Thioredoxin-1 (Trx1) : a new target in the treatment of cardiovascular diseases

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DEDICATION

Dedication

This dissertation is dedicated to the soul of my beloved father. It is also dedicated to my lover, my family, and my friends who have wholeheartedly supported me all the way without their love and encouragement, my studies could not be completed.

Finally, this dissertation is dedicated to all those who believe in the richness of learning.

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SUMMARY

Summary

The cardiovascular diseases (CVDs), resulting from complications of atherosclerosis, remain the leading cause of morbidity and death worldwide. Atherosclerosis as a chronic inflammatory disease, involves both innate and adaptive arms of immunity in which macrophages play the orchestral role in modulating lesion initiation, progression, and potentially devastating thrombotic complications. Available evidences support the notion of a central role of oxidative stress, due mainly to the imbalance between antioxidants and reactive oxygen species (ROS) in CVDs. Furthermore, the pathology is frequently associated with dynamic changes in macrophage activation, with classically activated M1 cells implicated in initiating and sustaining inflammation and M2 or M2-like cells associated with resolution or smoldering chronic inflammation. Among endogenous antioxidants, the thiordoxine-1 (Trx1) plays a central role in several diseases including CVD. Thus, the ubiquitous Trx1 has been reported to exert a myriad of beneficial roles. Indeed, it regulates not only cellular redox homeostasis and acts as a principal antioxidant defense system, but it also affects energy metabolism, modulates the immunological and inflammatory responses, and controls cell growth and survival. In contrast, its truncated form (Trx-80), exerts an opposite effects. However, several studies reported the beneficial role of Trx system in CVDs but the detailed molecular mechanism is not addressed yet. Therefore, the present study aims to investigate the role of both Trx1 and Trx80 in the biology of atherosclerosis through the modulation of macrophage polarization and the implicated signaling pathways as well.

Our *in vitro* major findings, using human macrophages and murine peritoneal macrophages, revealed that Trx1 on one hand promoted the polarization of anti-inflammatory M2 macrophages through downregulation of $p16^{INK4a}$ and suppressing nuclear translocation of activator protein-1 (AP-1) and Ref-1 as evidenced by the expression of the CD206 and IL-10 markers. On the other hand Trx1 also reduced the lipopolysaccharide (LPS)-induced differentiation of inflammatory M1 macrophages, as indicated by the decreased expression of the M1 cytokines, tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP¨-1). By contrast, Trx80 treatment attenuated the polarization of anti-inflammatory M2 macrophages induced by IL-4 or IL-4/IL-13 even it potentiated LPS-induced M1 activation. To validate our obtained *in vitro* results, hyperlipoproteinemic C57Bl/6.ApoE2.ki mice and human atherosclerotic vessel specimens from patients undergoing vascular surgery were used. Consistently, Trx1 and Trx80 affected macrophage phenotype in thymus, liver and atherosclerotic lesions. As a consequence, Trx1 reduced whereas Trx80 increased the aortic lesion area in mice. Plasma levels of cholesterol and triglycerides did not changed by the treatment. To further explore our results, the implicated signaling pathways has been studied and it was found that both Trx1 and Trx80 activated Akt. Furthermore, Trx80 uses mTOR signaling pathway to exert its effect in polarizing macrophages toward M1 phenotype since it activated mTOR in a dose-dependent manner as demonstrated by the increased phosphorylation of P70S6K. Based on our results, Trx1 antagonizes whereas Trx80 potentiates atherosclerosis through changing M1/M2 phenotypes. Therefore, Trx1 represents a promising target for therapeutic interventions. To avoid the proinflammatory effects of Trx80, since Trx1 can be cleaved by α-secreatase, thus developing Trx mimetic peptides that can be used as biochemical probes can potentially be developed into therapeutics.

Résumé

Les maladies cardiovasculaires (MCV), résultant de complications de l'athérosclérose, restent la principale cause de morbidité et de mortalité dans le monde. L'athérosclérose, considérée comme une maladie inflammatoire chronique, implique à la fois les systèmes immunitaires inné et adaptatif. Les macrophages jouent un rôle majeur dans l'initiation de la lésion, sa progression et les complications thrombotiques potentiellement dévastatrices. Un grand nombre de données rapporte l'implication du stress oxydatif, conséquence d'un déséquilibre entre antioxydants et espèces réactives de l'oxygène, dans les maladies cardiovasculaires. En outre, ces pathologies sont fréquemment associées à des changements dynamiques de l'activation des macrophages, soit vers le phénotype pro-inflammatoire M1 (activation classique), soit vers le phénotype anti-inflammatoire M2 (activation alternative). Parmi les antioxydants endogènes, la thiorédoxine-1 (Trx1) est une protéine ubiquitaire qui exerce différents effets physiologiques. Outre son rôle dans l'homéostasie redox cellulaire et comme puissant antioxydant, cette protéine est également impliquée dans le métabolisme énergétique, les réponses inflammatoires, la croissance cellulaire et la survie. En revanche, sa forme tronquée (Trx80) exerce des effets opposés. Il est à noter que plusieurs études ont rapporté le rôle bénéfique du système de la Trx1 dans les MCV mais les mécanismes moléculaires n'ont toujours pas été décrits. Par conséquent, notre étude porte sur le rôle des Trx1 et Trx80 dans le développement et/ou la régression de l'athérosclérose, en particulier dans la modulation de la polarisation des macrophages et dans les différentes voies de signalisation impliquées dans ces processus.
Nos principaux résultats obtenus *in vitro* sur des cultures primaires de macrophages humains ou de macrophages péritonéaux murins, ont révélé d'une part que la Trx1 induit la polarisation des macrophages vers le phénotype anti-inflammatoire M2 suite à la régulation négative de $p16^{INK4a}$ et l'inhibition de la translocation nucléaire de la protéine activatrice-1(AP-1) et de Ref-1; d'autre part, que la Trx1 inhibe la polarisation des macrophages vers le phénotype pro-inflammatoire M1 induit par le lipopolysaccharide (LPS). Par contre, la Trx80 inhibe la polarisation anti-inflammatoire M2 induite par IL-4 ou IL-4/IL-13 mais potentialise le phénotype M1 induit par le LPS. Pour valider *in vivo* les résultats obtenus *in vitro*, nous avons utilisé comme modèles expérimentaux, des souris C57Bl/6.ApoE2.ki hyperlipoprotéinémiques et des vaisseaux athérosclérotiques de patients ayant subi une chirurgie vasculaire. Injectées en intraveineuse, la Trx1 et la Trx80 affectent le phénotype des macrophages dans le thymus, le foie et les lésions athérosclérotiques. Chez la souris, la Trx1 réduit la surface des lésions aortiques alors que la Trx80 l'augmente. Enfin le traitement aussi bien par la Trx1 que par la Trx80 n'affecte pas les niveaux de cholestérol et de triglycérides plasmatiques. Pour explorer davantage nos résultats, nous avons étudié les voies de signalisation impliquées dans ces processus. Nos résultats montrent que la Trx1 et la Trx80 activent toutes les deux Akt. La Trx80 utilise la voie de signalisation mTOR pour exercer ses effets dans la polarisation M1 des macrophages puisqu'elle active mTOR de manière dose-dépendante comme l'indique l'augmentation de la phosphorylation de p70S6K. De plus, la Trx1 antagonise l'athérosclérose alors que la Trx80 la potentialise par suite des changements des phénotypes M1/M2, ce qui fait de la Trx1 une cible thérapeutique prometteuse. Toutefois, la forme entière de la Trx1 ne peut pas être utilisée puisqu'elle peut être potentiellement clivée par deux α-sécrétases pour générer la Trx80, protéine pro-inflammatoire. C'est pourquoi, le développement de peptides modifiés de la Trx1 pourrait être utilisé comme moyen thérapeutique dans la protection contre les réactions inflammatoires et les maladies cardiovasculaires.

