks Road, Oxford, OX1 3PT, UK

²Cardiovascular Division, Kings College London, Rayne Institute, St Thomas' Hospital, London, SE1 7EH, UK

³Centre for Molecular and Cellular Biology of Inflammation (CMCBI), Division of Immunology, Infection and Inflammatory Disease, King's College London, London SE1 1UL, UK

⁴Department of Biochemistry, Physiology and Pharmacology, University of Bristol, School of Medical Sciences, University Walk, Bristol, BS8 1TD, UK.

⁵UCL-institute of Child Health, London, WC1N 1EH, UK

⁶Centre for Advanced Biomedical Imaging (CABI), Department of Medicine and Institute of Child Health, University College London (UCL), London, WC1E 6DD, UK

⁷Centre for Mathematics and Physics in the Life Sciences and Experimental Biology (CoMPLEX), UCL, London, WC1E 6BT, UK

⁸MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College London, London, W12 0NN, UK

Abstract

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 (H_2O_2) 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 β 4-Sulfoxide inhibits interferon- γ , and increases monocyte dispersal and cell death, lies downstream of H_2O_2 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, T β 4-SO inhibits IFN- γ and increases monocyte dispersal and cell death, likely by stimulating superoxide production. Thus, T β 4-SO is a

*To whom correspondence should be addressed: Department of Physiology, Anatomy and Genetics, Sherrington Building, University of Oxford, South Parks Road, Oxford, OX1 3PT Tel. +44 (0) 1865 282366. Fax. +44 (0) 1865 272469. paul.riley@dpag.ox.ac.uk.

AUTHOR CONTRIBUTIONS MAE contributed experiments for MS/MS post-zebrafish tail wounding, mouse MI and ROS assays on spleen monocytes and analysed data. NS contributed data on mouse leukocyte infiltration after MI; KD, SB and JEC performed MI surgeries; HE and LST contributed human monocyte culture data; RR (heart), ML (tail/flank) and PM contributed bead-wounding data and movies in zebrafish; KM performed MS/MS analyses to detect T β 4-SO in wounded zebrafish larvae; JR, ANP and ML performed MRI to assess scarring and cardiac function in T β 4-treated mouse hearts. PRR devised the initial hypothesis, supervised experiments and collaborations and wrote manuscript.

CONFLICTS OF INTEREST The authors declare no disclosures or conflicts of interest.

putative target for therapeutic modulation of the immune response, resolution of fibrosis and cardiac repair.

INTRODUCTION

Cardiac injury activates an innate immune mechanism (reviewed in¹) 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 (H₂O₂)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 unknown³.

Thymosin 4 (T 4) is an actin-binding peptide which exerts both anti-inflammatory and anti-fibrotic effects in the context of tissue wounding; notably following alkali eye injury⁴ and blebomycin-induced damage in the lung⁵. T 4 is one of the first proteins to be up-regulated after wounding⁶ and has been shown to modulate oxidative stress, targeting cardiac fibroblasts and cardiomyocyte survival following myocardial infarction^{7,8}. There are two naturally occurring derivatives of T 4, the tetrapeptide Ac-SDKP, an N-terminal cleavage product known to have anti-fibrotic effects during hypertension⁹ and which can reverse inflammation and fibrosis in rodents post-MI¹⁰ and Thymosin 4-sulfoxide (T 4-SO), formed by oxidation of the methionine-6 residue and thought to be the biologically active, secreted form of the parent peptide. T 4-SO is largely unstudied to-date, but has been shown to inhibit neutrophil chemotaxis *in vitro* and attenuate inflammation in carrageenan-injected mice¹¹. We sought to investigate whether T 4-SO might act as the immune cell dispersal signal during cardiac wound healing to prevent chronic inflammation.

Here we identify T 4-SO as downstream of H₂O₂ acting to disperse inflammatory macrophages and promote healing following tail, body flank or heart injury in zebrafish. This function is conserved in mice, where T 4-SO, via a direct effect on leukocytes at key time points within healing myocardium, contributes to reduced fibrosis and scarring in the injured heart. Extrapolating these findings to humans, we show that T 4-SO blocks the production of pro-inflammatory IFN- to reduce the adhesion of activated monocytes and increases cell death downstream of elevated reactive oxygen species (ROS) production.

RESULTS

Tβ4-SO acts downstream of H₂O₂ in the wounded zebrafish

We initially examined whether mechanical wounding of the zebrafish larval tail fin² would lead to production of T 4-SO *in-vivo*. The zebrafish has five -thymosin isoforms arising from successive rounds of gene duplication¹² one of which has been described as the orthologue of T 4 (Fig. 1a), however, there are no previous reports describing T 4-SO in the zebrafish. Larvae at 72 hours post fertilisation (hpf) were pre-incubated in the presence or absence of the small molecule inhibitor of H₂O₂ diphenyleneiodium (DPI), followed by surgical removal of the tip of the tail fin (Fig. 1b). Levels of T 4-SO increased rapidly, one hour post-wounding, by approximately 5-fold from 2.0% +/- 1.4% SEM to 10.4% +/- 1.96% SEM (n=100 wounded tail fins/treatment, n=3 experimental replicates; Student's t-test, p = 0.01; Fig. 1c). Pre-treatment with DPI resulted in a 3-fold increase (6.4% +/- 0.8% SEM) which was significantly less than that observed in vehicle pre-treated control fish (Student's t-test, p = 0.05; Fig. 1d). To investigate whether ectopic administration of T 4-

