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Tenascin-C is increased in atherothrombotic stroke patients and has an anti-inflammatory effect in the human carotid artery.

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Abstract

Tenascin-C (Tn-C) is an endogenous ligand of toll-like receptor-4 (TLR-4); a key signalling molecule associated with chronic inflammatory conditions. Both Tn-C and TLR-4 are increased in unstable human atheroma, but their effects on local inflammatory conditions have not been investigated. The aim of the present study was to investigate the association and functional implications of Tn-C/TLR-4 signalling in large artery atherosclerotic stroke.

Plasma Tn-C was measured by ELISA and found to be higher in recent stroke patients (n=336; median 12.77 μg/mL, inter-quartile range 10.23-15.74 μg/mL) than in controls (n=321; median 11.31 μg/mL, inter-quartile range 8.89-13.90 μg/mL), $p<0.001$. Plasma Tn-C was also independently positively associated with stroke (odds ratio for highest Tn-C quartile 2.27, 95% confidence interval 1.37-3.76). Assessment of Tn-C associated chronic cytokine secretion was performed *in vitro* using paired, human, macroscopically disease matched, carotid atheroma tissue biopsies obtained from five patients undergoing carotid endarterectomy. A 4 day incubation with specific Tn-C blocking antibodies (Abs) increased secretion of TLR-4 associated cytokines, interleukin (IL)-8, IL-1β, tumour necrosis factor (TNF)-α and C-C motif chemokine (CCL)3 and expression of TLR-4 in the tissue. These results suggest with Tn-C blockade another endogenous TLR-4 ligand upregulates TLR-4 expression and subsequent cytokine secretion. Titration of the Tn-C Abs also dose dependently increased secretion of IL-6, IL-8, IL-1β and CCL3 in mixed, healthy, primary vascular cell culture. In summary, circulating concentrations of Tn-C are higher in patients with a recent history of atherosclerotic stroke and may play an anti-inflammatory role by reducing pro-inflammatory cytokine release from atheroma.

1. Introduction

Chronic inflammation is believed to play a key role in the complications of atherosclerosis, however the factors controlling these immune mechanisms are poorly understood [1-6]. Toll-like receptor-4 (TLR-4) is a key signalling molecule in the immune response to self and infection and has been implicated in atherosclerosis through its ability to promote inflammation, proteolysis and thrombosis [7-16].

Tenascin-C (Tn-C) is a matricellular protein which binds to TLR-4 through its fibrinogen-like domain (FBG) [17]. The FBG domain of Tn-C also acts as a binding site for aortic smooth muscle cells [18] and fibroblasts [19,20]. The expression of both Tn-C and TLR-4 are increased in unstable human atheroma [8,9,21,22]. The effect of Tn-C on inflammation within human atheroma has not been previously investigated [23]. However, in patients with rheumatoid arthritis (RA), studies suggest that Tn-C modulates TLR-4 activity and could therefore influence release of cytokines, such as interleukin (IL)-6, IL-8, and tumour necrosis factor (TNF)-α, from inflammatory cells infiltrating the atheroma [17].

Tn-C is present within the human circulation and high blood concentrations have been reported in a range of cardiovascular diseases (CVDs), including myocardial infarction [24], cardiomyopathy and pulmonary thromboembolism [25]. The association of circulating Tn-C concentration with large artery atherosclerotic (LAA) stroke in humans has not been previously studied [23].

The aims of this study were twofold: To assess if plasma concentrations of Tn-C were associated with LAA stroke and to determine if Tn-C influenced *in vitro* secretion of TLR-4 derived pro-inflammatory cytokines from both cultured human carotid atheroma tissue and healthy, primary vascular cells stimulated by serum.

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2. Methods

2.1. Patients

Cases with a recent LAA stroke (n=336) were obtained from a study undertaken between 2003 and 2008 at two acute stroke units in New South Wales, Australia, which has been previously described in detail [26,27]. Exclusion criteria for the original study included patients aged <18 years; a diagnosis of haemorrhagic stroke or transient ischaemic attack (TIA); and patients unable to undergo baseline brain imaging. Computerised tomography and/or magnetic resonance imaging brain scanning were performed on all cases to confirm an ischemic brain infarct. Other investigations obtained included echocardiography and carotid duplex. For the current study, patients were included if plasma samples were available and investigations indicated a LAA stroke had occurred. LAA stroke was defined by Trial of Org 10172 in Acute Stroke Treatment (TOAST), an etiologic system for categorization based on subtypes of ischaemic stroke [28]. Control subjects (n=321) were obtained from the Hunter Community Study cohort [29]. This study recruited healthy community dwelling men and women aged 55-85, randomly selected from the New South Wales (NSW) Electoral Roll during 2004-2007. Controls were excluded if they reported a history of any type of stroke and did not have stored plasma samples. Written informed consent was obtained from each participant as per the University of Newcastle and Hunter New England Area Health ethics committee requirements. Ethics approval was provided by the appropriate committee (Hunter New England Human Research Ethics Committee, 13/10/16/5.05).

Patients for the *in vitro* explant study were recruited from those selected to undergo primary carotid endarterectomy at The Townsville and Mater Hospitals, Queensland, Australia between September 2011 and June 2012. Inclusion criteria included: 1) verbal and written informed consent; 2) carotid

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stenosis of ≥ 70% identified on duplex imaging using criteria previously described [16]; and 3) no previous carotid artery intervention. Ethics approval was provided by the appropriate committee (Townsville Health Service District, 61/05). Patients with a history of TIA, stroke with good recovery and those with an asymptomatic stenosis selected for surgery were included.