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AAA	Abdominal Aortic Aneurysm
AD	Alzheimer's disease
ARE	Antioxidant Response Elements
ASK1	Signal-Regulated Kinase 1
CaMKII	Ca2+-Calmodulin-Dependent Kinase II
CHD	Coronary Heart Diseases
CVD	Cardiovascular Disease
DAMPs	Damage Associated Molecular Patterns
HIF1-α	Hypoxia-Inducible Factor-1 α
JNK	C-Jun N-Terminal Kinases
MAPKs	Mitogen-Activated Protein Kinases
mmLDL	Minimally Modified LDL
NLRs	Leucine-Rich Repeat-Containing Receptors
NLS	Nuclear Localization Signal
Nrf2	Nuclear Factor Erythroid-2 Related Factor 2
ORE	Oxidative Strss Responsive Element
PAMPs	Pathogen Associated Molecular Patterns
PD	Parkinson's disease
PDX-1	Pancreatic Duodenal Homeobox Factor-1
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PRX	Peroxiredoxins
PTEN	Phosphatase And Tensin Homology
Ref-1	Redox Factor-1
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
Sp1	Specificity Protein 1
TBP-2	Trx-Binding Protein-2
TLRs	Toll-Like Receptors
Tregs	Regulatory T Cells

Trx	Thioredoxin
TrxR	Thioredoxin Reductase
TSS1	Transcription Start Site
TXNIP	Trx-Interacting Protein
VDUP1	Vitamin D3 Up-Regulated Protein 1
VEGF	Vascular Endothelial Growth Factor

INTRODUCTION

1. Cardiovascular Diseases (CVDs)- the main cause of death in Europe

Over the past half century, advances in public health (emphasis on healthy diet, exercise, and smoking cessation), clinical cardiology (chest pain units, coronary stenting, and cardiac defibrillation), and scientific discoveries (lipid-lowering statins, angiotensin-converting-enzyme inhibitors) have contributed to a steady decline in deaths from CVDs [1,2]. Unfortunately, despite these improvements, CVD, as a dysfunction of heart and blood vessels, remains the leading cause of morbidity and mortality worldwide accounting for more than 80% of all fatalities occurring in western countries [3-6]. According to the *European Cardiovascular Disease Statistics* report in 2012, CVDs are the main cause of death in Europe: accounting for over 4 million deaths each year.

The term CVD encompasses an array of diseases such as coronary artery disease, hypertension, congestive heart failure, and stroke, all of which are complications of atherosclerosis that begins early in life and progresses gradually, remaining usually asymptomatic for a long period of time [7]. The risk factors for CVD can be either non-modifiable such as age, sex, ethnicity, and genetics or modifiable, such as elevated serum lipids, high blood pressure, physical inactivity, obesity, and smoking [8,9].

1.1.**Atherosclerosis - a chronic inflammatory disease**

Atherosclerosis, a chronic inflammatory disease, involves both innate and adaptive arms of immunity which modulate lesion initiation, progression, and potentially devastating thrombotic complications [10,11]. Atherosclerosis is characterized by the accumulation of lipids and fibrous elements in the large arteries. Normal large artery consists of three morphologically distinct layers (Figure 1). The tunica intima, the innermost layer, is bounded by a monolayer of endothelial cells on the luminal side and a sheet of elastic fibres, the internal elastic lamina, on the peripheral side. The normal intima is a very thin region and consists of extracellular connective tissue matrix, primarily proteoglycans and collagen. The tunica media, the middle layer, consists of smooth muscle cells (SMCs). The tunica adventitia, the outer layer, consists of connective tissues with interspersed fibroblasts and SMCs [12].

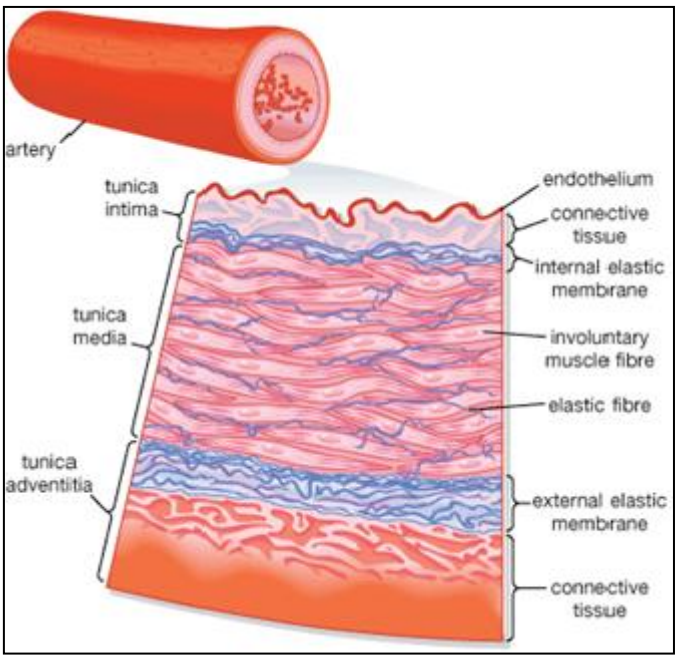


Figure 1: Structure of a normal large artery.

A large artery consists of three morphologically distinct layers. The intima, the innermost layer, is bounded by a monolayer of endothelial cells on the luminal side and a sheet of elastic fibres, the internal elastic lamina, on the peripheral side. The normal intima is a very thin region and consists of extracellular connective tissue matrix, primarily proteoglycans and collagen. The media, the middle layer, consists of smooth muscle cells (SMCs). The adventitia, the outer layer, consists of connective tissues with interspersed fibroblasts and SMCs.

On the basis of animal experiments and observations in human specimens, most contemporary schemes of atherogenesis (Figure 2) posit an initial qualitative change in the monolayer of endothelial cells that lines the inner arterial surface. Arterial endothelial cells, which normally resist attachment of the white blood cells streaming past them (Figure 2-a), prone to the permeation and then subendothelial accumulation of apolipoprotein B-containing lipoproteins, such as low-density lipoprotein (LDL) and remnant lipoproteins [13]. The overlying endothelium is activated when it is subjected to irritative stimuli (such as dyslipidaemia, hypertension or pro-inflammatory mediators) to secrete chemokines and express adhesion molecules that attract and bind monocytes [12,14]. Parallel changes in endothelial permeability and the composition of the extracellular matrix beneath the endothelium promote the entry and retention of cholesterol-containing low-density lipoprotein (LDL) particles in the artery wall [13]. These processes are followed by entry of the monocytes into the subendothelial space (Figure 2-b). Once in the subendothelium, the monocytes differentiate into macrophages and ingest the retained lipoproteins [12-14]. Lipoprotein uptake promotes the intracellular accumulation of various lipids, including cholesterol, oxysterols

and fatty acids, which promote the accumulation of lipid droplets in the cytoplasm of macrophages. This accumulation causes the macrophages to become foam cells (Figure 2-c) and induces an inflammatory response [12-14].