SO might directly affect the presence of leukocytes, latex beads pre-adsorbed with synthetic T 4-SO, or vehicle control (PBS), were implanted into *Tg[mpo:GFP]* zebrafish (Fig. 1e, f). Bead implantation acted as an injury stimulus resulting in recruitment of L-plastin+ (MPO-) macrophages¹³ in the control setting (Fig. 1g). Imaging of fish at 3 and 6 hours, thereafter, indicated a significantly reduced number of macrophages proximal to the T 4-SO bead relative to controls (3 hrs: co: 20 +/- 4.65 SEM versus T 4-SO: 8.25 +/- 2.39 SEM and 6 hrs: co: 17.25 +/- 4.05 SEM versus T 4-SO: 8.66 +/- 2.03 SEM (n= mean cell count from 5 bead implanted fish per treatment; Student's t-test, p = 0.01; Fig. 1h, i), suggesting ectopic T 4-SO 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 in¹⁴), suggesting T 4-SO was able to alter macrophage incidence despite the presence of neutrophils. To address the issue of whether T 4-SO affected leukocyte recruitment versus dispersal, we generated time lapse movies of the inflammatory cell response in *Tg[LysC:dsRed]*¹⁵ larval zebrafish up to 4 hours following bead implantation. Whilst similar numbers of LysC+ leukocytes were recruited to the bead wound in the control and T 4-SO treated setting at 1 and 2 hours post-injury, those that localised to a PBS bead were retained beyond 3 hours post-injury, whereas those that encountered a T 4-SO-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 hours resulted in the release of H₂O₂ 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 T 4-SO or PBS control via microinjection or bathing of the larvae in T 4-SO-containing medium, resulted in significantly reduced incidence of infiltrating leukocytes (L-plastin+) at both 3 and 6 hours post-injury (3 hrs: co: 30.4 +/- 2.96 SEM versus T 4-SO: 22.5 +/- 1.50 SEM; Student's t-test, p = 0.05 and 6 hrs: co: 35.3 +/- 3.19 SEM versus T 4-SO: 18.2 +/- 1.90 SEM; Student's t-test, p = 0.01; n= mean cell count from 4 injured fish per treatment; Fig. 2b-f).

Tβ4 and Tβ4-SO reduce inflammatory cell infiltration after MI

To investigate the effect of T 4-SO on leukocyte infiltration during wounding in mammals we utilised the mouse MI model. It is known that two distinct phases of monocytes participate post-MI in the mouse: inflammatory monocytes (Ly-6C^{hi}) 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-6^{lo}) dominate later (from days 4 -7) during the wound healing phase II¹⁶. Hence, we focused on these key time-points and on the potential for the parent peptide T 4 and derivative T 4-SO to augment the transition from phase I to II.

We initially treated animals with T 4 immediately post-MI and compared against both vehicle-treated controls and T 4 global knock-out (equivalent to loss of T 4-SO 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. T 4 treatment attenuated this increase with fewer MPO+ cells present (co: 70 + 14.5 S.E.M versus T 4: 30 + 19.1 SEM, p = 0.15) at the site of injury at both stages (Fig. 3a-d). Global knockout of T 4 significantly increased MPO+ cell numbers at the earliest stages of infiltration (day 2; wild type: 20.5 +/- 4.6 SEM versus T 4 KO: 47.7 +/- 5.0 SEM; 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 cascade¹⁷. TNF- α was

significantly reduced (6.2-fold reduction) after 2 days of T 4 treatment post-MI as compared to 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 a role in mitigating inflammatory injury during the early stages post-infarct, without interfering with subsequent myocardial healing, T 4 stimulated the expression of the inhibitory cytokine IL-10 at 2 days post-MI (8.1-fold increase compared to 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 T 4 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 SEM versus T 4: 3 +/- 1.4 SEM; Student's t-test, p = 0.05; Fig. 3m). In T 4 knockout mice we observed the opposite effect, such that F4/80+ cells increased continuously to around 5-fold that of control mice by day 7 post-MI (21 +/- 3.4 SEM versus 135.8 +/- 12.4 SEM; Student's t-test, p = 0.001; Fig. 3k, l, m). The observation that T 4 efficiently cleared the macrophage-rich infiltrate by day 7 post-MI was consistent with the zebrafish data for T 4-SO (Fig. 1h, i).

We next investigated the anti-inflammatory activity of T 4-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% SEM versus T 4-SO: 32.0% +/- 2% SEM; n=4; Student's t-test, p = 0.05; Fig 4a), which represented an increase in the proportion of macrophages, since the total number of monocyte (CD11b^{hi}, [Ly6G, B220, CD49b, CD90.2, NK1.1]^{Low}) cells remained unaltered (Fig. 4b). Conversely, 4 days post-injury the total number of monocytes was significantly reduced compared to controls (co: 5.9% +/- 0.95% SEM versus T 4-SO: 2.7% +/- 0.4% SEM; n=4; Student's t-test, p = 0.05; Fig. 4c). T 4-SO treatment of infarct suspensions did not, however, alter the relative proportions of monocyte Ly-6^{lo} versus Ly-6^{hi} 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 T 4/T 4-SO. During the so-called debris-removal phase I (day 2) T 4/T 4-SO increased macrophage numbers by directing transformation of the stable monocyte pool and subsequently enhanced the transition to the anti-inflammatory phase II (day 4) by signalling monocyte/macrophage clearance from the injury site.

