The biological role of Tribbles proteins
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The link between inflammation
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PUBLICATIONS RELATED TO THE THESIS

I.   LDL uptake by monocytes in response to inflammation is MAPK dependent but independent of tribbles protein expression
Katalin Eder[*‡], Hongtao Guan[†], Hye Y. Sung[*], Sheila E. Francis[*], David C. Crossman[*] and Endre Kiss-Toth[*]
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II.   Human Tribbles-1 controls proliferation and migration of smooth muscle cells via MAPK signalling pathways
 H. Y. Sung[§], H. Guan[¶], A. Czibula[ẞ], A. R. King[§], K. Eder[£], E. Heath[§], S. K. Suvarna[€], S. K. Dower[¶], A. G. Wilson[¶], S. E. Francis[§], D. C. Crossman[§] and E. Kiss-Toth
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III.   Tribbles-2 is a novel regulator of monocyte activation in Coronary Artery Disease
Katalin Eder, Hongtao Guan, Hye Y. Sung, Paul J. Sheridan, Jon Ward, Allison Morton, Adrienn Angyal, Michelle Janas, Gabriella Sarmay, Erno Duda, Martin Turner, Steven K. Dower, Sheila E. Francis, David C. Crossman and Endre Kiss-Toth
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Introduction

In Western societies, some of the most common causes of death are the various complications of atherosclerosis. Many known genetic and environmental risk factors contribute to the development of atherosclerosis. These include elevated levels of modified low density lipoproteins (LDL), free radicals arising from oxidative stress and cigarette smoking, obesity, hypertension, diabetes mellitus, genetic alterations, hyperactive monocytes/platelets, chronic infections such as by microorganisms like herpes viruses, *Chlamydia pneumonia* or others, and obviously combinations of these or as yet unrecognized factors (1).

Atherosclerosis is a multifactorial, complex pathological process. Cell-cell and cell-matrix interactions and communications involving macrophages, vascular smooth muscle cells, vascular endothelial cells, and lymphocytes are all likely to be involved. Three cellular components of the circulation, monocytes, platelets, and T lymphocytes, together with two cell types of the artery wall, endothelial and smooth muscle cells (SMC), interact in multiple ways to generate atherosclerotic lesions (2).

Atherogenesis appears to depend on the local presence of high amounts of modified LDL. Normally, LDL is transported trough the LDL receptors, which is a feedback inhibited process by the accumulation of cholesterol within the cell. However, if the LDL is chemically (most frequently oxidized) or enzimatically modified the LDL receptor can not transport it (1).

Modified LDL has the capacity to induce the expression inflammatory factors, in response to which monocytes migrate into the intima and differentiate into resident macrophages. Macrophages are able to take-up the modified LDL by scavenger receptors (SR) (3, 4).

This unregulated uptake of modified LDL forms leads to foam cell formation (5, 6). At the early stage of atherosclerosis foam cells form clusters. The intensive aggregation of these cells leads to the formation of the atheromatous core, and to the development of fatty streak lesions.

This process is accompanied by the migration of smooth muscle cells to the intima. They proliferate, and secrete extracellular matrix proteins that form a fibrous plaque (7).

This phase of plaque development is influenced by interactions of macrophages and T cells and it is result of both humoral and cellular responses. (8). Similarly, macrophages, endothelial cells, and smooth muscle cells are activated and produce pro-inflammatory cytokines, such as TNF-alpha, IL-6, MCP-1 or IL-8 (9). If prolonged, these inflammatory processes can induce the cell death of lipid-loaded macrophages and smooth muscle cell by apoptosis or necrosis (10).

The above processes lead to the formation of the necrotic core and the accumulation of extracellular cholesterol. Around the necrotic core, macrophages and macrophage foam cells produce and release metalloproteinases that facilitate plaque destruction (4). Plaque rupture exposes blood components to tissue factor, initiating coagulation, the recruitment of platelets, and the formation of a thrombus.

All these steps of atherosclerosis are controlled by several cytokines and chemokines. A number of cytokines have been shown to induce monocyte recruitment, cell migration or alter the scavenger receptor and adhesion molecule expression. They regulate the immune responses also; they have an influence on dendritic cell maturation and Th1/Th2 development. Cytokines generate cellular responses through activation of intracellular signal transduction networks. In atherosclerosis, the NF-κB and Mitogen Activated Protein Kinase (MAPK) pathways are amongst the most prominent ones.

The aim of my studies was to elucidate the biological effects of a novel family of MAPK regulators, called tribbles, in inflammatory monocyte function in the context of atherosclerosis.

Atherosclerotic Plaque Formation

LDL modification

Modification of LDL is one of the key triggers in the initiation and the progression of atherogenesis. Cholesterol is transported in the circulation by plasma lipoproteins. The principal cholesterol carrier, LDL, serves as an exogenous source of cholesterol and other cellular nutrients for hepatic and various extrahepatic tissues, where it is taken up by receptor-mediated endocytosis. LDL particles have a cholesteryl-ester rich core and a surface

dominated by free cholesterol, phospholipids and apolipoprotein B100 (apoB), the normal ligand for the LDL receptor (11).

Under most circumstances, LDL circulating in plasma is protected from oxidation by the presence of antioxidants. However LDL does not only occur in its native form. It can be trapped in the extracellular matrix of the artery wall, thus being subjected to a variety of enzymatic and chemical modifications. Many of these modified forms of LDL, including oxidized LDL are thought to be pro-atherogenic (12).

The oxidative modification process of LDL can be divided into three stages. The first is known as the initiation of lipid peroxidation, the second stage being the propagation stage of oxidation and the final step is the modification of the apoB moiety of LDL. After modification, apoB is no longer recognized by the LDL receptor but, instead, is recognized by scavenger receptors (13).

The precise entity responsible for the initiation of lipid peroxidation in biologic systems is currently unknown. A number of species have been proposed that include hydroxyl radical, $Fe^{2+}/Fe^{3+}/O2$, peroxynitrite, tyrosyl radical, and even enzymes, such as lipoxygenase.(14) Other processes of LDL modification include glycation and incorporation in immune complexes (13).

Modified LDL enhances monocyte adhesion to the endothelium by inducing expression of adhesion molecules such as integrins. Modified LDL also activates platelets, thereby promoting platelet-monocyte aggregation. LDL increases monocyte recruitment to the vascular wall also by directly acting on monocytes (13).

Modified LDL induces not only monocyte adhesion and recruitment, but it provokes/triggers the production of chemotactic and growth factors in various vascular cell types. Thus, for instance, in endothelial cells modified LDL can induce the expression and release of CCL2 (MCP-1) and macrophage colony stimulating factor (M-CSF) as well as CXCL8 (IL-8) (13).

Monocyte recruitment

The interaction of monocytes with activated luminal endothelium is a central event leading to atherosclerotic alteration of the arterial intima. Monocytes migrate into the subendothelial layer of the intima, where they differentiate into macrophages or dendritic cells.

The adherence of monocytes to the endothelium and their subsequent migration into the arterial wall are facilitated by the presence of cellular adhesion molecules on the surface of endothelial cells (15, 16). Monocytes express both the β1 integrin CD49d/CD29 and the β2 integrins CD11a/CD18, CD11b/CD18 and CD11c/CD18. In resting monocytes, most of CD11b/CD18 and CD11c/CD18 are stored in intracellular granules. Stimulation of monocytes with inflammatory mediators rapidly mobilizes these integrins, leading to their trafficking to the cell surface, where they become accessible for chemokine-dependent activation to mediate firm adhesion (15).

The strong attachment of monocytes to the luminal surface of the endothelium is mediated by the interactions of the integrins with ligands that belong to the immunoglobulin superfamily; in particular, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (17).

Modification of LDL in the intima leads to the upregulation of adhesion molecules on the endothelial cells (EC), particularly vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Thus monocyte and leukocyte recruitment is initiated, leading to enhanced transmigration of monocytes.

Activated monocytes roll on the surface of luminal endothelial cells and adhere to the endothelial cells activated in response to signals originating from the intima. Rolling of monocytes along the endothelial monolayer is mediated by the selectin family of adhesion proteins. L-selectin is expressed on the surface of monocytes while P-selectin and E-selectin are expressed on the luminal surface of activated endothelium (15, 16).

Following their adherence to the endothelium, monocytes migrate across the endothelium into the subendothelial space of the arterial intima. It is known that the process of extravasation involves not only a range of adhesion molecules but also soluble and immobilized chemokines that both stimulate adhesion and guide the adherent monocytes across the endothelium. The interaction with specific chemokine receptors first causes the arrest of leukocytes rolling along the endothelium through the activation of adhesion receptors. Then, chemokines stimulate the transendothelial migration. Once entered in the arterial wall, monocytes undergo differentiation (18).

LDL uptake and foam cell formation

In the arterial intima, monocytes differentiate into macrophages. Macrophages are normally protected from the accumulation of toxic cholesterol load by multiple mechanisms, notably the downregulation of surface LDL receptor molecules in response to repleted intracellular cholesterol stores (19). However, as the LDL accumulates, their lipids and proteins undergo oxidation and glycation and modified LDL may be taken up by alternate "scavenger" or "oxidized LDL" receptors that are not similarly downregulated, when the cholesterol load is in excess.

Monocyte-derived macrophages in arteries express cell surface receptors for LDL (LDL-R) as well as scavenger receptors for modified LDL (SR-A, CD36, CD68). The majority of macrophages avidly accumulate lipids in their cytoplasm and this leads to their transformation into foam cells. Macrophage-foam cells in human arteries display a remarkable heterogeneity with some foam cells containing mostly membrane-bound cytoplasmic lipid droplets while others contain membrane-free lipid droplets (20, 21).

Once LDL is modified and taken up by the macrophages, it activates the nascent foam cells, thereby inducing release of inflammatory mediators such as TNF-α, IL-1β, IL-8, and M-CSF. This, in turn, leads to increased transcription of the LDL-receptor gene and consequently enhanced binding of LDL to the endothelium and smooth muscle cells. Consequently, SMCs can also turn into foam cells (22, 23), feeding the process even further.