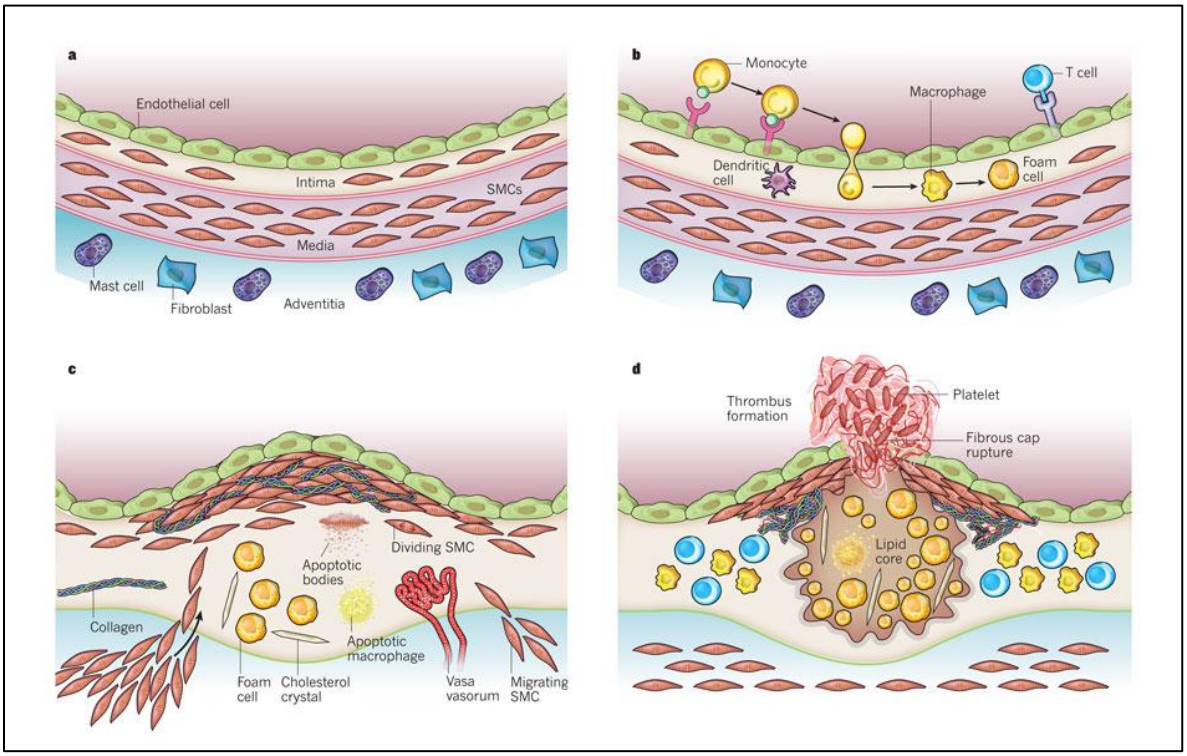


Figure 2: Stages in the development of atherosclerotic lesions (from [15]).

a, Normal large artery. **c**, The initial steps of atherosclerosis include adhesion of blood leukocytes to the activated endothelial monolayer, directed migration of the bound leukocytes into the intima, maturation of monocytes (the most numerous of the leukocytes recruited) into macrophages, and their uptake of lipid, yielding foam cells. **c**, Lesion progression involves the migration of SMCs from the media to the intima, the proliferation of resident intimal SMCs and media-derived SMCs, and the heightened synthesis of extracellular matrix macromolecules such as collagen, elastin and proteoglycans. Plaque macrophages and SMCs can die in advancing lesions, some by apoptosis. Extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque, often denoted the lipid or necrotic core. Advancing plaques also contain cholesterol crystals and microvessels. **d**, Thrombosis, the ultimate complication of atherosclerosis, often complicates a physical disruption of the atherosclerotic plaque. Shown is a fracture of the plaque's fibrous cap, which has enabled blood coagulation components to come into contact with tissue factors in the plaque's interior, triggering the thrombus that extends into the vessel lumen, where it can impede blood flow.

Chemoattractant mediators direct the migration of the bound leukocytes into the innermost layer of the artery, the tunica intima. The localized distribution of atheromatous lesions in the arterial tree, despite a systemic rise in risk factors such as increased LDL levels or blood pressure, probably reflects differing haemodynamics in different segments of the arterial tree,

distinction in the regional development of arteries [16] and the ability of normal laminar shear stress to elicit an atheroprotective program of gene expression by the endothelium [17]. Once resident in the artery wall, monocytes — the most numerous white blood cells in plaques — differentiate into tissue macrophages. In the nascent atheroma, these mononuclear phagocytes engulf lipoprotein particles and become foam cells — a term that reflects the microscopic appearance of these lipid-laden macrophages.

In mice, a pro-inflammatory subset of monocytes induced by hyperlipidaemia may preferentially furnish the precursors of lesional foam cells, but the fates and functions of this monocyte subset and its human equivalent remain under intense exploration [2,18]. Macrophages in the atheroma may also have a pro-inflammatory palette of functions, characteristic of M1 macrophages [19]. Some mononuclear phagocytes in plaques have the characteristics, and probably the antigen-presenting functions, of dendritic cells. Other leukocyte classes (such as lymphocytes) and mast cells also accumulate in atheromata, but less abundantly than phagocytes. Lesional T cells, although far fewer in number than macrophages, probably have key regulatory functions in plaques [15].

Atheroma formation also involves the recruitment of smooth muscle cells (SMCs) from the tunica media — the middle layer of the artery wall — into the tunica intima. Unlike that of most experimental animals used to study atherosclerosis, the intima of human arteries (including the coronary arteries) contains resident SMCs. During atherogenesis, other SMCs migrate from the media into the intima, and proliferate in response to mediators such as platelet-derived growth factor. In the intima, the SMCs produce extracellular matrix molecules, including interstitial collagen and elastin, and form a fibrous cap that covers the plaque [15]. This cap typically overlies a collection of macrophage-derived foam cells (Figure 2-c), some of which die (for example, by apoptosis) and release lipids that accumulate extracellularly. The inefficient clearance of dead cells — a process known as efferocytosis — can promote the accumulation of cellular debris and extracellular lipids, forming a lipid-rich pool called the necrotic core of the plaque [13] (Figure 2-d). In the worst-case scenario, an occlusive thrombus forms, leading to acute oxygen and nutrient deprivation of distal tissues fed by the artery. When these events occur in the coronary arteries, the region of heart muscle tissue fed by the involved artery becomes injured, and the result is unstable angina, myocardial infarction or sudden cardiac death [13,20].