A T β 4/T β 4-SO-depleted monocyte infiltrate reduces scarring

The functional consequences of the T 4/T 4-SO depleted monocyte infiltrate were examined by serial magnetic resonance imaging (MRI) of mice treated with T 4, to reflect sulfoxide activity, for 7, 14 and 28 days post-MI. T 4-SO was significantly increased in hearts following the addition of T 4 across the time course of the study: percentage oxidised T 4 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 ANOVA; p = 0.001). Increase in infarct volume (late gadolinium enhancement; LGE) was significantly higher in PBS compared to T 4-treated mice; persisting up to day 28 post-MI (co: 43.3 +/- 4.9 μ l; n=5 versus T 4: 22.0 +/- 5.3 μ l; 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 T 4: 18 +/- 4%; n=4; Student's t-test, p = 0.05; Fig. 4d) indicating that T 4/T 4-SO reduced infarct size and scarring. The reduced fibrosis was confirmed in the free ventricular wall following T 4 treatment (Fig. 4e, f) which, in turn, directly correlated with improved cardiac function as indicated by elevated ejection fraction (EF; Fig. 4g).

T β 4-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 T 4-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 (PBMC) of healthy individuals and initially excluded any effect of T 4 or T 4-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 T 4-SO lead to a significant reduction in the release of the pro-inflammatory cytokine IFN- γ , relative to controls (co: 1830 pg/ml +/- 365.3 pg/ml SEM versus T 4-SO: 1271.5 pg/ml +/- 483.1 SEM, 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 in¹⁹). CD14+ cells treated with T 4-SO were significantly less adherent than their control counterparts, even in the presence of recombinant IFN- γ (co: 219167 +/- 25834 SEM versus T 4-SO: 89167 +/- 13411 SEM; n=3; Student's t-test, p 0.05; Fig. 5b, c). We next assessed whether effects on CD14+ cell adhesion/dispersal might be accompanied by elevated cell death and observed T 4-SO significantly increased the number of trypan blue positive cells compared to IFN- γ and control treated cultures (T 4-SO: 135833 +/- 22928 SEM versus IFN- γ : 55833 +/- 2203 SEM versus co: 33333 +/- 833 SEM; n=3; p 0.05; Fig 5d). To determine a potential mechanism, we investigated whether T 4-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 T 4-SO followed by dihydroethidium (DE) a fluorescent indicator of ROS²². Activating IFN- γ treatment alone resulted in increased ROS, which was significantly augmented by the addition of T 4-SO (n=3; Student's t-test, p 0.05; Fig. 5e, f); consistent with T 4-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 T 4, 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 oxidised derivative, T 4-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. T 4-SO is formed by methionine oxidation of T 4 in the presence of H₂O₂ which alongside the observed up-regulation of T 4 at wound sites^{6,23} established the sulfoxide variant as a potential candidate for immune cell dispersal. We initially generated a simple zebrafish tail fin resection model² and revealed by QTOF-MS that T 4-SO is significantly up-regulated 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, T 4-SO may function as the elusive dispersal signal to prevent chronic inflammation and promote wound healing. Extrapolating to mouse MI, both the parent molecule T 4 and sulfoxide derivative were necessary and sufficient to ensure an elevated presence of macrophages, within the stable (Ly-6C^{hi}) 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 injury¹⁶. These findings collectively suggest T 4 through conversion to T 4-SO can function to prevent monocytoysis and, therefore, excessive granulation tissue formation as an immediate downstream consequence¹⁶. Functionally, the altered inflammatory infiltrate likely contributes to the reduced fibrosis and scarring, as determined by MRI, and as previously described¹⁶. However, T 4 is also known to promote a range of reparative functions post-MI which will have a significant impact here; including the maintenance of cardiomyocyte survival via Akt signalling²⁴, the induction of neo-vascularisation and coronary angiogenesis^{25,26} and reactivation of adult epicardial cells to contribute to myocardial regeneration²⁷. Thus, the anti-inflammatory role described for T 4-SO is one of a number of contributory factors reflecting the pleiotropy of T 4 in the context of cardiac wound healing and repair. Mechanistically, we observed no effect of either T 4/T 4-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- 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 wounds²⁸; 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, characterised by excessive granulation tissue/scar, attenuated repair and long term pathological remodelling of the injured heart (reviewed in^{29,30}). Consequently, timely resolution of the inflammatory infiltrate is essential for optimal infarct healing. Our data are consistent with the T 4-SO, formed as a bi-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

72hpf zebrafish were pre-treated for 30 minutes with either 10μM DPI or DMSO followed by resection of the caudal fin. The fish were recovered for 1 hour followed by removal of a small section of tissue immediately proximal to the wound. The tissue was reconstituted in 20μl 100mM Tris, pH 7.8, containing 8M Urea, vortexed for 1 hour, and 1.5μl 0.2M Dithioerythritol (DTE) in 100mM Tris, pH 7.8 was added, vortexed again and the sample incubated at room temperature for 1 hour. 3μl of 0.2M Iodoacetamide in 100mM Tris, pH 7.8 was added, vortexed and incubated for 30minutes at room temperature. Finally, 165μl of H₂O containing 1μg of proteomics grade trypsin (Sigma) was added and incubated for 16h at 37°C. Separation of samples and standards was performed using a 2795XE high 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, diverted to waste for the first 1.5 min after sample injection, to minimise accumulation of endogenous compounds on the ionisation source. The HPLC was coupled to a triple quadrupole tandem mass spectrometer (MicroMass Quattro; Waters, UK) operating in negative-ion mode using the following settings: capillary 3.2 kV, source temperature 150 °C, desolvation temperature 350 °C, cone voltage 30 V, cone gas flow rate 90 l/h and desolvation gas flow rate 900 l/h. The MRM mode was used for quantification and data were acquired and processed using MassLynx software (Waters, UK).