2.2. Blood samples

Peripheral blood was collected into 3.2% Na citrate Vacuette containers (Greiner Bio-One GmbH, Frickenhausen, Germany) and platelet-free plasma was prepared by serial double centrifugation (15 min at 2100g) within 2 hours of collection. For stroke cases blood samples were collected within 8 hours of admission. All samples were aliquoted and stored at -80°C.

2.3. Carotid atheroma samples

A conventional endarterectomy was performed, developing a deep medial plane and removing the specimen *en bloc* [16]. Specimens were placed in chilled culture medium immediately after excision, kept chilled and transferred to the laboratory for processing within 6 hours [16]. Specimens were dissected aseptically for the explant culture intervention studies.

2.4. Explant culture

Atheroma explant culture was carried out as described previously [16,30,31]. Two disease matched pairs were obtained from each of the five patients undergoing carotid endarterectomy. We have previously established that: i) Carotid endarterectomy samples remain viable in culture for at least 8 days, as assessed by histology, immunohistochemistry, cytokine release, and measurement of tissue ATP [32]; ii) Adjacent paired biopsies have similar features of plaque instability *in vitro*. Tissue factor (TF) concentration and activity were similar in adjacent biopsies with a coefficient of variation for TF activity <5% (n=6) [16]; iii) Carotid endarterectomy samples emulate the patient

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response to signalling pathway interventions. Angiotensin II (AII) levels increase in the blood of patients receiving chronic angiotensin receptor blocker (ARB) or angiotensin converting enzyme inhibitor (ACEi) drug therapy. In the *in vitro* explant cultures AII levels also increased in the culture supernatant in response to these drugs [31]. Paired adjacent, disease matched biopsies were cultured with media containing a blocking antibody (Ab) against Tn-C and the matched control sample was incubated with media alone. The Tn-C polyclonal goat Ab used in this study has been raised against a peptide from within amino acids 2140-2190 of Tn-C, which is a region within the TLR-4 binding FBG domain of Tn-C [17] (20 μg/mL; Santa Cruz #SC-9872). This Ab has been designed for use in competition studies and can be blocked with a specific, commercially available Tn-C peptide. Conditioned media was harvested (30,000g, 30min, 4°C) and stored at -80°C until assayed. Explant tissue was stored in culture media/10% DMSO at -80°C until processed and assayed.

2.5. Primary vascular cell culture

Due to the nature of the carotid endarterectomy procedure the range of cell types present in the atheroma explant tissue culture include: endothelial cells (ECs), smooth muscle cells (SMCs), inflammatory cells which have infiltrated the atheroma tissue from the blood and red blood cells on the surface of the tissue. Mixed, healthy, primary vascular cell cultures comprising human aortic ECs (HAEC; Lonza; #CC-2535; seeded at $2.6x10^{2}$ cells/mL) and human aortic SMCs (HASMC; Lonza; #CC-2571; seeded at $1.5x10^{4}$ cells/mL) were established in triplicate the day before and allowed to adhere overnight. Total blood derived cells, including inflammatory cells and red blood cells (RBCs), (isolated from heparin plasma and rinsed 3 times with Hanks Balanced Salt Solution to remove intrinsic clotting factors) and donor matched serum were added to the healthy, primary EC/SMC with titrated Tn-C FBG Ab (0, 0.67, 2, 6, 18μg/mL) and cultured at 37°C in a humidified 5% CO_2 atmosphere for 4 days. At the end of the culture period the RBCs flowed freely when

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pipetted and there was no formation of a bolus. Conditioned media was harvested (30,000g, 30min, 4°C) and stored at -80°C until assayed. The blood was collected from four healthy, male volunteers and pooled. Verbal and written, informed consent was received. Ethics approval for these control samples was provided by the appropriate committee (James Cook University, H4109).

2.6. Assessment of plasma and supernatant protein concentrations

Plasma Tn-C was measured by ELISA using a commercially available kit following the manufacturer's instructions (USCN, Wuhan, China). The intra- and inter-assay co-efficients of variation for this assay were 5.4 and 16.9%, respectively in our laboratory. The concentrations of IL-6, IL-8, IL-1β and CCL-3 within culture supernatants were measured by commercially available ELISA kits (R&D Systems, Minneapolis, USA). The appropriate conditions for each assay were determined by sample titration. Based on these preliminary studies the following volumes and dilutions of conditioned media and plasma were assayed: IL-6 (8µL of 1/100 explant and 30µL 1/100 primary cell culture supernatant; #DY206), IL-8 (30µL of 1/100 explant and 10µL 1/10 primary cell culture supernatant; #DY208), IL-1β (100µL neat culture supernatant; #DY201), TNF-α (100µL neat culture supernatant; #DY210), CCL3 (30µL of neat explant and 10µL 1/10 primary cell culture supernatant; #DY270) and Tn-C (45µL of 1/1000 plasma or 20µL of 1/100 explant culture supernatant; #E91975Hu). As the explant culture system is a closed system the total amount of cytokine secreted by each sample was determined and used in the subsequent analyses.

2.7. Assessment of atheroma protein concentrations

Following completion of the explant studies, protein was extracted from the atheroma and quantified by the Bradford assay as previously described [31]. Total protein content of each cultured tissue sample was used to normalise between the treated and untreated control samples. Atheroma concentrations of the key TLR-4 pathway proteins involved in cytokine synthesis, TLR-

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4, myeloid differentiation primary response protein (MyD88), and nuclear factor-KB (NF-KB) [33], were measured in the explant tissues by ELISA using commercially available kits (USCN, Wuhan, China). The appropriate conditions for each assay were determined by sample titration. Based on these preliminary studies the following volumes and dilutions of tissue lysate were assayed: TLR-4 (100μL of 1/384 lysate; #E0753Hu), MyD88 (100μL of 1/24 lysate; #E1707Hu) and NF-KB (100μL of 1/24 lysate; #E1824Hu). As the explant culture system is a closed system the total amount of TLR-4 pathway protein expressed within each sample was determined and used in the subsequent analyses.