1.2. **Lipoproteins- Role of Oxidized LDL in Atherosclerosis**

Lipids have a central role in the pathogenesis of plaques, but the mechanistic links between lipids and atherogenesis remain unclear. Observational data support a strong association between plasma lipid levels and the risk of cardiovascular disease [15]. Clinically, the most important plasma lipids are cholesterol and triglyceride (TG; also called triacylglycerol) [21]. Cholesterol has numerous roles: it is a component of cell membranes; the precursor for steroid hormones and vitamin D, and for oxysterols and bile acids (both of which are activators of nuclear hormone receptors involved in sterol metabolism); and it is required for the activation of neuronal signaling molecules [21 22]. Only a small amount of circulating cholesterol originates from the diet; ~80% is derived from endogenous synthesis, for which 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) catalyses the rate-limiting step. Most cholesterol in the circulation is esterified, with free cholesterol constituting a minor fraction. TG is a key energy source that is made up of free fatty acids (FFAs) that are ester-linked to a glycerol backbone. TG is synthesized in intestinal and liver cells and is then transported through the plasma and, after lipolysis at the endothelial surface, delivers FFA to peripheral cells for β-oxidation or storage [23].

The insolubility of cholesterol and TG in plasma requires that they are transported in spheroidal macromolecules called lipoproteins, which have a hydrophobic core containing phospholipid, fatsoluble antioxidants and vitamins, and cholesteryl ester, and a hydrophilic coat that contains free cholesterol, phospholipid and apolipoprotein molecules. The main TG-carrying lipoproteins are chylomicron (CM) and very low-density lipoprotein (VLDL). The main cholesterolcarrying lipoproteins are LDL and high-density lipoprotein (HDL) [23]. Lipoproteins are distinguished from each other by size, density, electrophoretic mobility, composition and function [23].

Chronic excess of plasma LDL interferes with arterial relaxation, and LDL particles that are not catabolized through regulated receptor-mediated endocytosis are recognized by scavenger receptors on arterial-wall macrophages [12,24]. Lipids that are engulfed by macrophages become oxidized, generating toxic intermediates, which induce cytokine production and chemotaxis of inflammatory cells [12,24].

Dysfunctional lipid homeostasis plays a central role in the initiation and progression of atherosclerotic lesions. Modified or oxidized LDL cholesterol induces endothelial dysfunction with focal inflammation which in turn causes increased expression of atherogenic signaling

molecules that promote the adhesion of monocytes and T lymphocytes to the arterial endothelium and their penetration into the intima. Monocytes accumulate in the subendothelial space and undergo transformation into macrophages which express scavenger receptors that allow them to take up modified lipoproteins. Ultimately, when net influx of cholesterol exceeds efflux, these macrophages become lipid-overloaded foam cells [25]. Foam cell accumulation leads to fatty streaks. Eventually engorged foam cells undergo secondary necrosis and form the lipid core of advanced atherosclerotic plaques [13]. Several ATP-binding cassette (ABC) transporters play a pivotal role in lipid trafficking [26]. ABC transporters are integral membrane proteins that carry diverse solutes across lipid bilayers, often against a concentration gradient. They are critically involved in cholesterol and phospholipid efflux and reverse cholesterol transport (RCT), processes that maintain cellular cholesterol homeostasis and protect arteries from atherosclerosis. RCT, the removal of cholesterol from peripheral cells (including vascular macrophages) for transport to the liver, is of particular significance for atheroprotection [27]. Moreover, CD36, a multiligand class B scavenger receptor expressed by monocytes/macrophages, has been shown to be a key player in atherosclerosis development through oxidized LDL (oxLDL) binding and internalization, thereby leading to macrophage-derived foam cell formation and to their accumulation into fatty streaks [28]. Therefore, to date, CD36 has received the most attention in this context. Although SR-A and CD36 mediate the majority of modified LDL uptake by macrophages *in vitro*, their roles in developing atherosclerosis in mice remain complex and conflicting depending on published studies [29,30]. These controversial results could be attributed to the possible differences in the genetic background, regional context or inflammatory features studied.

Foam cell formation involves phagocytosis of matrix-retained lipoproteins and fluid-phase pinocytosis of aggregated lipoproteins by macrophages, but substantial evidence also suggests that oxidative modification of subendothelially accumulated LDL triggers the inflammatory process underlying atherosclerosis. Precisely how oxLDL and associated lipids instigate the atherogenic process remains intensively investigated, but an ill alliance of various pathways is emerging. Close links between lipid and inflammation biology, via different signaling pathway, scontinue to emerge as the pathogenic interface providing several possibilities for therapeutic intervention (Figure 3).

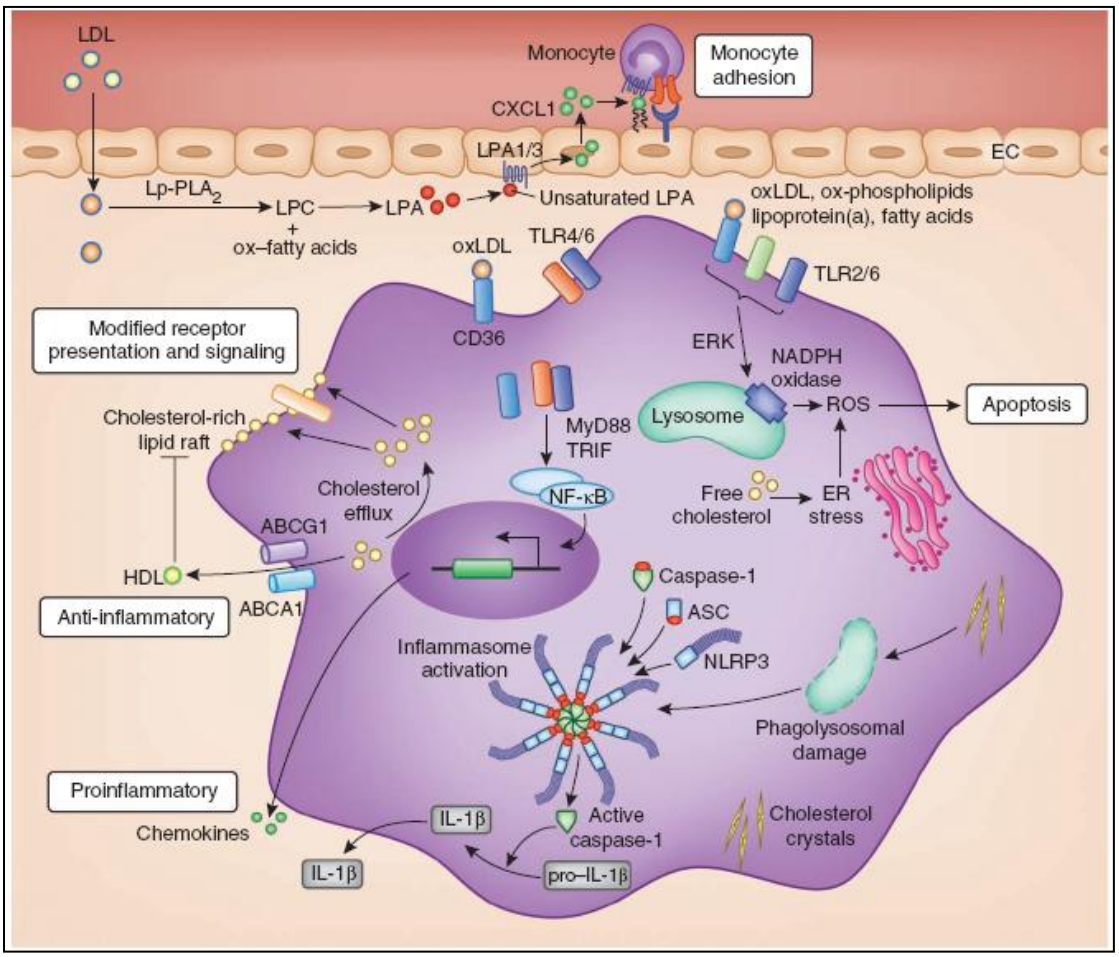


Figure 3: Lipid mediators affect inflammation and atherogenesis through diverse signaling pathways (from [31]).