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 (A(SP)A; Home Office, UK). Wild-type, Tg(mpo:GFP), Tg[LysC:dsRed] or Tg(myl7:GFP) zebrafish larvae (72 hpf) were anaesthetised in MS-222 and placed in 3% methyl cellulose in 0.3% Danieau's solution on a glass slide. Heparin-Ceramic HyperD M Hydrogel Composite beads (Sigma-Aldrich, H0532) were rinsed for 5-10 minutes in 0.3% Danieau's solution five times and pre-soaked in 100 µg/ml T 4-SO or PBS for 30 min+ before being inserted through the skin and muscle of the flank region using a fine tungsten needle (World Precision Instruments).

Heart injury was either via microinjection delivery of T 4-SO (3 nl of 100 µg/ml) into the heart and pericardial space or implanting of a bead into this region, as described above. Control fish were injected with PBS vehicle or beads soaked in PBS vehicle. For laser injury, fish were pre-treated with 100 µg/ml T 4-SO or PBS in Danieau's solution for at least 2 hours, embedded in low-gelling point Agarose (Sigma-Aldrich, A9414) and wounded using a nitrogen ablation laser (Spectra-Physics) attached to a Zeiss AxioPlan 2 widefield imaging system. For imaging of H₂O₂ production, 72 hpf Tg(myl7:GFP) larvae were incubated in Danieau's medium containing 200 mM DMPO (Enzo Life Sciences) for five hours, wounded and then sacrificed at 30 mpi. Larvae were fixed in 4% paraformaldehyde overnight at 4 °C and subject to whole-mount immunostaining using rabbit anti-L-plastin (1:500), rabbit anti-DMPO (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 taken using a Leica SP5 confocal imaging system attached to a Leica DMI 6000 inverted microscope. For live imaging of LysC:dsRED+ positive leukocyte recruitment to a wound, fish were anaesthetised 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 (A(SP)A; Home Office, UK). Myocardial infarction was induced in isoflurane-anaesthetized mice by permanent ligation of the left anterior descending artery (LAD). On recovery, animals received interperitoneal (i.p.) injection of either T 4 (6mgkg⁻¹), T 4-SO (6mgkg⁻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 AxioImager and cells were counted using the 'cell counter' plugin in Image-J: at least 4 fields of view per section were quantified from 3 sections per heart.

Western blotting

Post-MI hearts were lysed in Lamni 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% 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, RA3-6B2, Anti-CD49b-PE, DX5, Anti-NK1.1-PE, PK136, Anti-Ly-6G-PE, 1A8, Anti-CD11b-APC, M1/70, Anti-F4/80-biotin, C1:A3-1 (AbD Serotec), Anti-CD11c-biotin, HL3, Anti-I-A^b-biotin, AF6-120.1, Anti-Ly-6C-FITC, AL-21, Strep-PE-Cy7 was used to label biotinylated antibodies. Monocytes were identified as CD11b^{hi} (CD90/B220/CD49b/NK1.1/Ly-6G)^{lo} (F4/80/I-A^b/CD11c)^{lo} Ly-6C^{hi/lo}. Macrophages/dendritic cells were identified as CD11b^{hi} (CD90.2/B220/CD49b/NK1.1/Ly-6G)^{lo} (F4/80/I-A^b/CD11c)^{hi} Ly-6C^{lo}. The sub-population percentage 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 (10ng/ml) and T 4-SO (100ng/ml) for 16h and then isolated on ice for 30 minutes, followed by centrifugation. Cells were then washed once in PBS and stained using a standard protocol for flow cytometry with the following: Anti-Ly-6G-PE, 1A8 (BD Biosciences), Anti-CD11b-APC, M1/70 (BD Biosciences), DAPI (Sigma), dihydroethidium (DE) (1 μ m). 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 Bioscience), Anti-CD14-APC (Serotec), Anti-CD163-FITC (Santa Cruz), Anti-CD206-PE (Immunotec), Anti-CD25-PE (Miltanyi Biotech), Anti-CD28-FITC (Serotec), Anti-CD69-FITC (Serotec) and Anti-Ox40-PE (Biolegend). Anti-CD16 AF488 (Biolegend), Anti-CD14 APC-cy7 (Biolegend), Anti-CD40 PE (Serotech), Anti-HLA-DR PerCP-cy5.5 (BD), Anti-CD86 Pacific Blue (Biolegend), Anti-CD54 AF647 (Biolegend). Murine and human samples were acquired on a BD LSRII equipped with 4 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 T 4 or vehicle, were subjected to MRI 7 days after LAD ligation. 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 \pm 0.5 $^{\circ}$ C with oxygen and anaesthetics (1–2% isoflurane), supplied via a nose cone (1lmin⁻¹). Cardiorespiratory monitoring and gating were performed using an MR-compatible system (SA Instruments) with needle electrodes inserted into the front limbs and a respiratory pillow placed on the chest. Imaging was performed using a 9.4T VNMRS horizontal bore scanner (Varian) with a shielded gradient system (1,000mTm⁻¹) using a 39mm diameter volume coil (Rapid Biomedical GmbH). An electrocardiogram and respiratory gated spoiled gradient echo sequence was used to acquire cine cardiac images with the following parameters for standard cine acquisitions: Time to echo (TE), 1.18ms; time to repetition (TR), 4.5ms; flip angle, 20 $^{\circ}$; slice thickness, 1mm; no slice separation, field of view (FOV), 25.6 \times 25.6mm²; matrix size, 128 \times 128; number of signal averages (NSA), 2. Twenty cine-frames were recorded to cover the cardiac cycle. Infarct size was assessed using late gadolinium enhancement (LGE): 0.6mmolk⁻¹ Gd-DTPA was