2.8. Data Analysis

The risk factors of stroke cases and controls were compared with Pearson Chi-squared test for nominal variables. Continuous variables were presented as medians with inter-quartile ranges and compared by Mann-Whitney U test. Binary logistic regression analysis was used to assess the association of plasma Tn-C with stroke adjusting for other risk factors including age, gender, history of smoking, atrial fibrillation, myocardial infarction, diabetes and hypertension. SPSS software (IBM, Austin, USA) was used for these analyses.

The normalised amount of pro-inflammatory cytokines in supernatants (n=10 pairs for the diseased explant studies) and TLR-4 pathway proteins in tissue of atheroma exposed to Tn-C blocking antibody were presented as mean ± SEM of the relative ratios in paired, experimental and control samples [16,31]. For some sample pairs (particularly TNF-α) the protein was undetectable in the untreated control sample but detectable in the treated sample. In this case the untreated control sample was assigned the value of the lowest detectable concentration in the assay for further analysis. Differences between experimental and control samples were assessed using Wilcoxon's paired test. Values for experimental samples >1 implied the blocking Ab enhanced activity or

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expression while values =1 implied the blocking Ab had no effect. Correlations of expression of various proteins in the explant cultures were assessed using Spearman's rho correlation. Prism 5 software (GraphPad, San Diego, USA) was used for all the *in vitro* cultured sample analyses. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Plasma Tn-C concentration is positively associated with LAA stroke

The risk factors of stroke cases and controls are shown in Table 1. Stroke cases were older and more likely to have a history of hypertension, diabetes and atrial fibrillation. Plasma Tn-C concentration was significantly higher in stroke patients (median 12.77 μg/mL, inter-quartile range 10.23-15.74 μg/mL, n=336) than controls (median 11.31 μg/mL, inter-quartile range 8.89-13.90 μg/mL, n=321), $p<0.001$ (Table 1). Tn-C was independently, positively associated with stroke after adjusting for other risk factors, and patients with plasma Tn-C in the highest quartile were more than twice as likely to have had a stroke compared with those with plasma Tn-C in the lowest quartile (odds ratio for highest Tn-C quartile 2.27, 95% confidence interval 1.37-3.76; Table 2).

3.2. Heterogeneity of atheroma samples from different patients

Concentration of IL-6, IL-8, IL-1β, CCL3 and Tn-C present in the untreated control atheroma supernatant and TLR-4, MyD88 and NF-KB in the untreated control atheroma tissue were measured using ELISA. The mean normalised amount of cytokine or TLR-4 pathway protein ± SEM in the untreated control cultures (n=10) were as follows: IL-6, 530.7±321.4 ranging from 42-3283 ng/mg; IL-8, 175.1±62.4 ranging from 36.5-669.1 ng/mg; IL-1β, 17.7±11.8 ranging from 0.2-120.4 pg/mg; CCL3, 216.0±92.3 ranging from 2.7-855.0 pg/mg; Tn-C, 1707±403.6 ranging from 246.2-3628 ng/mg; TLR-4, 18448±4817 ranging from 7158-56309 ng/mg; MyD88, 79.3±13.7 ranging from 22.3-158.5 ng/mg; NF-KB, 77.0±3.4 ranging from 60.6-90.0 ng/mg (Figure 1a-h). Characteristics

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of the five patients from which carotid atheroma samples were obtained are shown in Table 3.

Table 1: Patient characteristics of subjects with and without stroke used in the biomarker assessment

Characteristic	Stroke	Control	P value
Number	336	321	
Age (years)	74 (67-78)	66 (61-73)	<0.001
Men	188 (56.0%)	167 (52.0%)	0.313
Hypertension	249 (74.1%)	160 (49.8%)	<0.001
Diabetes mellitus	69 (20.5%)	37 (11.5%)	0.002
Ever smoker	172**(52.9%)	154 (48.0%)	0.209
Myocardial infarction	37#(11.4%)	33 (10.3%)	0.642
Atrial fibrillation	91*(27.2%)	42 (13.1%)	<0.001
Plasma Tn-C (μg/ml)	12.77 (10.23-15.74)	11.31 (8.89-13.90)	<0.001

Nominal variables are presented as numbers (%) and compared by chi-squared. Continuous variables are presented as median (inter-quartile range) and compared by Mann Whitney U test. Data missing on *1, **11, and #12 subjects.

Table 2: Independent risk factors for stroke in the Tn-C biomarker assessment

Characteristic	Odds ratio	95% CI	P value
Plasma Tn-C in μg/ml*			
<9.685	1.00	Reference	
9.685-11.866	1.71	1.04-2.79	0.033
11.8661-14.998	1.74	1.06-2.86	0.028
>14.998	2.27	1.37-3.76	0.001
Ever smoker	1.50	1.05-2.14	0.028
Male gender	1.04	0.73-1.50	0.819
Atrial fibrillation	1.81	1.15-2.84	0.010
Age above 70.0 years†	3.53	2.47-5.03	<0.001
Previous myocardial infarction	0.78	0.45-1.37	0.393
Diabetes mellitus	1.61	0.98-2.62	0.052
Hypertension	2.45	1.70-3.51	<0.001

For nominal variables the comparisons are to subjects without the risk factor. * Men with citrate plasma Tn-C concentrations in the top, third and second quartiles were compared with subjects who had citrate plasma Tn-C in the lowest quartile. †Approximately median value.