OxLDL binds CD36, inducing intracellular CD36-TLR4-TLR6 heterotrimerization, which activates NF-κB and chemokine expression by lesional macrophages. Atherogenic lipid mediators, such as oxLDL, oxidized phospholipids and lipoproteins and fatty acids, also trigger NADPH oxidase activation through a CD36-TLR2-TLR6 pathway, resulting in a sustained oxidative burst and apoptosis of ER-stressed cholesterol-overloaded foam cells. Furthermore, an intracellular excess of free cholesterol, for example due to defective cholesterol efflux by ABC transporters, can modify receptor presentation and signaling by increasing lipid raft formation, which in hematopoietic stem cells induces myeloproliferation due to enhanced IL-3 and GM-CSF-signaling. Also, intracellular cholesterol crystals can exert proatherogenic effects by stimulating IL-1β production by macrophages through NLRP3 inflammasome activation. Lipids can also affect endothelial cell functions—the phospholipase Lp-PLA2 hydrolyzes oxLDL-associated phospholipids into oxidized fatty acids and lysophosphatidylcholine (LPC), and the derivative lysophosphatidic acid (LPA) triggers endothelial cells to secrete and present CXCL1 for monocyte adhesion. Thus, oxLDL and associated lipids act on diverse cell types to instigate inflammation and proatherogenic processes.

2. Macrophage Polarization- a response to the microenvironment

Macrophages are a heterogeneous cell population involved in diverse physiological processes including anti-microbial defense, wound resolution, inflammation, tissue remodeling, plaque formation in atherosclerosis and promotion of tumor growth [32-34]. Diversity and plasticity are hallmarks of the monocytes-macrophage system. Mononuclear phagocytes develop into morphological and functional distinct cell types in response to the tissue microenvironment (eg, lung alveolar macrophages, Kupffer cells, decidual macrophages). Moreover, inflammatory signals of microbial or leukocyte origin drive different forms of macrophage activation and polarization [33]. The role of macrophages in atherosclerosis is extensively reviewed recently [35]. Macrophages also play a major role in the resolution of inflammation by producing anti-inflammatory cytokines and chemokines and eliminating tissue debris. Macrophages can thus exhibit pro- and anti-inflammatory properties, depending on the disease stage and the signals they receive, i.e. the inflammatory balance in the microenvironment [36].

Despite their heterogeneity, macrophages can be broadly divided into M1 (classically activated) or M2 (alternatively activated) phenotypes [33,37]. The M1 phenotype is induced in response to microbial products such as LPS or proinflammatory cytokines including IFN-γ, IL-1β or TNF-α. M1 macrophages produce further proinflammatory cytokines (TNF-α, IL-12 and CCL3) with reactive oxygen and nitrogen intermediates, which combine to give M1 macrophages potent antimicrobial, tumoricidal and inflammatory properties [34]. In contrast, the M2 phenotype results from exposure to anti-inflammatory molecules such as glucocorticoid hormones, IL-4, IL-13, IL-10 or immune complexes. M2 macrophages are anti-inflammatory and immunosuppressive in nature. They are highly phagocytic, preferentially activate the arginase pathway, promote angiogenesis, tissue remodeling and have pro-tumoral activity. However, *in vivo*, the division between M1 and M2 cells may be blurred, with the above phenotypes likely representing two extremes in a continuum of macrophage functional states [33,37,38].

Interestingly, macrophage polarization is a dynamic process in which they can switch from an activated state to another, being either classical or alternative, upon a specific signal. This versatility of macrophage activation may greatly enhance tissue macrophage ability to resolve inflammation quickly without new monocyte/macrophage recruitment and may

provide new insight into the inflammatory process as well as new fields of investigation for cell trafficking [36].

Mononuclear phagocytes are diverse and can express a continuum of differentiation/activation programs in response to environmental signals. In response to tissue signals, macrophages in plaques can express pro- and antiatherogenic programs. Available information is compatible with a "macrophage balance" view of mononuclear phagocytes in atherosclerosis. Tipping the balance toward pro- or antiatherogenic functions affects pathogenesis, evolution, and complications [33].

Large body of evidences supports the notion of a central role of oxidative stress in most of the atherosclerotic process and the correlation between increased oxidative stress and vascular disease in as much as most risk factors lead to dramatic increases in ROS [9,39-45].

3. **Oxidative Stress - The Underlying Cause of Many Diseases**

Free radicals, characterized by an unpaired electron, are highly reactive molecules generated predominantly during cellular respiration, metabolism, and phagocytosis (Figure 4). In addition, low levels of free radicals produced in response to growth factors and cytokines are key components of cellular metabolism [7,46]. The mitochondrial respiratory chain is the major intracellular source of ROS, oxygen-containing chemically reactive molecules, and free radicals under normal physiologic and pathologic conditions [47].

The ROS and reactive nitrogen species (RNS) are both physiologically necessary and potentially destructive. Moderate levels of ROS play specific roles in the modulation of several cellular events, including signal transduction, proliferation, and gene expression [49]. High ROS/RNS levels can cause damage to key cellular components such as lipids, proteins, and nucleic acids, possibly leading to subsequent cell death by necrosis or apoptosis [50](Figure 5). Oxidation of any of these substrates, if uncontrolled, can theoretically contribute to disease development. Indeed, an increasing body of evidence suggests that the production of ROS/RNS, and subsequently oxidative/nitrosative stress and damage, is linked to either the primary or secondary pathophysiological mechanisms of multiple disorders, including cancer, diabetes, atherosclerosis, coronary heart disease, and cataract. Interestingly, these diseases are degenerative processes involved in the rate of aging and in age-related diseases [51].