Scavenger receptors

A number of scavenger receptors for modified LDL (SR-A, SR-B, SR-C SR-D, SR-E, SR-F, SR-PSOX) have now been identified not only in macrophages but also non-macrophage cells.

Oxidised LDL induces the production of M-CSF by various vascular cells and macrophages in atherosclerotic lesions. M-CSF increases the synthesis of type I and type II class A macrophage scavenger receptors (MSR-A I, II) (24), creating a positive feedback loop. SR-A type I and type II were the first identified members of the scavenger receptor family (25, 26). Several studies reveal important roles of these receptors in atherogenesis. SR-A gene knockout mice crossed with either the atherosclerosis-susceptible apolipoprotein E (apoE)-knockout mice (27) or LDL-receptor knockout mice (28) show a marked reduction in the size

of atherosclerotic lesions (~50% reduced relative to apoE-/- and ~20% reduced relative to LDLR-/-, respectively), suggesting that SR-A has an important pro-atherogenic function *in vivo*. However, MSR-A I, II deficiency does not induce the complete block of uptake of these ligands by macrophages, implicating that receptors other than MSR-A are also play a role in the uptake of these ligands by macrophages (27). However, a recent experiment using peritoneal macrophages obtained from double knockout mice of SR-A and CD36 clearly showed that SR-A and CD36 account for 75–90% of degradation of acLDL or oxLDL, providing solid evidence that SR-A and CD36 are the major receptors, responsible for endocytic uptake of modified LDL by macrophages or macrophage-derived cells (29). CD36 belongs to the class B scavenger receptor family, which includes the receptor for selective cholesteryl ester uptake, scavenger receptor class B type I (SR-BI), and lysosomal integral membrane protein II (LIMP-II). It is a highly glycosylated 88 kDa protein that binds to various ligands such as fatty acids, collagen, thrombospondin, anionic phospholipids, and oxLDL (30). CD36 expression is broad and includes microvascular (but not large vessel) endothelium, adipocytes, skeletal muscle, dendritic cells, epithelia of the retina, breast, and intestine, smooth muscle cells, and hematopoietic cells, including erythroid precursors, platelets, megakaryocytes, and monocytes/macrophages (30).

LOX-1 (lectin-like OxLDL receptor-1) is a C-type lectin identified as a novel scavenger receptor for oxLDL which is highly expressed on endothelial cells (31), and mediates endocytic uptake and subsequent lysosomal degradation of oxLDL. LOX-1 is highly expressed by endothelial cells covering early atherosclerotic lesions (32), indicating a crucial role as an initiator as well as accelerator for the formation of atherosclerotic lesions.

The macrophage scavenger receptor SR-PSOX was identified by expression cloning from a cDNA library. Human SR-PSOX is a type I membrane protein of 254 amino acids that shows no homology to other scavenger receptors. SR-PSOX recognizes phosphatidylserin and oxLDL and is predominantly expressed in lipid laden macrophages of human atherosclerotic lesions (33).

Necrotic core

Dysregulated uptake of modified forms of LDL by scavenger receptors leads to foam cell formation (5). At the early stage of atherosclerosis, foam cells form clusters. The

intensive aggregations of these cells lead to the formation of an atheromatous core within the wall and to the development of fatty streak lesions. This process is accompanied by the migration of smooth muscle cells to the intima. These proliferate and secrete extracellular matrix proteins and collagen and form a fibrous cap overlying the central core that together called as fibrous plaque (7). This phase of the development of atherosclerosis is influenced by interaction of macrophages and T cells expressing Th1 and Th2 cytokines resulting in both humoral and cellular responses. In addition to macrophages, endothelial cells and smooth muscle cells are also activated and produce proinflammatory cytokines such as TNF-alpha, IL-6 and MCP-1. Later, these inflammatory processes and the toxicity of oxidized LDL for macrophages (10) can cause the death of lipid-loaded macrophages and smooth muscle cells by apoptosis and/or necrosis.

In early atherosclerosis, macrophage apoptosis is a frequently occurring event. This early lesional macrophage apoptosis modulates the cellularity of the lesion and decreases as lesion development progresses. In late lesions, macrophages also undergo apoptosis, albeit by mechanisms that are probably unique to these lesions, such as free cholesterol (FC) loading and exposure to oxysterols. In this setting, however, phagocytic clearance is not efficient and secondary necrosis of the macrophages ensues (34). This event leads to the generation of the necrotic cores consisting of lipids, cholesterol crystals and cell debris. Inflammatory factors in the necrotic core may stimulate surrounding macrophages to secrete matrix-degrading metalloproteinases (MMP) in atheroma that facilitate plaque destruction (35).

Despite this, lesions may remain quiescent in fibrous plaque state for many years. Such lesions may never produce any symptoms during the course of a patient's lifetime. At some point, lesion activation is initiated by rupture of the atherosclerotic plaque, leading to the rapid onset of clinical symptoms, including Acute Coronary Syndromes or stroke.

Plaque rupture

Once a fibrous cap is formed over an atherosclerotic lesion, it becomes subject to a number of stresses due to both blood pressure and shear on the luminal surface of the artery. This fibrous cap can remain thick and replete with smooth muscle cells that produce collagen adding to the stability of the plaque. Under these circumstances, shear forces and mechanical stress from arterial pressure on the plaque are not met with any structural failure, and the lipid core remains isolated from the circulation. However, in certain cases, often associated with

inflammation and cytokines, components of the plaque may change such that the shoulder regions of the atherosclerotic plaque become increasingly populated by macrophages and T cells. Once inside the vessel wall, infiltrating cells interact with ECM, oxidized lipids, and with each other. All of these interactions have been shown to increase production of pro-inflammatory cytokines such as IL-1 and TNF-α and matrix-degrading enzymes (36, 37). These enzymes include serine proteases, tissue-type and urokinase-type plasminogen activators, plasmin, the matrix metalloproteninases (MMPs), and cysteine proteases (14). Cytokines also promote the apoptosis of vascular smooth muscle cells. In this scenario, the plaque becomes more acellular and contains less interstitial collagen. When the structural components of the fibrous cap are injured and degraded by a variety of MMPs, the plaque contents are exposed to the luminal surface of the artery. Since the lipid core contains a number of prothrombotic components, contact with blood leads to a thrombotic response that may precipitate a vascular insufficiency with catastrophic consequences such as heart attack or stroke.

Atherosclerosis as a chronic inflammation

Russell Ross published a remarkable review in the *New England Journal of Medicine* entitled: "Atherosclerosis: a chronic inflammatory disease" in 1999 (38). The view that atherosclerosis is indeed a chronic inflammatory disease initiated by monocyte/lymphocyte adhesion to activated endothelial cells (EC) is now widely accepted and substantiated by experimental and clinical observations.

The presence of immune cells and their products in the human and/or experimental atherosclerotic lesion indicates their participation in lesion biology. A number of immune cell types have been detected in human atherosclerotic plaques, including macrophages, dendritic cells, CD4+, CD8+ T cells (39), and several distinct pro-inflammatory (TNF-α, IL-1β, IL-12, IL-18, IFNγ) and anti-inflammatory (TGFβ, IL-4, IL-10) cytokines (40). These observations support the hypothesis that both innate and adaptive immune mechanisms are involved in atherogenesis.

Innate immunity provides the first line of defence for the host and its activation generates fast and blunt responses. It is characterized by a natural selection of germline-encoded receptors,

which focus on highly conserved motifs in pathogens. It involves several cell types not only specialized cells. In the case of atherosclerosis, endothelial cells, vascular smooth muscle cells, and most importantly macrophages (and dendritic cells) are all involved in generating innate immune responses. These cell express a limited repertoire of highly conserved pattern-recognition receptors (PRRs), such as scavenger receptors, Toll-like receptors (TLRs) and IL-1 receptor (IL-1R) (41, 42) The importance of innate immunity in atherogenesis has been verified in knockout animal experiments. Chi *et al.* showed that the complete absence of IL-1R1 markedly reduces the progression of atherosclerosis in ApoE-/- mice. This effect of IL-1R1 absence persisted whether the inciting factors were genetic, dietary, or infectious, alone or in combination; thus, ApoE+/-/IL-1R1-/- mice have reduced atherosclerotic lesions compared with ApoE+/-/IL-1R1+/- mice, whether fed chow or an high fat diet, and whether inoculated with an oral pathogen, *P. gingivalis* or not (43). The recruitment of monocytes is also essential for lesion formation, as hypercholesterolemic mice that are deficient in MCP-1 or in expression of CCR2 (its cognate receptor on monocytes) have a greatly reduced incidence of atherosclerosis (44, 45). Similarly, hypercholesterolemic *Op/Op* (*Csf1−/−*) mice, which lack monocyte colony-stimulating factor and therefore lack differentiated macrophages in their tissues, show minimal atherosclerosis (46). Macrophages and dendritic cells are important in adaptive immunity in their capacity to ingest pathogens and present antigens to initiate adaptive immune responses.

In contrast, adaptive immunity is more precise but slower in mounting an adequate response to challenge. Specific molecular structures on antigens are recognized by antigen receptors, such as T-cell receptors (TCRs) and B-cell receptors (BCRs), which provide great specificity and affinity to the antigen. Dendritic cells and macrophages can activate T cells presenting the antigen through their class I or class II major histocompability complex (MHC). T cells are prominent components of both the early and late lesions. Most T cells in lesions bear CD3 and CD4 markers and ab-TCR. These represent about two-thirds of all CD3+ cells in advanced human lesions and more than 90% of T cells in lesions of *ApoE −/−* mice (47). In addition, moderate numbers of CD8+ T cells and relatively few B cells can also be found, although they mainly localised in the adventitia surrounding lesions (48, 49).