3.3. Correlation of pro-inflammatory cytokines with Tn-C and TLR-4 in human carotid atheroma culture *in vitro*

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The concentrations of Tn-C and a number of pro-inflammatory cytokines within the untreated atheroma culture supernatants were not correlated (IL-6, Spearman rho=-0.1030, P=0.7850; IL-8, Spearman rho=-0.4424, P=0.2044; IL-1β, Spearman rho=-0.1190, P=0.7930; CCL3, Spearman rho=-0.3333, P=0.3487) (Table 4). In contrast, the expression of TLR-4 within the atheroma tissue was correlated to the concentration of IL-8, IL-1β, TNF-α and CCL3 in the culture supernatant (Spearman rho=0.8045, P<0.0001; Spearman rho=0.7420, P=0.0004; Spearman rho=0.7455, P=0.0112; Spearman rho=0.7835, P<0.0001, respectively) (Table 4).

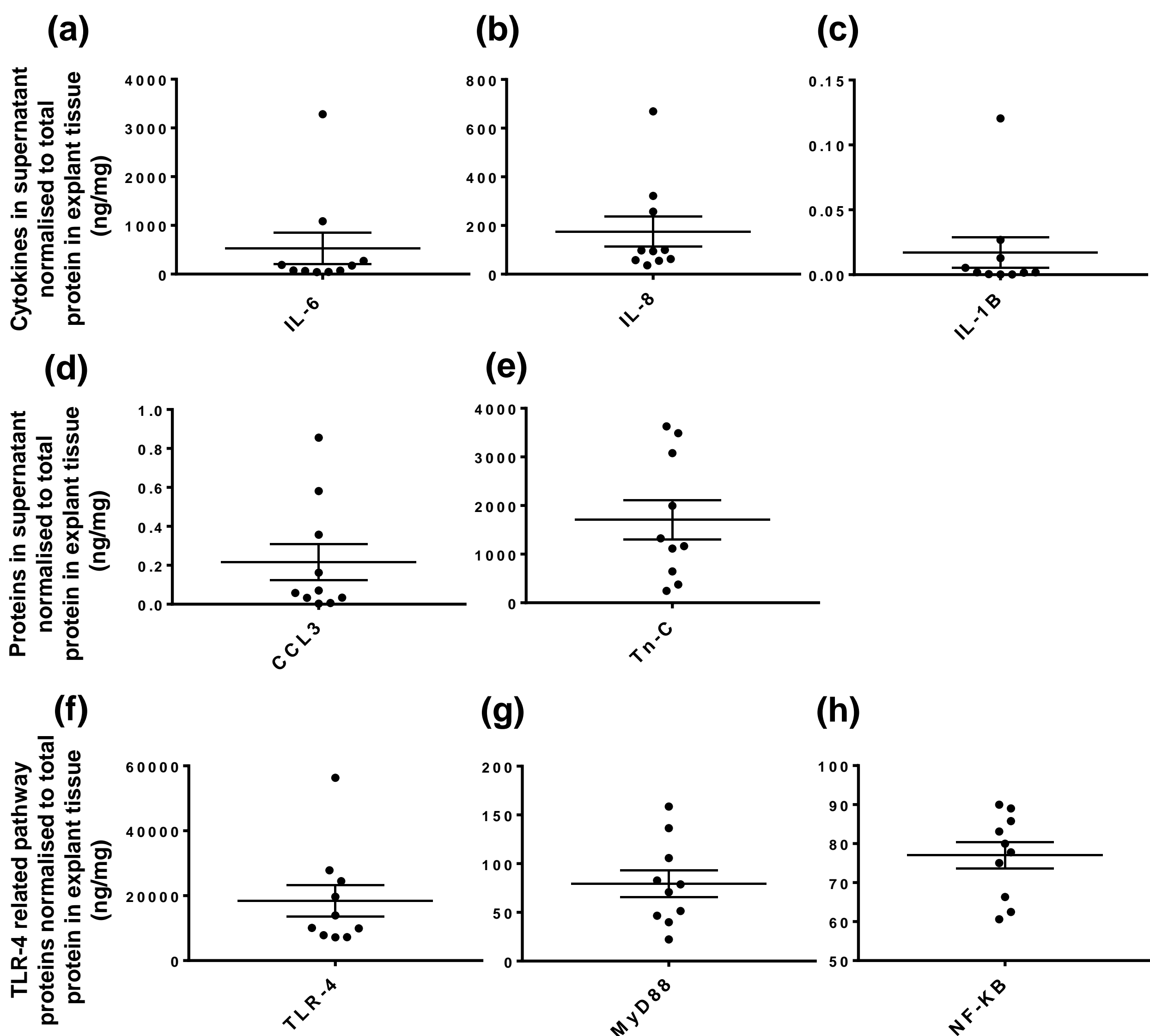

Figure 1. Patient heterogeneity for atheroma samples. Mean ± SEM of (a) IL-6, (b) IL-8, (c) IL-1β, (d) CCL3, (e) Tn-C, (f) TLR-4 (g) MyD88 and (h) NF-KB detected in the untreated control culture supernatant and tissue, normalised to the total amount of protein present in the explant tissue (n=10). Protein expression was measured by ELISA. Black circles indicate individual results.

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Table 3: Patient characteristics for the Tn-C FBG blockade culture studies

Characteristic	Diseased atheroma explant tissue
Number of patients, n	5
Age, years, mean±SD	65.7±4.7
Male, n	5
TIA, n	3
Stroke, n	0
Diabetes, n	0
Hypertension, n	3
IHD, n	2
Ever smoker, n	4

TIA indicates Transient Ischaemic Attack; IHD, Ischaemic Heart Disease

Table 4: Correlation of cytokines with Tn-C in the untreated explant culture supernatant and TLR-4 in the explant tissue.