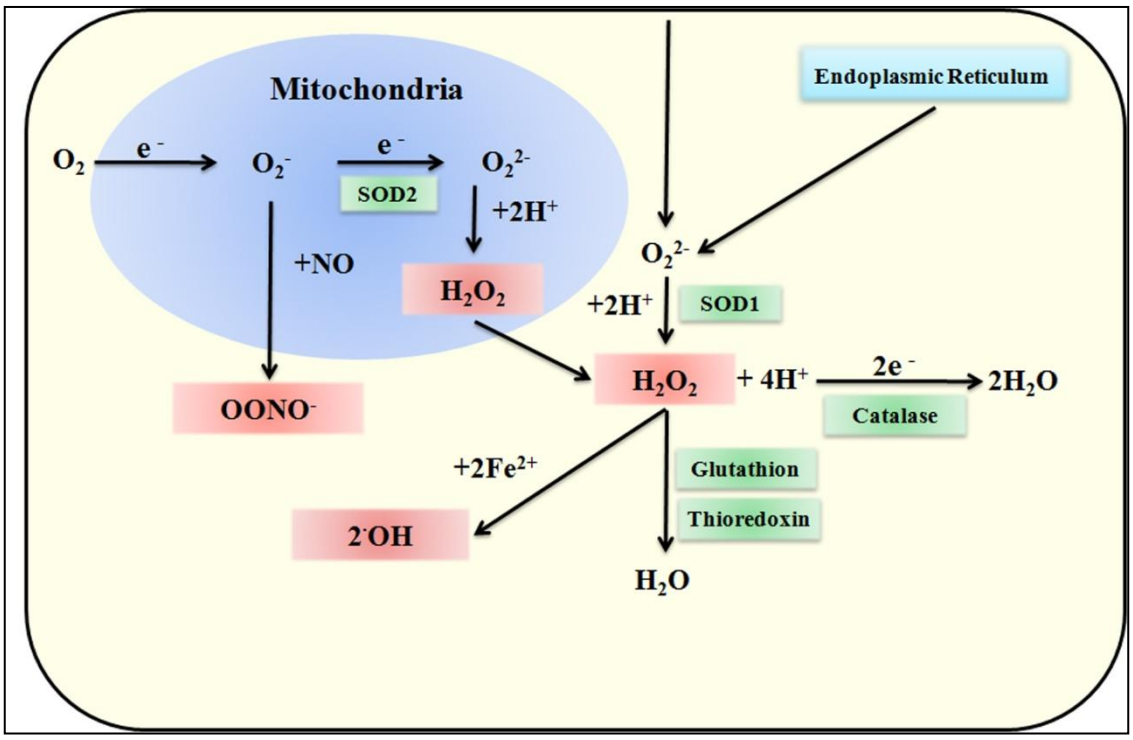


Figure 4: Generation of reactive oxygen species (ROS)

Mitochondria, the endoplasmic reticulum (ER) and the NADPH oxidase system are major generating sites of reactive oxygen species (ROS), oxygen-containing chemically reactive molecules, through cellular respiration, metabolism, and phagocytosis. Superoxide generated by mitochondria isconverted by superoxide dismutase 2 (SOD2) to H_2O_2**.** The later one, can be converted either to H_2O by catalase or to hydroxyl radicals (·**O**H) in the presence Fe^{2+}. Furthermore, H_2O_2 can also be reduced by antioxidants like glutathion, and thioredoxin [48].

An imbalance favoring the cellular production of free radicals in amounts exceeding the cellular defense capacities is referred to as oxidative stress [52-54]. Therefore, cells have evolved several strategies (enzymatic and non-enzymatic) to overcome free radical-induced oxidative stress including preventive and repairing mechanisms, physical barriers, and antioxidant defenses. Non-enzymatic antioxidants are ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, carotenoids, flavonoids, and other antioxidants. Enzymatic antioxidant defenses include superoxide dismutase, glutathione peroxidase, catalase, glutaredoxin, thioredoxin reductase (TrxR), and thioredoxin (Trx)[55].

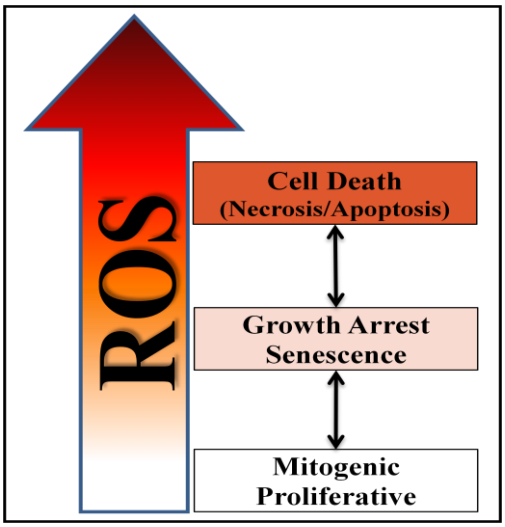


Figure 5: Cellular effects of reactive oxygen species (ROS)

Different levels of ROS, which change the cellular redox status, may result in different cellular responses; low levels of ROS, particularly of hydrogen peroxide, are mitogenic and promote cell proliferation, while intermediate levels result in either temporary or permanent growth arrest, such as replicative senescence. Very severe oxidative stress causes ultimately cell death either *via* apoptotic or necrotic mechanisms [48].

4. The Thioredoxin System

In 1964, Trx1 was first isolated from *Escherichia coli* by Laurent *et al* reporting that *E. coli* also contained an enzyme, TrxR, which catalyzes its reduction [56]. Through its redox-active cysteine residues, the dithiol Trx plays a vital role in maintaining the cellular environment in a reduced state. The dithiol moieties of Trx are reduced by receiving electrons from NADPH in the presence of TrxR. Reduced Trx in turn reduces proteins with disulfide bonds by transferring electrons from its reactive cysteines through thiol disulfide exchange reactions. Thus, NADPH, TrxR, Trx and thioredoxin interacting protein (TXNIP), the endogenous Trx inhibitor, are collectively termed the Trx system (Figure 6)[57-60]. There are three distinct forms of human Trx, encoded by separate genes; cytosolic Trx (Trx1), mitochondrial Trx (Trx2), and a Trx variant that is highly expressed in spermatozoa (SpTrx/Trx3)[61-63].

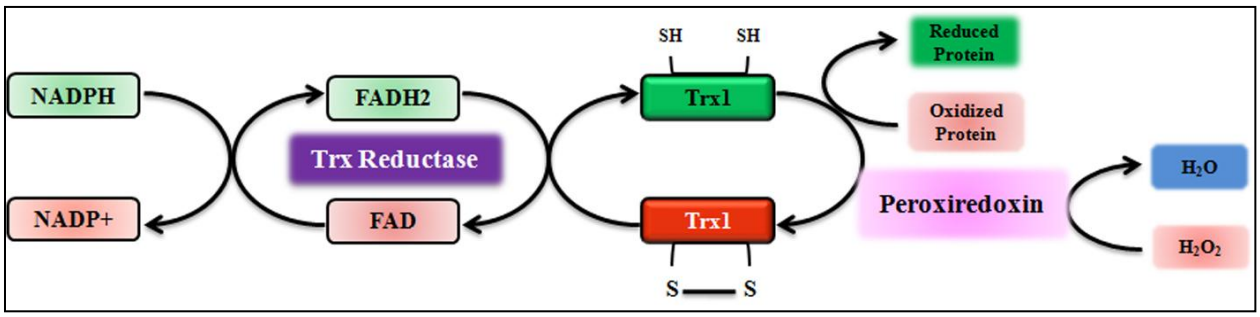


Figure 6: The Redox-Cycling Reactions of the Thioredoxin System

The dithiol moieties of Trx are reduced by receiving electrons from NADPH in the presence of thioredoxin reductase (TrxR). Reduced Trx1 in turn reduces oxidized proteins with disulfide bonds through thiol disulfide exchange reactions. Thus, NADPH, TrxR, and Trx are collectively termed the Trx system. Furthermore, Trx can scavenge free radicals indirectly through peroxiredoxin [48].