administered i.p. followed by a Look-Locker acquisition with multiple time to inversion (TI) to determine the optimum TI, and by a multi-slice inversion recovery (IR) acquisition with flip angle (FA) = 90° using the following imaging parameters: TE, 1.58ms; TR, ~500–600ms; FA, 90°; slice thickness, 0.5mm; 0.5mm slice gap; 7–8 slices; FOV, 25.6×25.6mm²; matrix size, 192×192; NSA, 2. A second stack of short-axis images offset by 0.5mm was acquired to generate a continuous data set. Randomized and anonymized images were analysed using the cardiac analysis software Segment (<http://segment.heiberg.se>). To estimate infarct size, endocardial and epicardial borders were segmented on LGE images automatically with manual adjustments followed by automatic delineation of infarct tissue using a built-in fraction of segment. Infarct size, expressed as percentage of left ventricular mass, was calculated as infarct volume/left ventricular volume (from cine data). Comparisons between groups were performed using a repeated measures one-way ANOVA, using R software²⁵ version 2.8.1.

Mouse peritoneal macrophage isolation and assessment of ROS

Adult wild-type C57BL/6J mice were humanely euthanized in accordance with A(SP)A 1986, injected i.p. with 5ml 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 600g for 10 minutes and washed once with PBS. Cells were then resuspended in RPMI medium 1640 supplemented with 1% penicillin/streptomycin, 1% glutamine, and 10% heat-inactivated FCS and allowed to adhere to the surface of a 24 well plate for 3 hours. Non-adherent (non-macrophage) cells were removed from the well with vigorous washing and macrophages were stimulated with IFN- γ (50ng/ml), PBS supplemented with 100ng/ml T 4-SO or PBS alone.

Peripheral Blood Cell Isolation

Ethical approval for the use of peripheral blood from healthy donors 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 cell sorting (Miltenyi Biotec) and confirmed by flow cytometry. Monocytes (>90% pure) were isolated by positive selection using anti-CD14 microbeads and T-cells also by positive selection using anti-CD4 microbeads (Miltenyi Biotec).

Human Cell Culture

Peripheral blood (PB) was collected from healthy volunteers, following written informed consent. Ethics approval for this study was given by the Bromley Research Ethics Committee (reference 06/Q0705/20). Mononuclear cells were isolated from PB using Ficoll-Hypaque (LSM 1077; PAA Laboratories) density-gradient centrifugation. Bulk CD4⁺ T cells (1×10^6 per ml) were cultured in 24-well plates in the absence or presence of monocytes in RPMI medium 1640 supplemented with 1% penicillin/streptomycin, 1% glutamine, and 10% heat-inactivated FCS (Cambrex, Nottingham, U.K.) and cultures were stimulated with 100 ng/ml anti-CD3 (OKT3; Ortho Biotech, Bridgewater, NJ). In co-culture 0.5×10^6 monocytes were cultured at a 1:1 ratio with CD4⁺ T cells for 3 days in 24-well plates. Where indicated, cells were also treated with T 4 (100ng/ml), T 4-SO (100ng/ml) or PBS.

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 prior to 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was generously supported by a Medical Research Council grant (reference G0701970).

REFERENCES

1. Frangogiannis NG. The immune system and cardiac repair. *Pharmacol.Res.* 2008; 58:88–111. [PubMed: 18620057]
2. Niethammer P, Grabher C, Look AT, Mitchison TJ. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. *Nature.* 2009; 459:996–999. [PubMed: 19494811]
3. Martin P, Feng Y. Inflammation: Wound healing in zebrafish. *Nature.* 2009; 459:921–923. [PubMed: 19536251]
4. Sosne G, et al. Thymosin β 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. *Exp.Eye Res.* 2002; 74:293–299. [PubMed: 11950239]
5. Conte E, et al. Thymosin β 4 protects C57BL/6 mice from bleomycin-induced damage in the lung. *European journal of clinical investigation.* 2013; 43:309–315. [PubMed: 23320620]
6. Malinda KM, et al. Thymosin β 4 accelerates wound healing. *J.Invest Dermatol.* 1999; 113:364–368. [PubMed: 10469335]
7. Kumar S, Gupta S. Thymosin β 4 prevents oxidative stress by targeting antioxidant and anti-apoptotic genes in cardiac fibroblasts. *PloS one.* 2011; 6:e26912. [PubMed: 22046407]
8. Gupta S, et al. Thymosin β 4 and cardiac protection: implication in inflammation and fibrosis. *Annals of the New York Academy of Sciences.* 2012; 1269:84–91. [PubMed: 23045975]
9. Rasoul S, et al. Antifibrotic effect of Ac-SDKP and angiotensin-converting enzyme inhibition in hypertension. *J.Hypertens.* 2004; 22:593–603. [PubMed: 15076166]
10. Yang F, et al. Ac-SDKP reverses inflammation and fibrosis in rats with heart failure after myocardial infarction. *Hypertension.* 2004; 43:229–236. [PubMed: 14691195]
11. Young JD, et al. Thymosin β 4 sulfoxide is an anti-inflammatory agent generated by monocytes in the presence of glucocorticoids. *Nat.Med.* 1999; 5:1424–1427. [PubMed: 10581087]
12. Edwards J. Vertebrate β -thymosins: conserved synteny reveals the relationship between those of bony fish and of land vertebrates. *FEBS Lett.* 2010; 584:1047–1053. [PubMed: 20138884]
13. Feng Y, Santoriello C, Mione M, Hurlstone A, Martin P. Live imaging of innate immune cell sensing of transformed cells in zebrafish larvae: parallels between tumor initiation and wound inflammation. *PLoS biology.* 2010; 8:e1000562. [PubMed: 21179501]
14. Soehnlein O, Lindbom L. Phagocyte partnership during the onset and resolution of inflammation. *Nat.Rev.Immunol.* 2010; 10:427–439. [PubMed: 20498669]
15. Hall C, Flores MV, Storm T, Crosier K, Crosier P. The zebrafish lysozyme C promoter drives myeloid-specific expression in transgenic fish. *BMC developmental biology.* 2007; 7:42. doi: 10.1186/1471-213X-7-42. [PubMed: 17477879]
16. Nahrendorf M, et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J.Exp.Med.* 2007; 204:3037–3047. [PubMed: 18025128]