Cytokines and chemokines

In 1989, Balkwill and Burke defined cytokine as "one term for a group of protein cell regulators, variously called lymphokines, monokines, interleukins, interferons, which are produced by a wide variety of cells in the body, play an important role in many physiological responses, are involved in the pathophysiology of a range of diseases, and have therapeutic potential"(50). Nowadays, the cytokine family consists of more than 50 secreted factors involved in intercellular communication and regulation of fundamental biological processes. Cytokines play an essential role in all of the steps of the development, progression and pathological complications of atherosclerosis. Their production is induced by a range of triggers. Primary triggers include modified LDL and lipid oxidation products such as oxidized phospholipids and lysophosphatidylcholine. Secondary triggers are heat shock proteins, infectious agents, matrix metalloproteinases, adipokines and platelet products. Once cytokine release is initiated and the atherosclerotic lesion developed, a number of factors can participate in maintaining and amplifying cytokine production.

Macrophages are a major source of cytokines in the atherosclerotic plaque. Their repertoire of expression is huge, including the pro-inflammatory cytokines TNF-α, IL-1, IL-6, IL-12, IL-15, and IL-18, as well as the anti-inflammatory cytokines IL-10 and TGF-β (51).

Although T and B lymphocytes, the detector cells of adaptive immune responses, differ entirely from those of innate immunity, the effector pathways used by both overlap to a great extent. Thus, T cell activation also leads to secretion of a range of cytokines. In atherosclerotic lesions, T cells express both Th1 and Th2 cytokines (8). Th1, the most prevalent type of CD4+ cells, induces macrophage activation and promotes inflammation by secreting interferon-γ (IFN-γ) and tumor necrosis-α (TNF-α), important pro-inflammatory cytokines. However, Th2 cells suppress inflammation and attenuate macrophage activity via several anti-inflammatory cytokines, including interleukin-4 (IL-4), interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). Hansson and colleagues (52) determined the expression profiling of Th1 and Th2 cytokines in advanced human atherosclerotic plaques. They found that IL-2 was present in 50% of plaques, and IFN-γ was detected in some but not all of the IL-2-positive plaques. In contrast, the expression of IL-4 and IL-5, both Th2 cytokines, and TGF-β is produced by both Th1 and Th2 cells. Others have found that both IL-10 (53) and TGF-β are abundantly expressed in all plaques (54). Both Th1 and Th2 cells play

a role throughout the development of atherosclerosis, Th1 being predominant during the initiation of lesion formation with a switch toward an pro-atherogenic Th2 response in the chronic phase of plaque development (55). The balance between pro-inflammatory and anti-inflammatory cytokines is a crucial determinant in disease development and progression.

Chemokines (chemotactic cytokines) are also produced in atherosclerotic plaques. Chemokines belong to a large superfamily of low-molecular-weight secreted proteins with a highly homologous three-dimensional structure. They have been classified into four subfamilies, CC, CXC, CX3C, and XC, depending on the relative position of the first two N-terminal cysteines. The receptors for chemokines were found to be seven transmembrane-spanning receptors that signal through G-protein interactions (56). Chemokines are produced by virtually all somatic cells, including the cellular constituents of the vessel wall, in response to inflammatory stimuli. Among the chemokines that are found in atherosclerotic lesions, monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8)/CXCL8 have attracted intense interest.

IL-8/CXCL8

Interleukin-8 (CXCL8, IL-8), a member of the CXC chemokine family, was originally identified as a potent chemotactic factor playing a central role in migration and activation of neutrophils. It is a multifunctional chemokine involved in many biological processes. Pro-atherogenic oxidized low density lipoprotein has been shown to induce IL-8 secretion in primary human peripheral blood monocytes (57) and IL-8 produced by macrophages as well as CXCR2 expression has been shown within atherosclerotic lesions (58, 59). IL-8 has been shown to be a potent chemotactic factor for both T lymphocytes and vascular smooth muscle cells and is also mitogenic for the latter. Migration and proliferation of smooth muscle cells are another hallmark of atherosclerosis, and neovascularization is also a commonly observed feature of atherosclerotic lesions (57).

Signalling events

Macrophages express receptors that recognize a broad range of molecular patterns foreign to the mammalian organism but commonly found on pathogens. These pattern

recognition receptors (PRR) include various scavenger (ScRs) and Toll-like receptors (TLRs) (60). Their ligands contain pathogen associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), surface phosphatidylserine, and aldehyde-derived proteins. Whereas ligation of scavenger receptors leads to endocytosis and lysosomal degradation of the recognized particles (61), engagement of TLR transmits transmembrane signals that activate nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways (62-64).

NF-κB and MAPK signal processing pathways are shared by all eukaryote species from unicellular organisms to mammals. The activation of these pathways is controlled via multiple mechanisms, including intracellular localisation of various components, expression and regulated degradation of inhibitory subunits and phosphorylation of effector members by upstream kinase components of the pathway. Similarly, a variety of mechanisms have been described to be key in the inactivation of the signalling systems. These include activation of phosphatases, degradation of active components and de novo expression of inhibitory proteins.

NF-κB pathway

The NF-κB pathway is one of the main signalling pathways activated in response to pro-inflammatory cytokines, including TNF-α, IL-1, and IL-18, as well as following activation of the Toll-like receptors (TLR) by the pattern recognition of pathogen-associated molecular patterns. Activation of this pathway plays a central role in inflammation through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes. Activated NF-κB has been identified in SMC, macrophages, and EC of human atherosclerotic lesions (51).

The recognition of lipopolysaccharide as a PAMP from Gram-negative bacteria is mediated via the three membrane proteins CD14, MD2, and TLR4. Although CD14 has numerous ligands, TLR4 and MD-2 provide greater specificity for LPS. Activation of TLR4 triggers several subsequent steps including the recruitment of intracellular scaffold proteins (such as MyD88, TIRAP, and Tollip), autophosphorylation of IRAK and ubiquitination of TRAF6 (family of proinflammatory signal-transducing adapter proteins.) TRAF6 associates with the

MAP3 kinase TAK1. From TAK1, two signalling pathways diverge; one ultimately leads to NF-kB activation and the other to MAP kinase activation (51).

MAPK pathway

MAPKs are evolutionary conserved enzymes connecting cell-surface receptors to critical regulatory targets within cells. One of the most explored functions of MAPK signalling modules is regulation of gene expression in response to extracellular stimuli. MAPKs activate transcription factors without affecting DNA binding and also regulate gene expression through post-transcriptional mechanisms involving cytoplasmic targets.

MAPKs regulate cell proliferation, control cell survival, death and cell motility. It has been demonstrated that activation of MAPK cascades occurs in response to a wide range of stimuli, including pro-inflammatory cytokines, growth factors, mechanical stimuli (stress) and integrin dependent cell/matrix interactions (65). Actual roles of each MAPK cascade are highly cell type and context dependent (66).

Many MAPKs activate specific effector kinases, MAPK-activated protein kinases (MAPKAPKs), and are inactivated by MAPK phosphatases. All MAPKs recognize similar phosphoacceptor sites composed of a serine or threonine followed by a proline, and the amino acids that surround these sites further increase the specificity of recognition by the catalytic pocket of the enzyme (66).

The protein kinases that form MAPK signalling modules may interact via a series of sequential binary interactions to create a protein kinase cascade. The signalling network comprises of a three level kinase cascade: MAPKKK (MEKKs and MLKs, for instance), MAPKK (MKK3, 6, 4, 7 and MEK1) and MAPK (p38, JNK and ERK). Three parallel cascades are now commonly described: p38, the extracellular signal regulated protein kinases (ERK), and stress activated protein kinase/c-Jun N-terminal kinases (JNK).

The insulin/mitogen-regulated extracellular signal regulated kinase (ERK) pathway was the first mammalian MAPK pathway to be identified. The kinase components of the ERK module contain a linear cascade consisting of the Raf (MAP3K), MEK (MAP or ERK kinase-

MAP2K) and ERK (extracellular signal-regulated kinase-MAPK) kinases. In mammalian cells monomeric GTPases of the Ras superfamily are potent upstream activators of signal transduction pathways, and Ras is pivotal to the activation of the ERK pathway. Ras regulates ERK predominantly through triggering the activation of the Raf-1. There are three members of the Raf-1 family (Raf-1, B-Raf, and A-Raf), two distinct MEK proteins (MEK1 and MEK2), and two ERK members (ERK1 and ERK2).

The second identified pathway was the stress-activated protein kinases/c-Jun NH2-terminal kinases, a family of MAPKs activated by environmental stresses and inflammatory cytokines of the TNF superfamily.

The p38 MAPKs are a second mammalian stress activated MAPK family. Originally described as a 38-kDa polypeptide, that underwent Tyr phosphorylation in response to endotoxin treatment, and osmotic shock. Potent upstream activators of JNK and p38 pathways are members of the Rho subfamily of the Ras superfamily. They regulate JNK through MKK4 and MKK7. p38 MAPK is activated by MKK3, MKK4, and MKK6.

Each MAPKK, however, can be activated by more than one MAPKKK, increasing the complexity and diversity of MAPK signalling. These MAP3Ks include TAK1 and members of the ASK1, MEKK, and MLK families (67, 68). This complexity extends to the MAP3K enzymes in these pathways, with multiple protein kinases with different selectivity for activation of JNK, p38 MAPK, or both of these.

Whilst Jun kinases (JNK) and p38 MAPKs have been implicated in responses primarily to stress (heat, hypoxia, chemical, oxidative, etc. and pro-inflammatory cytokines), extracellular signal regulated protein kinases (ERK) primarily respond to mitogenic stimuli such as growth factors (PDGF) (69), oxidised LDL (70) or Ang II (71) in vascular cells. In most cases a given stimulus will activate more than one group of MAPKs. The specific contribution of each MAPK pathway to a physiological response through different transcriptional factors varies from cell type to cell type. In some cases, MAPK pathways can co-operate, but they can antagonise in others (72, 73). Whilst activation of MAPK cascades occurs under physiological conditions, increased MAPK activity has been reported in a number of pathogenic conditions, including cardiovascular cellular responses (74, 75).