Depending on its subcellular localization, Trx exerts different roles (Table 1). It can be found in the extracellular environment, cytoplasm and the nucleus [64]. In the extracellular environment, Trx exhibits chemokine-like activity [60], while in the cytoplasm, it is regulating the cellular redox environment and also the activity of certain proteins. In the nucleus, Trx1 has been shown to interact with many transcription factors such as Ref-1, HIF-1α, NF-κB, p53, AP-1, Nrf-2, glucocorticoid receptor, estrogen receptor, and others [65-70], thereby regulating gene expression. Intracellular Trx1 localizes mainly in the cytoplasm. However, several factors induce nuclear translocation of Trx1, despite the lack of a nuclear localization signal. This nuclear translocation, through karyopherin-α [71], suggests that Trx1 may be associated with signaling molecules that bridge the cytoplasmic and nuclear compartment [62]. By contrast, the translocation of Trx1 to the membrane requires binding of TXNIP [72].

Trx-2 is a mitochondrial redox protein containing the Trx1 active site Trp-Cys-Gly-Pro-Cys. It is encoded by a nuclear gene, *TRX2*, with a mitochondrial targeting signal peptide. Initially, Trx2 has been cloned from a rat heart library by Spyrou *et al.* who reported that this gene encodes a 18 kDa protein [63]. Later Trx2 also has been cloned from human osteosarcoma cells [73] and human embryonal stem cells [74]. The *TRX2* gene is expressed in virtually all organs and tissues with the highest expression level in the brain [75,76]. Trx-2 plays important roles during embryonic development since $TRX2^{-/+}$ heterozygous mice appeared normal even though they displayed decreased levels of Trx2, whereas early embryonic lethality was seen in the $TRX2^{-/-}$ homozygous embryos [77]. Furthermore, overexpression of the Trx2 can facilitate development of resistance to ROS-induced apoptosis [74], as well as resistance of human embryo kidney cells to etoposide [73]. The Trx2 system appears to have a more important role

in preventing mitochondrial dysfunction than the mitochondrial GSH system in endothelial cells under conditions that mimic a septic insult [78] because inhibition of Trx2 resulted in higher mitochondrial metabolic activity, lower ATP/ADP ratios, lower oxygen consumption, increased lactate formation and evidence of caspase 3 and 7 activation [78]. Like Trx1, Trx2 is important for cell viability and prevents apoptosis-induced cell death since its knockdown in endothelial cell increases TNF/ASK-1-induced cytochrome c release and cell death [79]. Moreover, Trx-2 improves endothelial cell function and reduces atherosclerotic lesions in the apolipoprotein E-deficient mouse model via reducing oxidative stress and increasing NO bioavailability [80].

Among other isoforms of Trx, spermatid-specific thioredoxin-3 (SpTrx3) has been characterized, which is exclusively expressed in testis and localized to the Golgi apparatus of mammalian spermatocytes and spermatids. Furthermore, SpTrx3 is more abundant in defective spermatozoa from infertile men. In addition, it constitutes a novel sperm postobstruction autoantigen [81] (reviewed by [82]). In this study, we will focus mainly on the Trx1 isoform and the other Trx system members are discussed when required. In the following sections we use the abbreviation Trx referring mainly to all Trx isoforms and when the role is restricted to one isoform and not others, as it will be indicated.

4.1.**The Thioredoxin-1 Gene**

The gene encoding human Trx1 is located on chromosome 9 at bands 9q31. Its coding region spans over 13 kb and is organized in five exons separated by four introns. The Trx1 promoter contains two transcription start sites (Figure 7). Initial studies involving the Trx1 promoter region identified the first transcription start site (denoted as TSS1) at –110bp (with respect to the ATG start codon) by mapping the 5' end of cDNA clone to a genomic sequence [83]. Three Sp1 (specificity protein 1) binding sites were also located between –244bp and–183bp [83] and have been found to enhance transcription of the Trx1 gene [84]. In contrast, a second transcription start site has been previously mapped (using primer extension analysis) to –74bp with a TATA box (TATAAA) located at –102bp [85]. The TATA box is located downstream of TSS1 and is therefore only relevant for transcription initiation at TSS2. Luciferase assays, utilizing various wild-type and mutated Trx1 promoter fragments, revealed the roles for the ORE (oxidative stress responsive element), antioxidant response elements (ARE), three Sp1-binding sites and the TATA box in the activation of the Trx1 gene [86-89].

Table (1): Cellular occurrence and the subcellular distribution of Trx

Subcellular Distribution	Roles	Targets	Cells	References
Cytoplasm	Antioxidant	Oxidized Proteins, DNA, Lipids	All cells	57,90-96
Cytoplasm	Antiapoptotic	ASK1	Mv1Lu, L929, and 293 cells	97
Cytoplasm	Signaling Transduction	Akt and PTEN regulation	Neuroblastic neoplasms, and neuroblastoma cells lines	98
Cytoplasm	Anti-inflammatory	Downregulation of monocyte chemoattractant protein-1 (MCP-1)	Trx-1 Overexpressing/Knockdown EA.hy 926 and bovine aortic ECs	99
Cytoplasm	Immunomodulation	Regulation of regulatory T cells	Tregs	100
Translocation to Nucleus	Gene Regulation	Histone deacetylase 4	Cardiac myocyte	101
Translocation to Nucleus	Gene Regulation	Hypoxia inducible factor-1α	HeLa cells	102
Translocation to Nucleus		Glucocorticoid receptor	COS7, CV-1, and HeLa cells	103
Nucleus	Gene Regulation	NF-κB , AP-1, and Ref-1	L929 and HeLa cells	65,99
Cell Surface	Immunomodulation	Inhibition of complement deposition	HUVEC	104
Extracellular	Chemokine	Immunomodulation chemoattractant	*In vitro*: human monocytes, PMNs, and T cell *In vivo*: Air pouch in mice	90 60

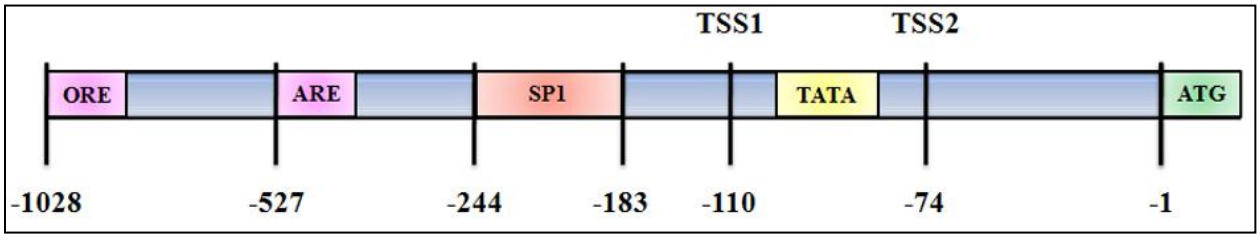


Figure 7: Map of the human Trx promoter region.

The first transcription start site (TSS1) is located at -110bp, while three Sp1 (specificity protein 1)-binding sites are also located between -244 bp and -183bp. The TSS2 is just at -74bp with a TATA box (TATAAA) located at -102bp. Oxidative stress responsive element (ORE), antioxidant responsive element (ARE) [48].