17. Frangogiannis NG, et al. Resident cardiac mast cells degranulate and release preformed TNF- α , initiating the cytokine cascade in experimental canine myocardial ischemia/reperfusion. *Circulation*. 1998; 98:699–710. [PubMed: 9715863]
18. Taams LS, et al. Modulation of monocyte/macrophage function by human CD4+CD25+ regulatory T cells. *Hum.Immunol*. 2005; 66:222–230. [PubMed: 15784460]
19. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *J.Leukoc.Biol*. 2004; 75:163–189. [PubMed: 14525967]
20. Simon HU, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis*. 2000; 5:415–418. [PubMed: 11256882]
21. Yermolaieva O, et al. Methionine sulfoxide reductase A protects neuronal cells against brief hypoxia/reoxygenation. *Proc.Natl.Acad.Sci.U.S.A*. 2004; 101:1159–1164. [PubMed: 14745014]
22. Rothe G, Valet G. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2,7 -dichlorofluorescein. *J.Leukoc.Biol*. 1990; 47:440–448. [PubMed: 2159514]
23. Smart N, et al. Thymosin β 4 induces adult epicardial progenitor mobilization and neovascularization. *Nature*. 2007; 445:177–182. [PubMed: 17108969]
24. Bock-Marquette I, Saxena A, White MD, Dimaio JM, Srivastava D. Thymosin β 4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. *Nature*. 2004; 432:466–472. [PubMed: 15565145]
25. Bock-Marquette I, et al. Thymosin β 4 mediated PKC activation is essential to initiate the embryonic coronary developmental program and epicardial progenitor cell activation in adult mice in vivo. *J.Mol.Cell Cardiol*. 2009; 46:728–738. [PubMed: 19358334]
26. Smart N, et al. Thymosin β 4 facilitates epicardial neovascularization of the injured adult heart. *Ann.N.Y.Acad.Sci*. 2010; 1194:97–104. [PubMed: 20536455]
27. Smart N, et al. De novo cardiomyocytes from within the activated adult heart after injury. *Nature*. 2011; 474:640–644. [PubMed: 21654746]
28. Schafer M, Werner S. Oxidative stress in normal and impaired wound repair. *Pharmacological research : the official journal of the Italian Pharmacological Society*. 2008; 58:165–171. [PubMed: 18617006]
29. Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. *Cardiovasc.Res*. 2002; 53:31–47. [PubMed: 11744011]
30. Aoki S, et al. Elevated peripheral blood mononuclear cell count is an independent predictor of left ventricular remodeling in patients with acute myocardial infarction. *J.Cardiol*. 2011; 57:202–207. [PubMed: 21168993]