A large body of literature suggests that MAPKs may be organised into multiprotein complexes to create a functional signalling module (67, 76-79). This organisation modulated

by scaffold proteins, which interact with each of the protein kinases and control the spatial organisation of MAPK signalosomes. These scaffold complexes allow for the precise regulation of MAPK signalling. Seminal studies of *Saccharomyces cerevisiae* have established that scaffold proteins are indeed physiologically relevant to the regulation of MAPK modules in yeast. Thus the mating response is regulated by a MAPK module that is coordinated by the scaffold Ste5p (78), the first MAPK cascade scaffold to be described (79). A number of mammalian MAPK scaffolds have been reported in recent years (67). Most of these proteins have been shown to associate one or more MAPKs, MAPKKs or MAPKKKs. The best known scaffold proteins are the JNK interacting proteins (JIP-1,2,3,4), kinase suppressor of Ras (KSR), and the MEK-partner 1 (MP1). The MAPK scaffolds are important signal transducers, facilitating the assembly and activation of MAPK signalling modules. Mathematical modelling indicates that a scaffold protein can confer complex kinetic regulatory properties on the function of a MAPK module (79, 80). In addition, scaffold complexes can prevent the activation of MAPK modules by irrelevant stimuli and can provide spatial and temporal control of MAPK signalling. Depending on their concentration, scaffolds may be able to potentiate or inhibit MAPK function (67, 80).

Tribbles

Our group described the important role of a recently identified family of proteins, tribbles, in the signal processes of monocytes and smooth muscle cells (81, 82). Tribbles are a novel family of proteins with MAPK modulator, scaffold-like function. Using a genome-wide functional screen for components of inflammatory signalling networks, Kiss-Toth *at al.* identified a novel protein, human homologue of Drosophila tribbles (TRB) (83, 84). A subsequent database search revealed the existence of three mammalian tribbles-like proteins (TRB-1, 2 and 3).

Tribbles was first identified in Drosophila as a gene that is required for gastrulation, oogenesis, and viability (85-87). Tribbles coordinates cell division during gastrulation by promoting turnover of the cell cycle protein string/CDC25, thereby inhibiting premature mitosis. In this context, loss of tribbles function was associated with increased proliferation, whereas overexpression of tribbles slowed the cell cycle (86). Tribbles also promotes the

degradation of slbo, the Drosophila homolog of the C/EBP family of basic region-leucine zipper transcription factors, during oogenesis (88).

In humans there are three tribbles homologues, TRB-1,-2,-3 (89).

TRB-3 is the best studied member of the mammalian tribbles family. Identification of this protein was first reported by two groups in 1999. Mayumi- Matsuda and colleagues (90) described it as a gene that is up-regulated in a neuronal cell line undergoing nerve growth factor withdrawal induced apoptosis. In a study published in parallel, Klingenspor *et al.*(91) screened for differentially expressed genes in the fatty liver dystrophy (*fld*) mouse. In this study, *Trb-3* was reported as *Ifld2*, a gene highly induced in new born animals. The human *TRB-3* gene is located on chromosome 20 at p13-p12.2.

TRB-2 was originally identified as a transiently expressed, mitogen induced, and highly labile cytoplasmic phosphoprotein, induced by thyroid hormone in dog thyroid cells (92). *TRB-2* is located on chromosome 2, at p24.3.

Human TRB-1 was first identified as a homologue of TRB-2 by Wilkin *et al*. (92), based on a partial cDNA sequence. The human gene is located on chromosome 8 at q24.1.

Hegedűs *et al.* recently undertook a systematic data mining to annotate tribbles orthologues and to characterise evolutionary relationships in tribbles protein family between the various eukaryotic species. The unicellular protozoa *Monosiga ovata* represented the earliest point on the evolutional tree where a tribbles-related sequence was identified. This finding is in line with the hypothesis that tribbles are ancient proteins and may have a key role in fundamental cellular processes. There is a single *tribbles* gene in invertebrates; the *Trb-1*, *Trb-2* and *Trb-3* subfamilies began to segregate in fish. Interestingly, neither amphibians nor birds have *Trb-3* related gene while mammals possess all three tribbles subfamilies. This suggests two possible explanations; independent gene duplication of mammals and fish genes then followed by convergent evolution, resulting in the appearance of similar *Trb-3* like genes in both cases or. The mammalian *Trb-3* gene may have evolved directly from the fish counterparts (93).

The proteins encoded by these *tribbles* genes may serve an important regulatory function in modulating the activity of various signalling pathways and transcription factors.

Based on amino acid sequence, tribbles resembles a serine-threonine kinase; however, it has a variant catalytic core that lacks a canonical ATP binding site, and their function is unknown.

Tribbles structure and localization

Tribbles proteins consist of 3 domains: N-terminal domain, kinase-like domain and C-terminal domain. Tribbles kinase-like domain is located in the middle of the protein, with relatively short N- and C-terminal flanking sequences. These N- and C-terminal segments proved to be the less conserved regions within the gene family.

The N-terminal (NT) segment of tribbles proteins is about 60–80 residues long in most of the investigated organisms. The most striking feature of the NT fragment is the very high serine (7–24%) and proline content (6–23%), mostly in the sequence stretch adjacent to the kinase-like domain. The kinase-like domain is highly conserved during evolution. The functionality of tribbles kinase-like domain was addressed in a study by Bowers *et al* (94). These tests did not detect kinase activity for tribbles. The C-terminal fragment of tribbles proteins is in most cases about 35–45 residues in length and is rich in charged amino acids. Some data suggest that this region could be important in establishing protein– protein interactions (89).

A number of studies have investigated intracellular localisation of the various tribbles family members. However, it is notable that all of the published data to date is based on overexpression.

When expressed as a GFP fusion protein, TRB-1 was localized in the nucleus of HeLa cells. The N-terminal, proline-rich domain is necessary for this localisation. Wilkin *et al.* used a myc-tagged expression construct of TRB-2 and found that the protein expressed was mainly localised in cytoplasmic granules. Our group has recently shown a distinct cytoplasmic localisation for TRB-2, again by the use of GFP fusion proteins.

TRB-3-GFP fusion protein is nuclear in Cos-7, GT1-7, CHO, HeLa, 293 cells, transfected with FLAG tagged TRB-3 also showed nuclear staining (89).

Cell type specific function

Many aspects of tribbles action appear to be cell type and/or organism specific. It has been shown that tribbles are able to bind to various MAPKKs and that their concentration regulates preferential activation of the different MAPK pathways, presumably leading to different cellular responses (95).

Sung *et al* demonstrated that the pattern of tribbles regulation is strongly cell-type dependent. They found whilst *TRB-2* expression is up-regulated at 6 h in monocytes, mRNA levels of the

same gene decrease sharply (and transiently) in response to an identical stimulus (IL-1β) in synovial fibroblasts. Similarly, *TRB-3* expression was differentially regulated in the various cell types tested. Whilst low mRNA levels are detected at 3 and 6h in synovial fibroblasts and in vascular smooth muscle cells (VSMC), *TRB-3* expression is up-regulated in THP-1 cells with the highest levels observed at 10 h. The dynamics of expression for these genes was also influenced by the cell types tested. *TRB-1* was rapidly and very transiently upregulated in VSMC and THP-1, whilst a profound but delayed activation was observed in synoviocytes. Their results also demonstrate cell-type specificity for tribbles action. Whilst both TRB-1 and -3 had the ability to block AP-1 activation in epithelial cells, only TRB-3 was active in macrophages and neither of these proteins interfered with AP-1 activation in fibroblast cells (82).

The hypothesis for the molecular mechanism of action is that tribbles may compete for the binding site with the MAPKs, thus regulating their activation. This model may explain why evolution preserved a catalytically inactive kinase domain from unicellular organisms to mammals (95).

Hypothesis and aims of the study

The hypothesis to be tested by my studies was that tribbles proteins act as regulatory molecules in inflammatory processes through modulation of MAPK signalling. Thus they may have a role in atherogenesis, especially in the inflammation induction role of macrophages.

Aims of my studies were to investigate the role of human tribbles, particularly human TRB-2, in human monocyte cells function *in vitro* and to elucidate related molecular mechanism by which tribbles interact with protein kinases and regulate activity of binding partners or activation of down stream protein molecules.

We examined:

- the uptake of modified LDL molecules by monocyte

- the role of MAPK pathways and tribbles in LDL uptake by monocytes

- the effect of modified LDL molecules on TRB-2 expression level

- the effect of TRB-2 on LPS induced IL-8 production

- and the role of the MAPK pathways in IL-8 production.

Materials and methods

Ethics:

Human samples were obtained under the ethical approval granted by the North Sheffield Research Ethics Committee. This study conforms to the principles outlined in the Declaration of Helsinki.

Cell culture:

Human monocytic leukaemia THP-1 cells were purchased from ATCC and maintained in RPMI (Gibco, Invitrogene) supplemented with 10% foetal calf serum (FCS), L-glutamine and penicillin-streptomycin.

THP-1 transfection with DharmaFECT

Transfections were performed using DharmaFECT 2 transfection reagent (Dharmacon, Inc., Lafayette, CO USA) according to the manufacturer's recommendation. For experiments $1,7x10^5$ cells were used on 6 well plates, per transfection.

THP-1 electroporation with siRNA

Transfections were performed using Nucleofector (Amaxa AG, Cologne Germany) using program U-001 and Cell line Nucleofector Kit V solution (Amaxa AG, Cologne Germany). For most experiments, 1.0×10^6 cells were used per nucleofection. siRNA SmartPool against human *TRB-1,2,3* and *MAPKK*s were purchased from Dharmacon (Dharmacon, Inc., Lafayette, CO.) and used according to the manufacturer's recommendation. After transfection, cells were incubated for 4 h. After 4 h cell were divided into 24well plate $1,5x 10^5$ cell per well.