Cytokine in culture supernatant	Mean concentration (range) in untreated explant culture	Correlation with Tn-C in untreated explant culture supernatant		Correlation with TLR-4 in explant culture tissue	
	ng/mL	Spearman rho	P value	Spearman rho	P value
IL-6	216.3 (34-1073)	-0.1030	0.7850	0.1744	0.4260
IL-8	99.2 (25-308)	-0.4424	0.2044	**0.8045**	**<0.0001**
IL-1β	0.018 (0.0003-0.115)	-0.1190	0.7930	**0.7420**	**0.0004**
TNF-α	Not detectable			**0.7455**	**0.0112**
CCL3	0.115 (0.002-0.531)	-0.3333	0.3487	**0.7835**	**<0.0001**
Tn-C	956.1 (163-1642)				

Spearman rho correlations between the cytokines and Tn-C were performed using the concentration in the untreated control supernatant (n=10). This enabled direct comparison with RA cell culture studies presented in the literature. As the explant culture system is a closed system and the amount of cytokine present in the culture supernatant is a direct result of the amount of TLR-4 present in the tissue the tissue TLR-4 correlations were determined using the total amount of TLR-4 and cytokine present within each culture well (n=20 and n=10 for TNF-α).

3.4. Effect of blocking Tn-C on chronic TLR-4 derived cytokine expression in diseased human carotid atheroma culture supernatants *in vitro*

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Blocking Tn-C (20 µg/mL Ab) binding to TLR-4 within human carotid atheroma *in vitro* (2 matched pairs per patient, n=10) upregulated secretion of all the TLR-4 associated inflammatory cytokines as follows: IL-6 by ~3-fold (P=0.1141); IL-8 by ~6-fold (P=0.0051); IL-1β by ~20-fold (P=0.0109); TNF-α by ~17-fold (P=0.0077) and CCL3 by ~75-fold (P=0.0051) (Figure 2a), with the increase in IL-8, IL-1β, TNF-α and CCL3 demonstrating a significant increase. The increased secretion of cytokines was associated with a concomitant increase in tissue expression of TLR-4 (n=10; P=0.0051) with TLR-4 expression increased ~17-fold (Figure 2b).

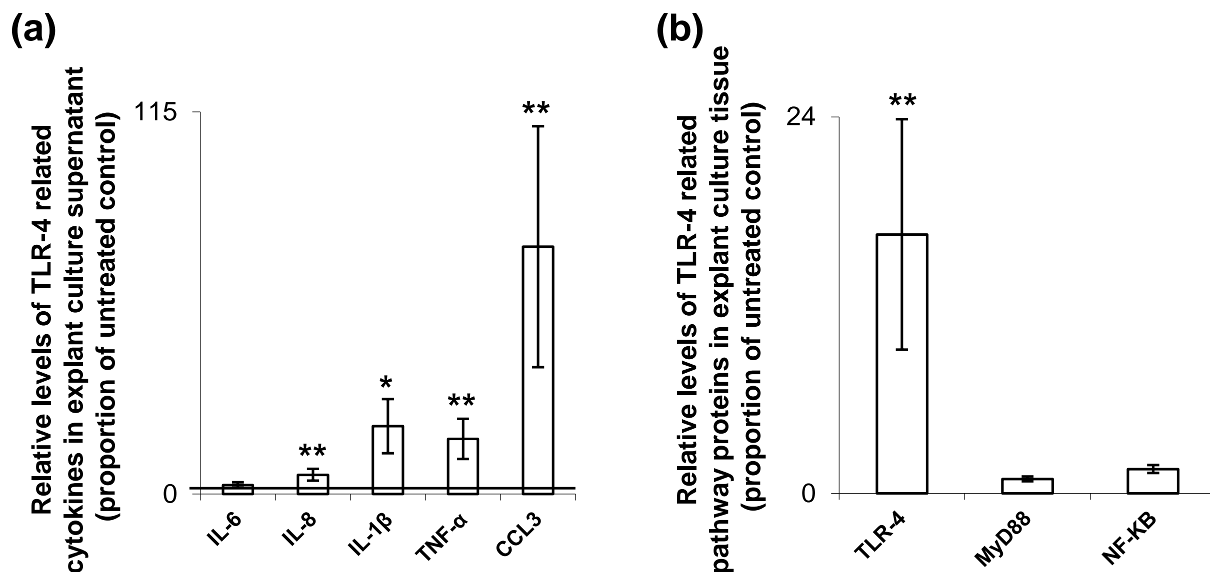

Figure 2. Changes in expression of TLR-4 related proteins following Tn-C blockade in diseased atheroma explant culture. Relative expression of TLR-4 related proteins: (a) cytokines in the supernatant and (b) TLR-4 pathway proteins in the tissue of diseased atheroma explant cultures after 4d of culture with and without Tn-C FBG domain blockade (Tn-C FBG Ab: 20 µg/mL, n=10 pairs). Shown are the mean values ± SEM of the ratio of cytokine or TLR-4 pathway protein (normalised to total protein for the explant study) expressed by paired human samples incubated with intervention relative to control. Protein expression was measured by ELISA **P<0.01; *P<0.05 using Wilcoxon's paired test. (1=no change-denoted by horizontal line, >1 increased with treatment)

3.5. Effect of blocking Tn-C on TLR-4 derived cytokine expression in mixed, healthy, primary vascular cells stimulated by human serum *in vitro*

The level of the cytokines IL-6, IL-8, IL-1β and CCL3 secreted by healthy cells of the same types found in the atheroma tissue increased with addition of normal human serum (Figure 3a-d).

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Titration of the blocking Tn-C Ab in the mixed, healthy, primary vascular cell (EC+SMC+BC+ser) culture also upregulated secretion of IL-6, IL-8, IL-1β and CCL3 in a dose dependent manner (Figure 4a-d).