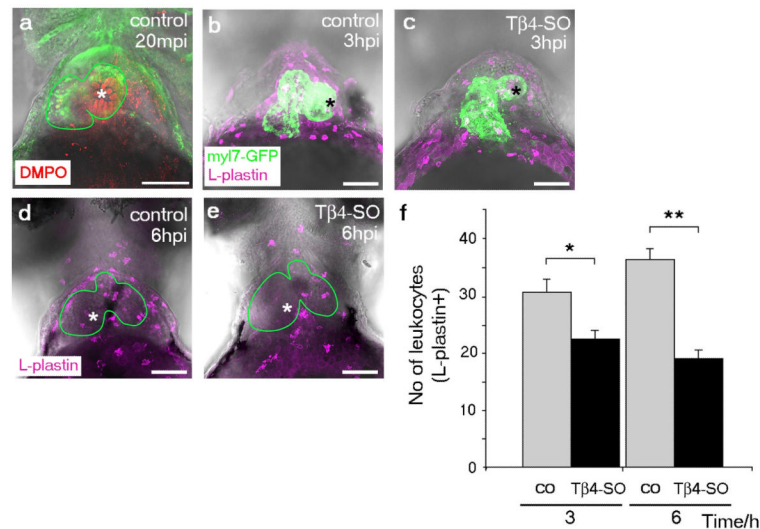


Figure 2. T 4-SO disperses leukocytes after cardiac injury in zebrafish

(a). Laser injury to the heart of a 72 hr zebrafish larvae immunostained for 5,5-Dimethyl-1-Pyrroline-N-Oxide (DMPO; red) revealed localised H_2O_2 production at the wound site (b). At 3 hours post laser heart injury (hpi), a reduced leukocytic response (revealed by L-plastin staining – magenta; c, d) was seen in T 4-SO treated fish within 500 μm of the focal point of injury (c) compared to controls (b). Larger, needle-induced wounds to the heart region of *Tg[myl7:GFP]* transgenic fish, resulted in a stronger inflammatory response in PBS-treated control hearts at 6 hpi (d), which was significantly reduced in T 4-SO treated fish (e). Total numbers of L-plastin+ leukocytes was significantly lower at 3 and 6 hpi for T 4-SO treated fish compared to controls; n=5 fish per treatment, error bars are standard error of the mean (SEM) (f). Asterisks mark wound site and green outlines delineate the heart as determined by myl7-GFP+. Scale bars: a-e, 100 μm . All statistics Student's t-test; * p < 0.05, ** p < 0.01.

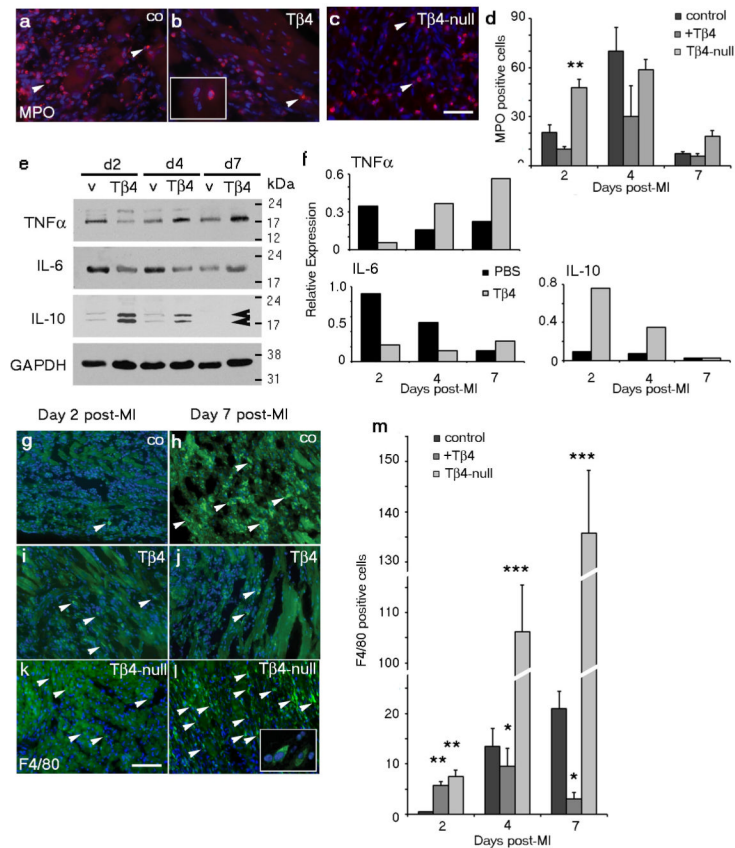


Figure 3. T 4/T 4-SO modulates the inflammatory response post-MI in the mouse heart
 Representative images, showing infiltration of the injured myocardium with neutrophils (white arrowheads highlight individual MPO+ cells; **a-c**) and macrophages (white arrowheads highlight individual F4/80 cells; **g-l**) in T 4-treated, control vehicle-treated and T 4-null myocardial infarction (MI) hearts. Significantly fewer cytotoxic myeloperoxidase (MPO)-peroxidase+ neutrophils were present in T 4-treated, compared with controls and T 4-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 T 4-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 T 4 as compared to vehicle controls (**e**) and quantification of protein expression by scanning densitometry (**f**). Black arrowheads indicate alternate glycosylated isoforms (21kDa and 24kDa) of IL-10. The upstream initiator of the acute inflammatory cascade, TNF- α , is down-regulated at early stages post-MI (2 days) following T 4 treatment along with the downstream inflammatory cytokine, IL-6; levels of both TNF and IL-6 subsequently become elevated after 7 days consistent with an early reduction in injury and enhanced repair at later stages (**e, f**). The anti-inflammatory cytokine IL-10 is up-regulated after 2 and 4 days of treatment with T 4 (**e, f**). Representative images, F4/80 positive macrophages in the scar region post-MI, in co (**g, h**), T 4 treated (**i, j**) or T 4 $^{-/-}$ animals (**k, l**); both at day 2 (**g, i, k**) and day 7 (**h, j, l**) post-MI, with cell counts quantified in (**m**). T 4-induced macrophage infiltration peaks at day 4 but is rapidly cleared, to a level significantly reduced relative to vehicle-treated by day 7; in T 4-null hearts the incidence of macrophages at the wound was significantly elevated relative to the controls as highlighted by cell counts (**m**). Scale bar: **c** represents 20 μ m (applies to **a-c**) and **K** represents 20 μ m (applies to **g-l**); v, vehicle; n=4-6 MI hearts analysed per time point; error bars are standard

error of the mean for all bar graphs; all statistics Student's t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