ELISA:

THP-1 cells were transfected by specific siRNA, stimulated for 36 h by 100 ng/ml LPS and the production of IL-8 was measured by ELISA (R&D Systems, Minneapolis, MN USA).

LDL uptake

Monocyte uptake of acLDL (Molecular Probes, Eugene, OR USA) was evaluated by flow cytometry using lipoproteins labelled with the fluorescent probe Dil (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate). $1,5x10^5$ THP-1 cells were treated with 5μg/ml Dil-labeled acLDL for 1, 2, 4 and 24 h. At the end of incubation cells were washed with PBS and then examined on FACScan cytometer (Becton Dickinson, Franklin Lakes, NJ USA). The Dil fluorescence was recorded on channel FL2 and analyzed by the CellQuest program.

MAPK inhibitor treatment

MEK1 inhibitor (PD98059), p38 MAPK inhibitor (SB203580) and JNK MAPK inhibitor (SP600125) were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany) and used at 20 μM for MEK1 and JNK MAPK inhibitors and 0,2 μM for p38 MAPK inhibitor. The cells were treated for 1 h with inhibitors before the LPS treatment.

RNA isolation and Quantitative Real-time PCR analysis

Total RNA was extracted from whole blood of patients with unstable angina and chronic stable angina using QIAamp RNA blood minikit (Qiagen Gmbh, Hilden Germany) according to the manufacturer's description. *TRB-2*, and *β–actin* genes were analyzed by Quantitative real time PCR using ABI prism 7900 sequence detection system (Applied Biosystems, Foster City, CA USA). The sequences of all primers and probes used are listed in Table1. To quantify transcript levels of the *TRB-2* gene, *β–actin* was used as a house keeping control, and each sample was normalized with respect to its *β–actin* transcript content.

Statistical Analysis

Experimental data was analysed by the PRISM (GraphPad) package, using the appropriate tests, as indicated in the figure legends.

Results

LDL uptake

Previous data shows that activation and subsequent recruitment of monocytes to the developing lesion of the arterial wall has an important role in the progression of atherosclerosis. Monocytes internalise LDL particles and this process leads to differentiation of monocytes into foam cells, which produce cytokines and chemokines. MAPK signalling pathways, which are involved in inflammatory responses and LDL uptake by monocytes, can be modulated by tribbles.

We first examined which cell activators affect LDL uptake. We treated the cells with 100 ng/ml LPS or 50 ng/ml PMA for 24 h. After the incubation we added 5 μg/ml Dil-acLDL to the cells. After 4 h incubation we measured the fluorescence of THP-1 cells by flow cytometry. Only the LPS treatment enhanced the LDL uptake. (Fig.1.)
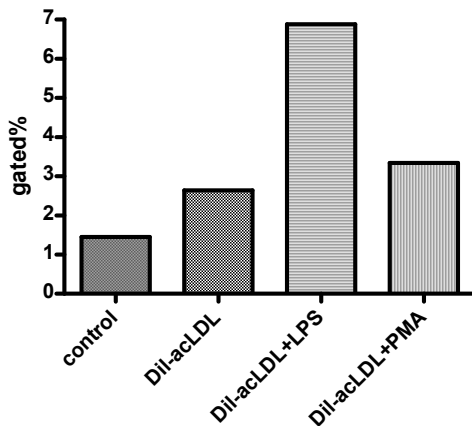

Figure 1. **LPS but not PMA potentiates AcLDL uptake of THP-1 cells**. The impact of cellular activation on acLDL uptake was measured by co-incubation of THP-1 cells and acLDL in the presence or absence of 100 ng/ml LPS or 50 ng/ml PMA for 24 h, as indicated. Flow cytometry was used to assess the level of LDL uptake.

To determine whether the fluorescence of cells was due to an active uptake or passive cell surface binding of LDL, we incubated cells with Dil-acLDL at 37 ^{o}C or 4°C for 4h. As the uptake is an active process it only occurs at 37°C, whilst binding can be readily detected at 4°C. As negative control we added unlabelled LDL to cells also. Uptake was quantified by flow cytometry. Fluorescence was enhanced in the 37°C treated cells only. (Fig.2.)
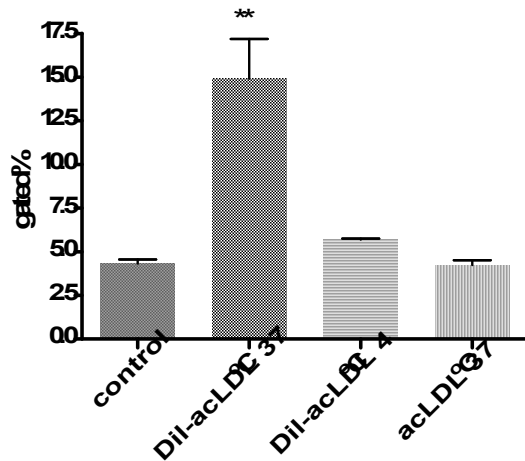
Figure 2. **Dil-acLDL is taken up by monocytes via an active molecular mechanism.** THP-1 cells were incubated with dil-acLDL for 4 h at 37 ∘C or 4 ∘C, as indicated. Uptake was quantified by flow cytometry. Student's t-test was performed to analyse the results. **: p<0.01

To characterize the dynamics of acLDL uptake by THP-1, LDL was added to the cells for 1, 2, 4 and 16 h. Activation of cells was achieved using 100 ng/ml LPS. There was a significant difference between LPS treated and non treated samples at each time point with the highest uptake observed after 16 h. The latter time point was therefore used in subsequent experiments. To determine optimal dose of LDL we added 5, 10 and 15 μg/ml Dil-acLDL to cells for 4 h. The difference between the uptake of LPS treated and non-treated cells was significant at all doses. We therefore used the lowest concentration for subsequent experiments. (Fig.3.)
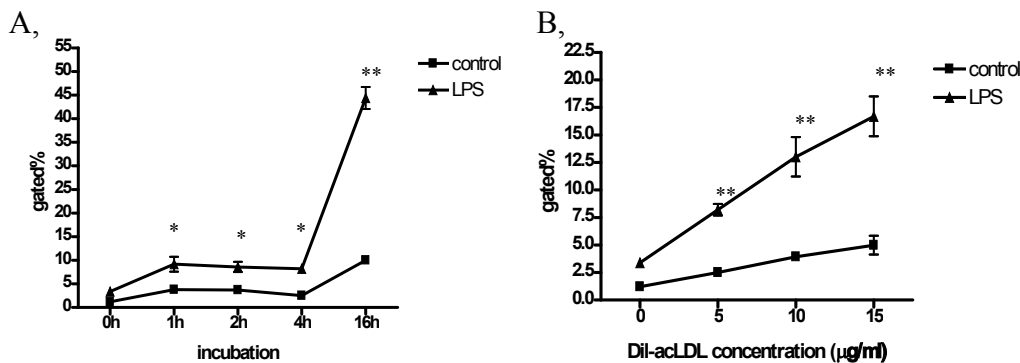
Figures 3. **The dynamics of acLDL uptake by THP-1 cells** was investigated by Dil labelled acLDL and analysed by FACS. (A) THP-1 cells were incubated with Dil-acLDL in the presence (black) or absence (white) of LPS for varying times and the dynamics of acLDL uptake was measured by flow cytometry. (B) The impact of LPS on acLDL uptake was assessed using an increasing concentration of Dil-acLDL, as indicated. Student's t-test was performed to analyse the results. * p<0.05, **: p<0.01

Role of MAPK pathway in acLDL uptake by THP-1 cells

Several reports suggest that expression of scavenger receptors is regulated via the MAPK pathways (96-98). However, the link between LDL uptake, inflammatory activation and MAPK signalling in monocytes is much less established. Therefore, the effect of pharmacological inhibition of specific MAPKs on acLDL uptake was tested. Cells were treated with MAPK inhibitors in the presence or absence of LPS (Figure 4). The results show that blocking the activation of JNK pathway lead to a marked elevation of acLDL uptake that was independent of inflammatory stimulation. Whilst the ERK and p38 pathways do not influence the uptake of acLDL under non-inflammatory conditions, both pathways led to a significant decrease in acLDL uptake after LPS stimulation. These observations demonstrate that the enhanced capacity to take-up acLDL by inflammatory activated THP-1 cells is mediated by specific MAPK pathways.
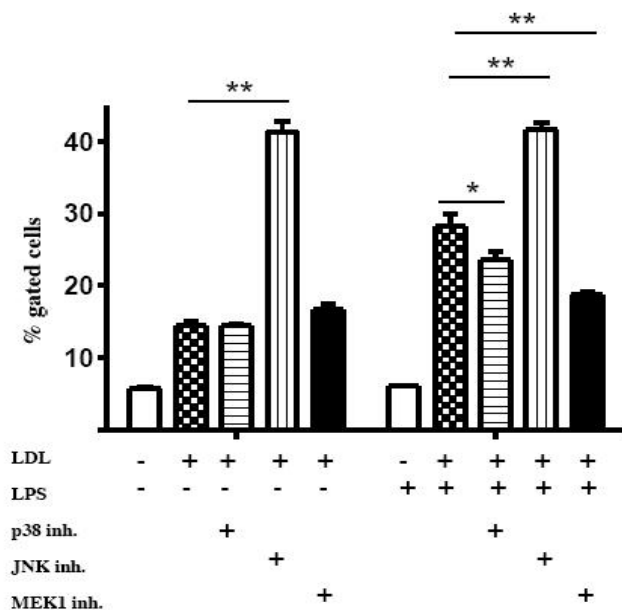

Figure 4. **acLDL uptake is regulated by MAPK signalling pathways.** Blocking the activation of JNK pathway lead to a marked elevation of acLDL uptake, this is independent of inflammatory stimulation. Whilst the ERK and p38 pathways influence the LDL uptake only under inflammatory conditions.