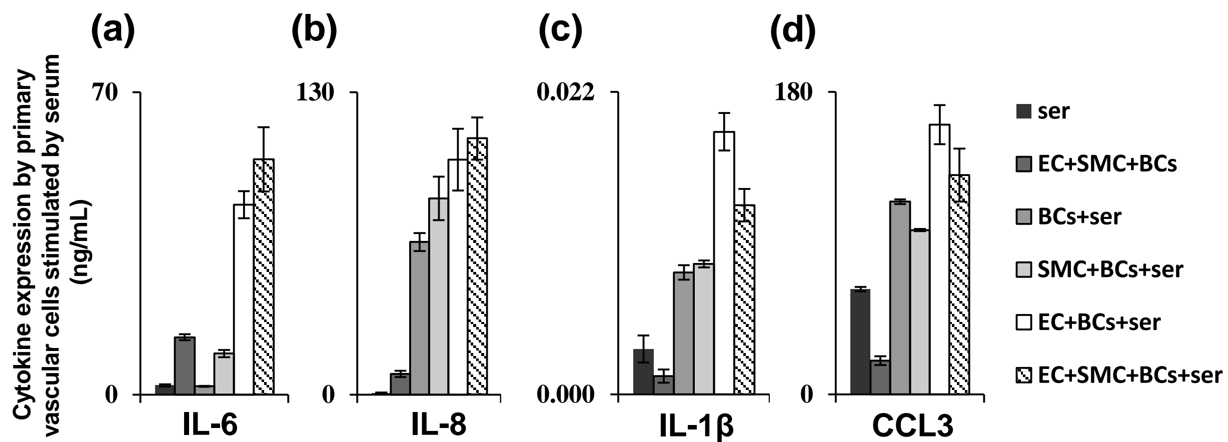

Figure 3. Expression of TLR-4 related proteins by healthy, primary vascular cell cultures. **E**xpression of TLR-4 related cytokines in the culture supernatant after 4d of culture using various combinations of healthy EC, SMC, BCs and serum: (a) IL-6, (b) IL-8, (c) IL-1β and (d) CCL3. Shown are the mean values from triplicate cultures ± SEM.

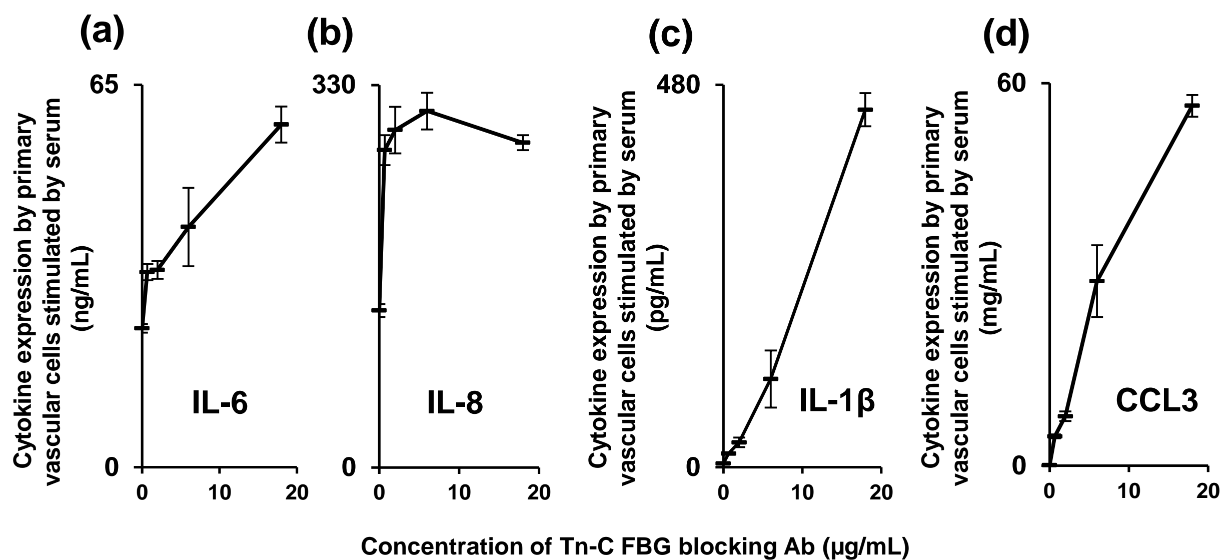

Figure 4. Changes in expression of TLR-4 related proteins following Tn-C blockade in mixed, healthy, primary vascular cell cultures. **E**xpression of TLR-4 related cytokines in the mixed, primary vascular cell culture (EC, SMC, BC and serum) supernatant after 4d of culture with titrated Tn-C FBG blockade: (a) IL-6, (b) IL-8, (c) IL-1β and (d) CCL3. Shown are the mean values from triplicate cultures ± SEM.

4. Discussion

There were two main significant findings from this study. Firstly, plasma concentrations of Tn-C

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were positively associated with LAA stroke. Secondly, blockade of Tn-C within diseased human carotid atheroma and also within mixed, healthy, primary vascular cells *in vitro* increased expression of the pro-inflammatory cytokines, IL-6, IL-8, IL-1β, TNF-α and CCL3. These findings suggest that Tn-C has an anti-inflammatory impact in the vasculature.