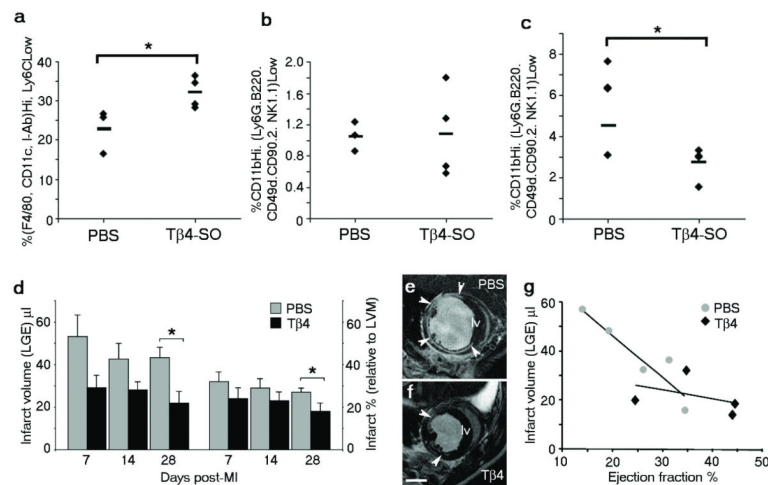


Figure 4. T 4/T 4-SO attenuates inflammatory cell infiltration, scarring and fibrosis

Following treatment with T 4-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**). T 4 treatment resulted in significantly increased T 4-SO levels (refer to main text) and reduced scarring and fibrosis, by 28 days post-MI, according to surrogate measures of infarct volume and % infarct relative to left ventricular mass (LVM); as determined by longitudinal MRI of late gadolinium enhancement (LGE); n=4 hearts per treatment per time point; error bars are standard error of the mean (SEM; **d**). Short axis MRI images of PBS control (**e**) and T 4 (**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 T 4 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 T 4 treated hearts (slope: -0.37; p=0.624) compared to PBS controls (slope: -1.76; p=0.0234) reflected reduced scar formation (**g**). Scale bars: **f** represents 2mm; Statistics repeated measures one-way ANOVA; *p < 0.05.

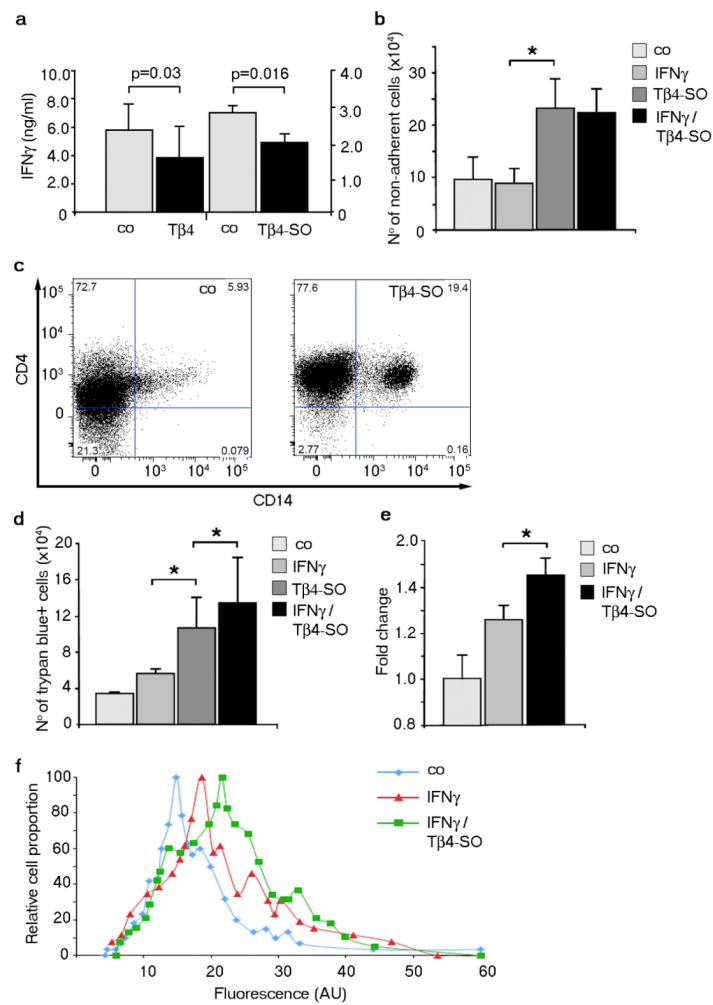


Figure 5. T 4-SO reduces adhesion and increases cell death of human monocytes via increased ROS

Co-cultures of human CD14 $^{+}$ monocytes and CD4 $^{+}$ T-cells treated with either T 4 or T 4-SO resulted in significantly decreased levels of IFN γ ; n=4 co-cultures per treatment; error bars are standard error of the mean (SEM; **a**). In both single CD14 $^{+}$ monocyte cultures and co-cultures with CD4 $^{+}$ T-cells, T 4-SO significantly reduced the adherence of the cells in the presence of IFN γ ; n=3 cultures per treatment; error bars are standard error of the mean (SEM; **b, c**); presence of cells in the right FACS quadrant reflects non-adherent monocytes when treated with T 4-SO (**c**). Vital dye staining (trypan blue) of CD14 $^{+}$ cultures demonstrated T 4-SO significantly increased monocyte cell death, which was further increased by the additive effect of IFN γ ; n=3 cultures per treatment; error bars are standard error of the mean (SEM; **d**). In isolated murine peritoneal macrophages T 4-SO resulted in a significant fold increase in reactive oxygen species (ROS); n=3 cultures per treatment; error bars are standard error of the mean (SEM; **e**) which was reflected by increased dihydroethidium (DE) fluorescence (**f**). Statistics: Wilcoxon matched-pairs signed rank test (**a**) and Student's t-test (**b, d, e**); * p < 0.05.