The effect of knockdown of tribbles on the uptake of acLDL in THP-1 cells

As stated above, tribbles regulate MAPK activation. The effect of trb on LDL uptake was therefore tested. THP-1 cells were transfected with siRNA against *TRB-1,-2,-3* using lipotransfection methods (DharmaFECT). After transfection Dil-labelled acLDL was added to

cells and LDL uptake measured by FACS. There were no significant differences in LDL uptake between si*TRB*s or siNc transfected cell.

To determine whether another siRNA transfection method would produce similar results, cells were electroporated with Nucleofector kit. This produced similar results and it can be concluded that reduced tribbles level do not affect the LDL uptake. (Fig. 5. a, b)
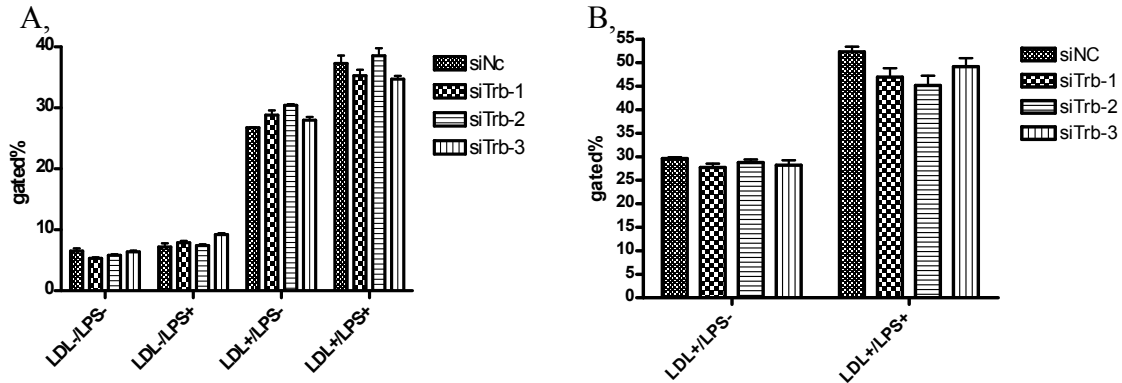

Figure 5. **si*TRB-2* lipofection (A) and electroporation (B) do not modulate the LDL uptake of THP-1 cells**. THP-1 cells were transiently transfected with control (siNC) or tribbles specific siRNA. The impact of reduced tribbles levels on acLDL uptake was measured in the presence or absence of LPS, as previously.

The effect of overexpression of tribbles on acLDL uptake

To complement the knockdown studies, THP-1 cells were transiently transfected with control or tribbles expression constructs and incubated with acLDL, in the presence or absence of LPS, as above. Similar to results obtained in knockdown experiments, overexpression of these proteins did not affect the ability of cells to take up acLDL (Fig.6.)
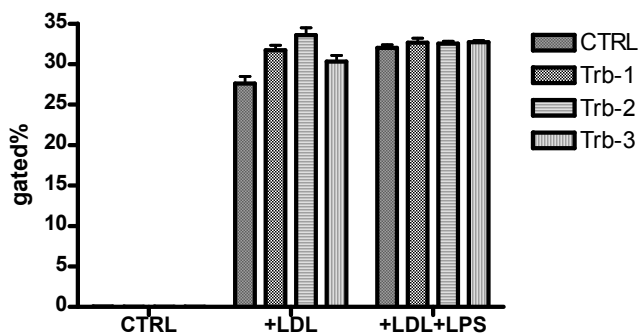

Figure 6. **Modulation of tribbles expression does not influence the LPS potentiated acLDL uptake of THP-1 cells.** THP-1 cells were transiently transfected with control (mock) or tribbles expression constructs. The impact of elevated tribbles levels on acLDL uptake was measured in the presence or absence of LPS, as above.

The effect of LDL on LPS induced IL-8 production and tribbles expression

Tribbles expression does not appear to regulate LDL uptake in THP-1 cells. In order to characterize the contrary effect we investigated the role of LDL uptake on cytokine and tribbles expression.

To investigate the effect of LDL uptake on cytokine expression, THP-1 cells were treated with or without acetylated LDL (acLDL) after LPS stimulation, and the amount of IL-8, as a biologically relevant marker of inflammatory activation of monocytes, was measured by ELISA. The uptake of acLDL significantly enhanced IL-8 production. (Fig.7.)

TRB-2 mRNA expression levels were also measured by using qRT-PCR and were found to be down-regulated by acLDL after 4 h and more efficiently after 24 h (Fig.8.a,).

hTRB-2	Forward	CATACACAGGTCTACCCCC
	Reverse	TCCGCGGACCTTATAGAC
	Probe	FAM-CTTCGAAATCCTGGGTTTT-TAMRA
β-actin	Forward	GGATGCAGAAGGAGATCACTG
	Reverse	CGATCCACACGGAGTACTTG
	Probe	FAM-CCCTGGCACCCAGCACAATG-TAMRA

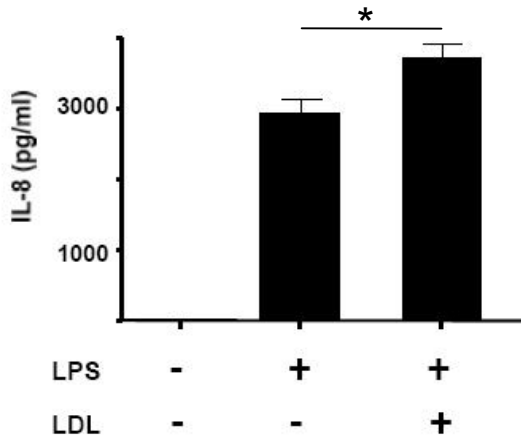*Table 1*. List of the secuences of primers and probes.

Figure 7. **AcLDL potentiates LPS induced IL-8 production.** The impact of 24hrs acLDL and LPS treatment alone and in combination was studied on IL-8 protein production in THP-1 cells. Cells were lysed and IL-8 levels were quantified by ELISA. One way ANOVA with Dunnett's Multiple Comparison Test was performed to analyse the results. *: $p < 0.05$
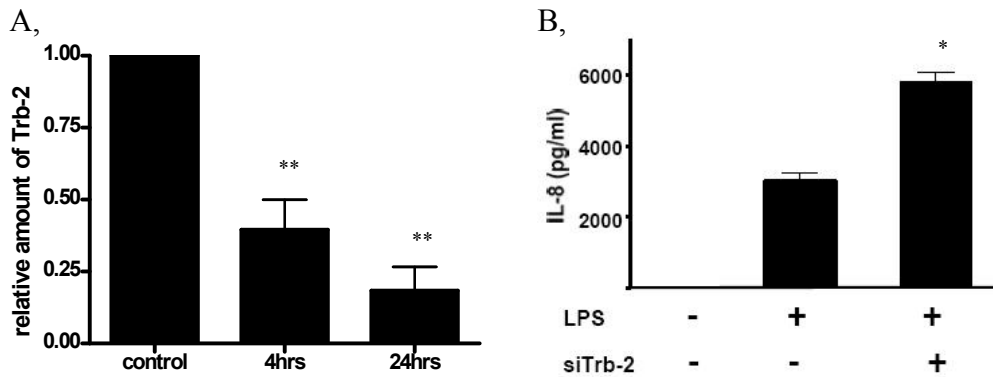
Figure 8. (A) **In THP-1 cells *TRB-2* mRNA level was down-regulated by acLDL treatment** after 4h and more efficiently after 24hours. *TRB-2* expression was measured in response to acLDL treatment by qRT-PCR. In order to assess statistical significance, one way ANOVA test with Dunnett's Multiple Comparison Test was performed. **: $p<0.01$ (B) **Reduced TRB-2 level enhanced the IL-8 production.** The impact of reduced TRB-2 levels on LPS induced IL-8 production was measured by ELISA. Student's t-test was performed to analyse the results. $p=0.041$

Role of JNK and ERK pathways in modulation of IL-8 production by TRB-2

To examine the effect of TRB-2 on cytokine expression TRB-2 levels were suppressed by transfection of siRNA against *TRB-2* and IL-8 release measured. IL-8 production was induced in siRNA transfected cells by LPS treatment, as above. The results in Figure 8.b, show that si*TRB-2* treated THP-1 cells produce significantly higher levels of IL-8, compared to cells transfected with control siRNA.

Mitogen-activated protein kinases (MAPK) are a family of serine / threonine specific kinases which, besides playing a role in regulating cell growth, migration and differentiation, are implicated in the development of atherosclerosis. Thus MAPKs, p38 MAPK and ERK 1/ 2, and JNK 1/2 appear to be implicated in the regulation of IL-8 by human macrophages. In addition previous data suggest that tribbles are mediators of MAPK signalling.