Our finding of higher circulating concentrations of Tn-C in patients who have recently had a LAA stroke is in keeping with many studies where Tn-C has been associated with chronic inflammation. These include associations between Tn-C and a range of inflammatory diseases such as acute coronary syndrome [22], osteoarthritis [34,35] and RA [36]. The area of Tn-C staining in coronary atheroma sections has been shown to be significantly higher in samples from patients with coronary events [22]. Similarly, the level of Tn-C in cartilage removed from the knees of osteoarthritic patients is higher compared to controls [34,35], and correspondingly elevated in synovial fluid from both osteoarthritis [34] and RA [17] patients. In all these studies the level of Tn-C was positively correlated with increased inflammation. The association of Tn-C with inflammation extends beyond chronic inflammatory diseases to also include acute events such as tissue injury and infection [36]. Tn-C is upregulated at day five of healing in human skin wounds [37], the most damaged regions of ruptured tendons [38], the most photodamaged region in sunburnt skin [39] and regions of newly formed fibrosis in human fibrotic disease [40]. Pathogenic infection by *Staphylococcus aureus* [41], various bacterial and viral agents [40,42] also lead to increased levels of circulating Tn-C in the first few days of the infection [43].

It is widely accepted that TLR-4 has a role in atherosclerosis impacting on inflammation, vascular remodelling and thrombosis [7]. Tn-C has been previously identified as an endogenous TLR-4 ligand and suggested to stimulate TLR-4 activity in arthritic joint disease [17,34,43]. Chondrocytes isolated from osteoarthritic cartilage stimulated with 10 μg/mL Tn-C secreted ~9 ng/mL IL-6 [34],

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peripheral blood monocytes differentiated to macrophages with 100 ng/mL macrophage colony stimulating factor stimulated by 1μM Tn-C secreted ~7 ng/mL IL-6 [17] and primary synovial fibroblasts from joints of patients with RA secreted ~8 ng/mL IL-6 when stimulated by 1μM Tn-C [17]. A mixed RA synovial cell culture secreted ~700 ng/mL Tn-C [43]. In comparison, the level of IL-6 secreted by the atheroma explant tissue was of a higher magnitude (216.3 ng/mL) than the levels determined in synovial cell monocultures stimulated by Tn-C. Tn-C levels in the atheroma explant supernatant were of a similar magnitude (956.1 ng/mL) to the mixed culture of synovial membrane derived cells. The mixed RA synovial cell culture also demonstrated a positive correlation between the levels of Tn-C (ranging from ~50-1250 ng/mL) and IL-6 (ranging from ~20-1500 ng/mL) in the culture supernatant (Spearman rho=0.8045; $P<0.001$) [43]. In contrast, there was no correlation between IL-6 (ranging from 34-1073 ng/mL) and Tn-C (ranging from 163-1642 ng/mL) secretion in the atheroma explant supernatants. These results suggest that the factors that determine the inflammatory response in the synovium are not the same as those that determine the inflammatory response in the vasculature.

Our current findings suggest that Tn-C may have an inhibitory impact on TLR-4 driven upregulation of the cytokines, IL-6, IL-8, IL-1β, TNF-α and CCL3 in atherosclerotic disease. TLR-4 derived pro-inflammatory cytokine secretion was significantly increased following the blockade of Tn-C binding to TLR-4 in diseased atheroma tissue. Further to this there was a concomitant increase in expression of TLR-4 (which drives the production of pro-inflammatory cytokines through the MyD88 dependent pathway [33]) in the explant tissue by approximately 17-fold, after Tn-C blockade. IL-8, IL-1β, TNF-α and CCL3 expression in the explant culture supernatant were also significantly correlated with TLR-4 expression in the tissue. A role for TLR-4 in the upregulation of these cytokines in atheroma is supported by these results. Overall these findings are consistent with Tn-C having an anti-inflammatory impact on TLR-4 driven cytokine upregulation in

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atheroma.

A crucial element of the explant culture work is the fact that all the proteins upregulated in the culture system are only derived from the cells of the tissue itself representing a chronic response. This demonstrates that TLR-4 in the tissue must be activated by endogenous ligands derived from the cultured explant tissue itself to induce expression of inflammatory cytokines, such as IL-6 or IL-8 (LPS was not detectable in the culture supernatants using the Etoxate assay from Sigma; data not shown). There are a number of other endogenous TLR-4 ligands that have been associated with atherosclerosis. These include fibronectin (FBN) [44], resistin (RSTN)] [45], fetuin A (Fet-A) [46] and hyaluronan [47]. Blockade of Tn-C binding to TLR-4 in the atheroma explant could possibly allow another atherosclerosis associated, endogenous ligand, which is more effective at upregulating the cytokines, access to TLR-4 leading to the increased TLR-4 in the tissue and consequent secretion of the cytokines demonstrated in the proposed model of the Tn-C blockade studies (Figure 5).

This study has some limitations. Firstly, the atheroma studies were limited to *in vitro* studies. The main limitation of this technique is that the circulating factors present in the *in vivo* context are missing so the *in vivo* relevance of these findings is uncertain. A previous study investigating angiotensin II (AII) signalling and interstitial collagenase expression in atheroma culture *in vitro* determined that the atheroma tissue response to angiotensin receptor 1 blockade and angiotensin converting enzyme 1 blockade were consistent with the *in vivo* response to these drugs with regards to AII secretion [31]. Secondly, there were no stroke victims amongst the donors that provided the atheroma tissue. To help further address these issues another culture study was performed using serum (providing the circulating factors) and healthy, primary vascular cells of the types potentially present in atheroma tissue. The results obtained were in accordance with the explant study with, IL-

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6, IL-8, IL-1β and CCL3, being upregulated in a dose dependent manner. The concordance of both sets of results, suggest that in this instance the findings are relevant to the *in vivo* context and that Tn-C may have an anti-inflammatory impact on the vasculature in general, not just in atheroma, and as a consequence would also be applicable to LAA stroke victims.