Selective MAPK inhibitors were used to block individual pathways and investigate their involvement in IL-8 production by THP-1 cells. These data demonstrate that blocking of JNK and ERK but not p38 pathways suppresses IL-8 production by monocytes. (Fig.9.) To further investigate the impact of MAPK on IL-8 production, THP-1 cells were transfect with si*TRB-2*, and treated with MEK1 and JNK inhibitor. These inhibitors attenuated the cytokine level to the same extent regardless of *TRB-2* levels.
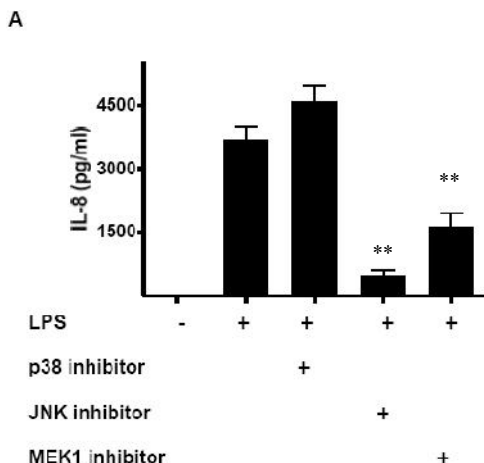
A

Figure 9. **The involvement of MAPK pathways in LPS induced IL-8 production** was measured by the use of inhibitors of specific MAPK pathways. Blocking of JNK and ERK but not p38 pathways suppresses the IL-8 level of monocytes. Cells were lysed and IL-8 levels were quantified by ELISA. One way ANOVA with Dunnett's Multiple Comparison Test was performed to analyse the results. **: $p < 0.01$

TRB-2 modulation of IL-8 production via interaction with MAPKKs

Previously, we demonstrated that TRB-1 and -3 proteins interact with MAPKKs and regulate their activity (95). Therefore, the interaction of TRB-2 with MKK4/SEK-1, MKK7 and MEK-1, which are known activators of JNK or ERK, was investigated. The results show that down-regulation of kinase levels led to impaired IL-8 production. These observations are compatible with a negative regulatory role for TRB-2 in control of ERK and JNK activation (Fig.10.)
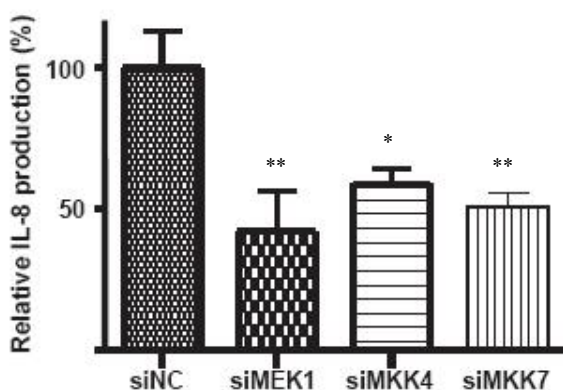
Figure 10. **Down-regulation of MAPKK levels attenuate IL-8 production**.THP-1 cells were transfected with si*MAPKK* or control siRNA (siNC), as indicated and stimulated by LPS. Production of IL-8 was detected by ELISA. One way ANOVA with Dunnett's Multiple Comparison Test was performed to analyse the results. *: $p < 0.05$, **: $p < 0.01$

Relationship of TRB-2 levels and IL-8 produced in response to LPS in primary monocytes

In order to investigate the potential *in vivo* relevance of our findings, responsiveness of primary monocytes from four healthy volunteers was assessed by stimulating cells with LPS or with the combination of LPS and acLDL. IL-8 levels in response to LPS alone were used

as a unit for normalisation and values measured in samples with LPS-acLDL costimulation were expressed relative to these. acLDL increased the amount of IL-8 produced in response to LPS in a manner similar to that seen in monocytic cell lines. (Fig.11.a) Furthermore, the level of IL-8 inversely correlated to the expression of *TRB-2* in primary monocytes. (Fig.11.b)
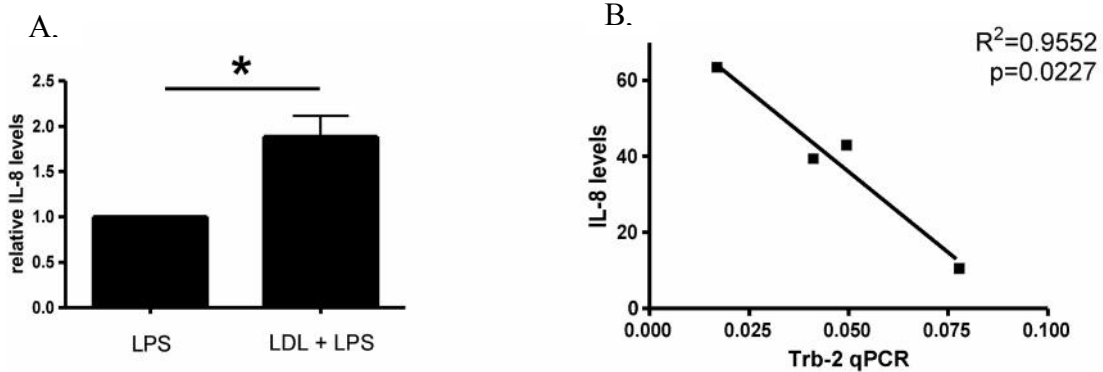

Figure 11. **TRB-2 levels are inversely correlated to the IL-8 produced in response to LPS in primary monocytes** (A) The ability of human primary monocytes to produce IL-8 was assessed. acLDL increased the amount of IL-8 produced in response to LPS (B) The relationship between the amount of IL-8 produced (LPS+acLDL treatment) and *TRB-2* expression was measured by linear regression.

Discussion

Elevated serum LDL levels have been implicated as a major risk factor for atherosclerotic vascular disease. Expression of inflammatory cytokines, chemokines by a variety of vascular cells, proliferation of vascular smooth muscle cell, migration of monocytes have all been linked to increased levels of modified LDL. The uptake of modified LDL by monocytes and differentiation to foam cells is a hallmark of the development of atherosclerotic lesions/plaques. A number of studies have demonstrated that MAPK pathways play a central role in both of these processes. Whilst the involvement of these MAPK pathways is relatively well characterised, the molecular mechanisms that modulate the ability of monocyte/macrophages to respond to inflammatory stimuli in an LDL-dependent fashion remain imprecisely understood. Therefore, the involvement of inflammatory signals in regulating the uptake of modified LDL by monocytes and the role of TRB-2 in monocyte biology has been investigated. The functional links between LDL and inflammatory activation of monocytes has also been explored.

In line with previous findings, inflammatory activation of THP-1 cells via LPS appears to substantially enhance the uptake of acLDL. Further, it was found that PMA stimulation, which is routinely used to differentiate THP-1 monocytes to a macrophage-like phenotype, does not influence this process, at least within the timeframe used in these experiments. Since a relatively short term exposure of monocytes to PMA was used, compared to most differentiation protocols (72-96 hrs), our results may imply that acute activation events, induced by PMA do not influence LDL receptor expression. Pharmacological inhibition of MAPK signalling pathways demonstrates that these signalling systems play a differential role in modulating acLDL uptake. However our results demonstrate that acLDL uptake in THP-1 cells is tribbles-independent.

The data shows clearly that TRB-2 is a novel regulator of MAPK signalling in the context of LPS-induced IL-8 release from a monocytic cell line and human peripheral blood monocytes. AcLDL uptake by monocytes is associated with a reduction in TRB-2 and potentiates LPS-induced IL-8 release. This effect was inhibited by pharmacological inhibition of the ERK and JNK pathways. In line with our previous findings that tribbles interact with MAPKKs and

regulate their activity (95), down-regulation of MEK1 and MKK4, -7 expression levels by siRNA led to impaired IL-8 production in response to LPS.

These data indicate that TRB-2 is an important negative regulator of monocyte IL-8 production in response to LPS and control the augmentation of this response by acLDL (Figure 12.). This is in keeping with the findings of others which support the mechanism of action of tribbles through physical interaction with other signal transduction proteins. Recent reports demonstrate that TRB-3 is able to interact with and modulate the activity of a number of key inflammatory signalling mediators, including p65/RelA (99) ATF4 (100) and Akt (101, 102).
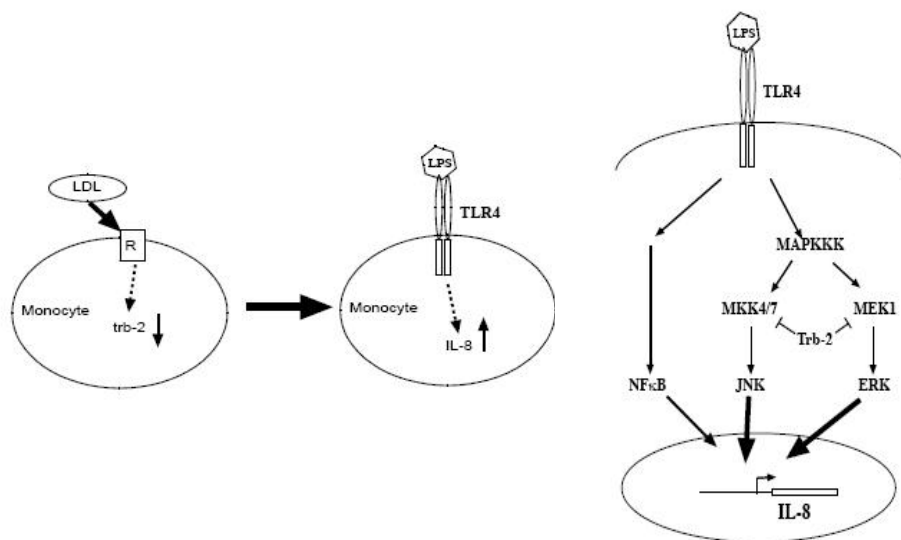
Figure 12. **A model for the role of TRB-2 in monocytes biology in inflammatory settings.** (A) acLDL uptake by monocytes triggers reduction of TRB-2 expression, resulting in a hypersensitive state towards inflammatory stimuli, as exemplified by LPS produced IL-8 production. **(B)** The molecular basis of TRB-2 regulatory function of MAPKK pathways in the expression of IL-8.

Increasing data indicate a convergence of innate immunity to pathogens and atherosclerosis. Several PAMPs can ligate TLRs and/or ScavRs. Some of these ligands participate not only in microbial pathogenesis, but in atherogenesis as well. It has been shown that nonspecific (endotoxin) stimulation of the immune system accelerates atherosclerosis in rabbits on a hypercholesterolemic diet (103). Some evidence has implicated microbial pathogens in human atherogenesis. Bacteria may induce innate immunity, molecular mimicry, and autoimmunity as well as direct infection of tissues. Several studies suggest a role for *Chlamydia pneumoniae*

in atherosclerosis. Interestingly, HSP60 of this microbe resembles human HSP60 and can elicit inflammatory responses (104). Not only the ligands but altered receptors have a role in atherogenesis. It was shown that TLRs are expressed in atherosclerotic lesions and may also participate in inflammatory signalling (105). Although TLR4 deficiency does not decrease atherosclerosis in cholesterol-fed *Apoe* /– mice (106), TLR4 activation by LPS increases atherosclerotic plaque formation in the apoE3*Leiden atherosclerotic mouse model (107). Human epidemiological data demonstrate that an Asp299Gly TLR4 polymorphism, which attenuates receptor signalling, is associated with a decreased risk of atherosclerosis and acute coronary events (108, 109). CD14, the non-transmembrane receptor for lipopolysaccharide, initiates inflammatory responses through interactions with TLRs. A polymorphism in the CD14 promoter, resulting in a significantly higher density of CD14 on monocytes, has been identified as a risk factor for myocardial infarction also (108). Dual stimulus of acLDL (scavenger receptor priming) and LPS (TLR-4 stimulation) of monocytes may be model the human coronary events.

In this study, human primary monocyte co-incubation with acLDL increased the amount of IL-8 produced in response to LPS, similar to that seen in monocytic cell lines, raising the possibility that TRB-2 may also be an important negative regulator of IL-8 production of primary monocytes, in response to LPS. These data are in line with previous findings that IL-8 has an important role in atherogenesis. IL-8 *in situ* hybridization experiments using human coronary atherectomy specimens showed that IL-8 mRNA is expressed in a macrophage-rich area of the lesion, consistent with expression in macrophage foam cells (110). It was also revealed that elevated plasma levels of IL-8 were associated with an increased risk of CAD in apparently healthy individuals (111).

The experiments in this study give evidence that tribbles, a novel group of proteins, can act as regulators of innate immune responses in monocytes. Tribbles appear not only to function in a cell type and possibly stimulus specific manner. Although from these results it is clear that tribbles action is highly specific; they regulate certain aspects of MAPK action (IL-8 expression) but not others (LDL uptake), within the same cell type. The molecular basis of this is currently unknown. Expression of other components of MAPK pathways and/or their state of activation may profoundly influence tribbles action.

Sung *et al* showed tribbles have a role in different aspect of atherogenesis. Their experiments demonstrated that TRB-1 is found in vascular smooth muscle cells *in vivo* and that expression levels are key in modulating the extent of VSMC proliferation and chemotaxis (81). Although tribbles are recently described proteins there are increasing data which show that they are important regulators in very different cellular processes and thereby influence several pathological processes. Their role in tumor cell growth was one of the first described functions of human TRB-3. Bowers *et al.* demonstrated that *TRB-3* mRNA is highly expressed in normal human liver tissue and multiple human tumor cell lines. Northern blot analysis of *TRB-3* mRNA expression in primary human tumor tissue shows that TRB-3 is overexpressed in specific tumor samples including lung adenocarcinoma and colon adenocarcinoma samples. Real-time RT-PCR of TRB-3 across a wide range of tumors from multiple tissues (breast tumors, colorectal tumors, lung tumors) indicates that TRB-3 is overexpressed in specific primary tumor types. They showed with in situ hybridization with a full-length TRB-3 probe that TRB-3 overexpression is not because of highly expressing infiltrating noncancerous cells, but rather is localized to specific cancer cell clusters within each tumor (94).

Keeshan *et al* showed that retroviral expression of *TRB-2* immortalized hematopoietic progenitors in vitro and induced fatal transplantable AMLs in murine recipients. Microarray analysis of a cohort of primary human AMLs identified elevated *TRB-2* mRNA expression in a cluster of tumors associated with a high frequency of C/EBPα mutations. Their studies identified TRB-2 as an oncoprotein that contributes to the pathogenesis of AML through the inhibition of C/EBPα function. This occurs through the interaction of TRB-2 with C/EBPα, resulting in the proteasomal-dependent degradation of C/EBPα. Although the mechanism of *TRB-2* upregulation in these leukemias has not yet been elucidated, their data suggests that TRB-2 overexpression is likely to be an important pathogenic mechanism in a subset of human AML (112).

Naiki *et al.* found that expression of TRB-2 and TRB-3 was immediately down-regulated in response to differentiation stimuli in 3T3-L1 preadipocyte. In fact, forced expression of TRB-2 and TRB-3 inhibited adipocyte differentiation in 3T3-L1 cells. TRB-2 and TRB-3 were shown to have anti-adipogenic effects. While TRB-3 suppressed adipogenesis by strong

inhibition of Akt activation, TRB-2 blocked adipogenesis through the inhibition of Akt activation and degradation of C/EBPβ and C/EBPδ (113).

These results support the observation that tribbles action is highly specific, cell type and stimuli dependent. Tribbles have a role in regulation of several different signalling pathway (MAPK, Akt, CEBP) thereby they may are essential participants in development of pathological processes.

As it was earlier mentioned the tribbles kinase-like domain is highly conserved during evolution. However the functionality of tribbles kinase-like domain was addressed in a study by Bowers *et al.* and these tests did not detect kinase activity for tribbles (94). One possibility might be a potential decoy function for tribbles. In this role, they may compete with active protein kinases for binding partners/substrates, thereby affecting the activation of kinase dependent signalling pathways. It could explain the fundamental question, why evolution maintains a complex family of proteins composed of almost just a single domain that is structurally highly similar to kinases but functionally lost its activity at the early stages of evolution.

Better understanding of the functions and mechanism of tribbles may contribute to comprehension of the regulation of signal processes and thereby some of the pathological disorders.

Summary

Atherosclerosis is a pathological condition that underlies several important adverse vascular events including coronary artery disease, stroke, and peripheral arterial disease, responsible for most of the cardiovascular morbidity and mortality in the Western world today. In general, the susceptibility to atherosclerosis depends on genetic factors, smoking, obesity, diabetes, and hypertension; however the.starting point is the local presence of high amount of modified LDL (low-density lipoprotein), which can cause an inflammation.

LDL is one of the groups of lipoproteins that carry the serum cholesterol. Normally LDL transport is through LDL receptors, which is feedback inhibited by the accumulation of cholesterol within the cell. However if the LDL is modified, for example chemically, or enzimatically, the LDL receptor can not transport it. This modified LDL leads to inflammation in the vessel and monocytes migrate to the intima and differentiate into resident macrophages. Macrophages express scavenger receptors for modified LDL via which they accumulate lipids in their cytoplasm and then transform into foam cells. Aggregation of foam cells leads to formation of fatty streak lesions. This process is accompanied by migration of smooth muscle cells to the intima where they can also turn into foam cell, feeding the process even further. They proliferate, and secrete extracellular matrix proteins that form a fibrous cap. These lesions may remain quiescent in fibrous plaque state for many years, however under certain circumstances become activated and leads to a thrombotic response such as heart attack or stroke.

Atherosclerosis is a chronic disease of the arterial wall where both innate and adaptive immuno-inflammatory mechanisms are involved. Inflammation is central at all stages of atherosclerosis. Cells involved in the atherosclerotic process secrete and are activated by soluble factors, cytokines such as TNF-α, IL-6, MCP-1and IL-8. They have a role in the development, progression, and complications of atherosclerosis. They are responsible for migration, expression of scavenger receptors, adhesion molecules; regulate maturation of monocytes to macrophages and dendritic cells, and influence the Th1/Th2 balance. Cytokines generate cellular responses through activation of intracellular signal transduction networks. In atherosclerosis, the NF-κB and Mitogen Activated Protein Kinase (MAPK) pathways are amongst the most prominent ones.

A recently described protein family, tribbles may serve regulatory function in different signal transduction pathways especially in MAPK pathway. Tribbles was described first in *Drosophila* as a gene that is required for gastrulation, oogenesis, and viability. In human are three tribbles homolog, TRB-1,2,3. Based on amino acid sequence, tribbles resembles a serine-threonine kinase; however, the kinase domain is highly conserved during evolution; it has a variant catalytic core that lacks a canonical ATP binding site and tests did not detect kinase activity. According to our current knowledge all three tribbles molecules have an important regulatory function in modulating the activity of various signalling pathways (MAPK, CEβP, PI3K) and transcriptions factors (AP-1).

My hypothesis was that tribbles proteins are regulator molecules in inflammatory processes, through modulating MAPK signalling so they may have a role in atherogenesis especially in the inflammation induction role of macrophages. During my PhD my aims were to investigate the role of human tribbles, particularly human TRB-2, in human monocyte cells function *in vitro* system and to elucidate related molecular mechanism on how tribbles interact with protein kinases and regulate activity of binding partners or activation of down stream protein molecules.

In line with previous findings, inflammatory activation of THP-1 cells via LPS appears to substantially enhance the uptake of acLDL. Pharmacological inhibition of MAPK signalling pathways demonstrated that these pathways play a differential role in modulating acLDL uptake. Blocking the activation of JNK pathway lead to a marked elevation of acLDL uptake that was independent of inflammatory stimulation. Whilst the ERK and p38 pathways did not influence the uptake of acLDL under non-inflammatory conditions, both of these pathways led to a significant decrease in acLDL uptake after LPS stimulation. However the results demonstrate that acLDL uptake in THP-1 cells is tribbles-independent.

I investigated the effect of LDL to cytokine expression also. I have shown that acLDL uptake by monocytes is associated with a reduction in TRB-2 and potentiate LPS-induced IL-8 release through the MAPK pathways. The negative regulatory role of TRB-2 on MAPKK stopped, by this means the activation the JNK and ERK pathways lead to elevated IL-8 production. I investigated the potential in vivo relevance of our findings on primary monocytes also, the TRB-2 levels were inversely correlated to the IL-8 produced in monocytes.

The experiments in this study give evidence that tribbles, a novel group of proteins, can act as regulators of innate immune responses in monocytes. Tribbles appear not only to function in a cell type and possibly stimulus specific manner. From my results it is clear that tribbles action is highly specific; they regulate certain aspects of MAPK action (IL-8 expression) but not others (LDL uptake), within the same cell type. The molecular basis of tribbles effects is currently unknown. Better understanding of the functions and mechanism of tribbles may contribute to comprehension of the regulation of signal processes and thereby some of the pathological disorders.

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