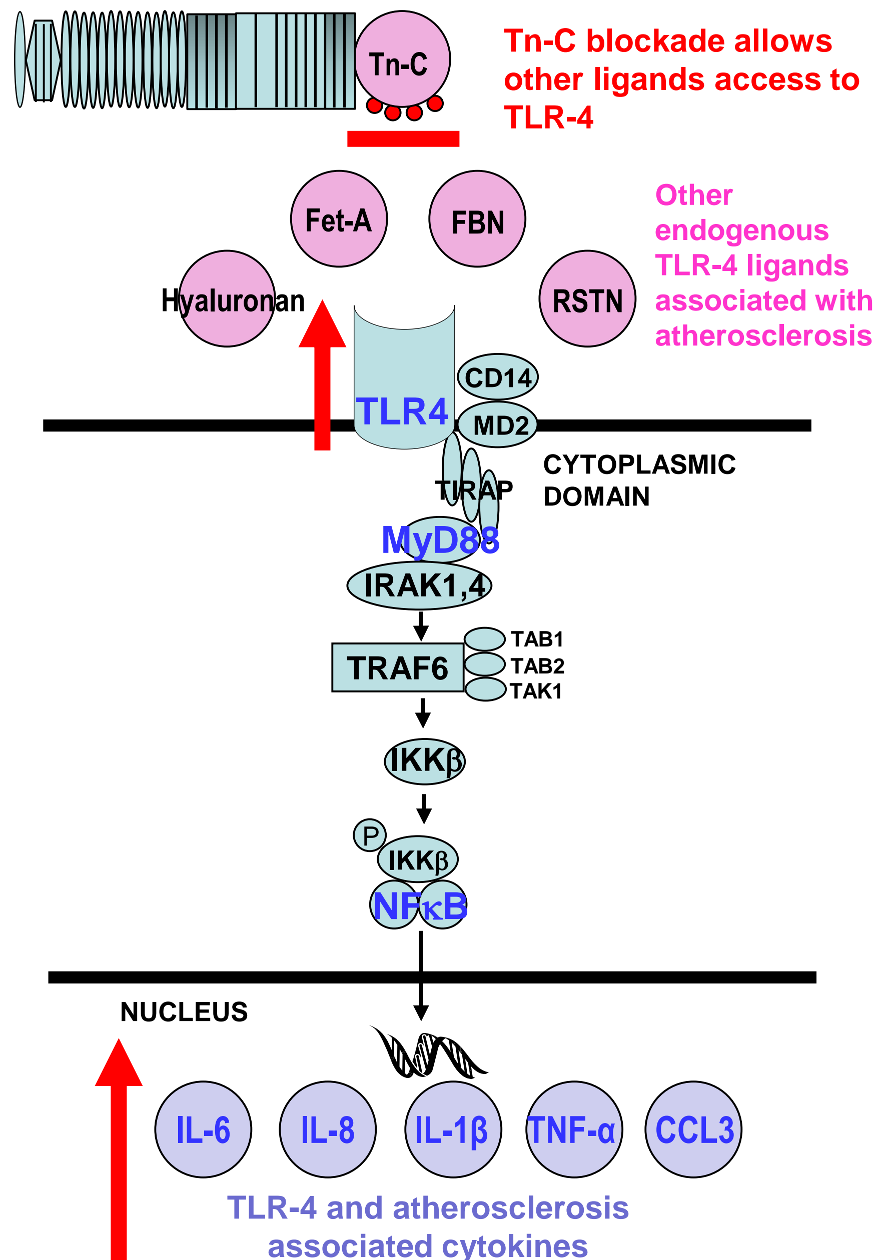

Figure 5. Model of Tenascin-C blockade effects on the TLR-4 signalling pathway. Endogenous ligand binding at the cell surface leads to recruitment of specific adaptor molecules to the TLR signalling domain on the cytoplasmic side of the receptor, which activates the downstream transcription factor, NF-KB, leading to increased expression of pro-inflammatory cytokines [33]. Blockade of the FBG domain of Tn-C that binds to TLR-4 led to an increase in secretion of pro-inflammatory cytokines and a concomitant increase in the expression of TLR-4 in the tissue. These

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results suggest blockade of Tn-C binding to TLR-4 enabled another endogenous ligand/s to upregulate expression of TLR-4 and these cytokines and that Tn-C binding reduces cytokine secretion resulting in an anti-inflammatory effect.

The third limitation of this study is the heterogeneity of the cultured tissue and the patients themselves. The patients included in these studies are heterogeneous in terms of co-morbidites and medication regimes. In order to minimise these effects we have developed a system of assessing paired biopsies from adjacent sites within macroscopically severe atheroma and including a 24h pre-incubation step before the intervention study itself. We have previously shown this technique minimised the heterogeneity between the paired treated and untreated samples [16,30,31]. For the plasma LAA stroke association assessment the stroke cases were significantly older and more likely to have a history of hypertension, diabetes and atrial fibrillation than the controls. To overcome this issue we adjusted for risk factor differences between cases and controls using logistic regression.

The heterogeneity of the tissue and the necessity to disease match the pairs as closely as possible restricted the number of pairs available to assessment with the just the Tn-C blocking Ab, which gave rise to the final limitation of the study, namely, omission of an isotype control to completely rule out the possibility that the cytokine increase in the blockade studies was due to a non-specific Ab response. The Tn-C Ab is designed for use in competition studies and the preparation is affinity purified to remove non-specific Abs, which makes it very unlikely that the Ab effects are non-specific.

In conclusion, this study suggests that Tn-C has an anti-inflammatory impact on TLR-4 driven cytokine upregulation in the vasculature and that the increased levels of Tn-C in LAA stroke patients could act to dampen the upregulation of inflammatory cytokines from the atheroma. Further validation of these findings in other cohorts and human samples are needed.

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