4.2. **Structure of Thioredoxin-1**

All Trx proteins in different organisms share a common structure, consisting of four α-helices and five β-sheets constituting a protein of 12 kDa [105,106]. They have a highly conserved active site sequence, Cys^{32}–Gly–Pro–Cys^{35}(CXXC)[107], which links the second β strand to the second α helix and forms the first turn of the second helix making it a compact globular protein with high temperature stability [108-111]. In addition to the two cysteine residues, human Trx1, but not bacterial Trxs, contain three other, critical structural cysteines residues, at positions -62, -69 and -73 providing Trx1 with unique biological properties (Figure 8)[112,113]. These cysteine residues of Trx1 can undergo post-translational modifications such as thiol oxidation, glutathionylation, and S-nitrosylation regulating its activity (see below). Both Cys62 and 69 are sites of S-nitrosylation [114-116] whereas Cys73 is a multimodification site, undergoing nitrosylation [117-119], glutathionylation [120], dimerization [116] or 4-hydroxy-2-nonenal modification [69].

Because the first N-terminal methionine is mostly removed after translation by an N-terminal methionine excision process, Trx1 found in human tissues consists mainly of 104 amino acids starting from the second N-terminal valine [89].

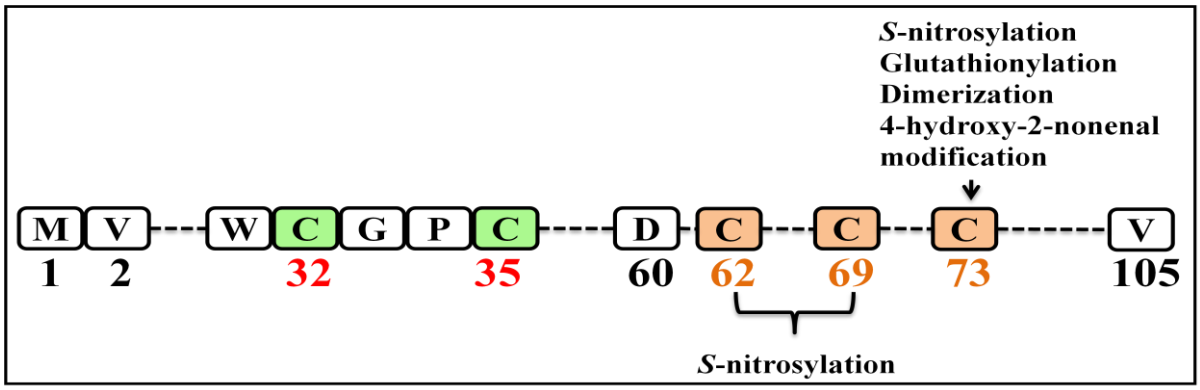


Figure 8: Cysteine residues of the highly conserved active site sequence Cys32–Gly–Pro–Cys35 (CXXC) of Trx.

In addition to the two cysteine residues, human Trx, but not bacterial Trxs, contain 3 other, critical structural cysteines residues, at positions -62, -69 and -73 providing unique biological properties to Trx. These cystine residues of Trx can undergo post-translational modifications. Both Cys62 and 69 are sites of S-nitrosylation, whereas Cys73 is a multimodification site, undergoing nitrosylation, glutathionylation, dimerization or 4-hydroxy-2-nonenal modification [48].

4.3.**Truncated Thioredoxin (Trx80)**

To date, only limited research has been performed on the biological roles of the truncated form of Trx (Trx80). Previously, it was termed eosinophil cytotoxicity-enhancing factor (ECEF) due to its eosinophil cytotoxicity and it has been first detected in the plasma of patients suffering from severe schistosomiasis [121-123]. Trx80 (10 kDa) is a natural cleavage product of Trx1 sharing the 80 or 84 N-terminal amino acids with Trx1 [124,125]. It has been suggested that the enzyme responsible for its cleavage would be an inducible protease [126].Very recent findings, indicated that the disintegrins and metalloproteinases (ADAM10 and 17), two α-secretases processing the amyloid β precursor protein, are responsible for Trx80 generation in brain [127]. Further work will have to establish other possible candidates in different tissues and under different pathophysiological conditions.

Macrophages are capable of cleaving full-length Trx1 to yield Trx80, which in contrast to the cytosolic localization of Trx1, is present mainly at the surface of monocytes [128]. Trx80 is expressed also by U937 monocytes, cytotrophoblasts and $CD4^+$ T cells [123,129]. Recently, human brain samples and human primary cultures were shown to produce Trx80 and polymerize it into very stable aggregates migrating at approximately 30 kDa in SDS–PAGE [127].

Whereas the levels of Trx80 varied from 2 to 175 ng/ml, those of Trx1 varied from 16 to 55 ng/ml without any correlation to Trx80 concentrations, which increased significantly under inflammatory conditions [130,131]. Trx80 activates monocytes and induces up-regulation of cell surface pathogen recognition receptors, molecules essential for T-cell activation and function [126] as well as release of proinflammatory cytokines evoking inflammation [90]. Induction of human monocytes is associated with phenotypic cell changes [124], which differentiate into a novel line type named by Cortes-Bratti *et al.* Trx80-activated-monocytes (TAMs) with an intrinsic function contributing to the first line of defense against foreign intruder and triggering innate immunity [130]. Thus, Trx80-activated monocytes could be more effective against bacteria and parasites *via* CD14 receptor up-regulation and other pathogen recognition receptors such as CD1 and the mannose receptor [128,130,131]. Rheumatoid arthritis (RA) synoviocytes express the truncated form Trx80, and treatment with the pro-inflammatory cytokines IL-1β and/or TNF-α increases Trx80 cell expression playing an important role in the establishment and/or the development of RA autoimmunity. Lemarechal *et al.* reported that synoviocytes represent important target cells of Trx80, which respond in a concentration-dependent manner [126]. Furthermore, in contrast to Trx1, Trx80 activates the

classical and alternative pathways of complement activation through binding to complement initiators C1q and properdin, leading to production of the anaphylatoxin C5a [104].

4.4.**The Thioredoxin Reductases**

Thioredoxin reductase (TrxR), a homodimeric, relatively thermostable, selenium-containing flavoprotein oxidoreductase [106,132], catalyzes the NADPH-dependent reduction of Trxs disulfide and of numerous other oxidized cell constituents [133]. TrxR is important for cell proliferation, antioxidant function, and redox signaling [134,135]. These enzymes have three isoforms in mammals: TrxR1 in the cytosol, TrxR2 in mitochondria, and TrxR3 or TGR (thioredoxin glutathione reductase) present primarily in testes [136]; in humans they are encoded by three different genes, *TNXRD1*, *TNXRD2*, and *TNXRD3*, respectively [133].

The C-terminal extension with the characteristic motif Gly-Cys-Se-Cys-Gly carrying the essential selenocysteine residue is unique for mammalian TrxRs and it forms a selenenylsulfide in the oxidized enzyme. Furthermore, this C-terminal extension enables TrxR to extend the electron transport chain from the catalytic disulfide to the enzyme surface and to prevent the enzyme from acting as a glutathione reductase by blocking the redox-active disulfide [137]. Due to their flexible C-terminal tail, TrxRs have a broad range of substrates including glutaredoxin 2, protein disulfide isomerase, granulysin, and also some nonprotein substratessuch as selenite, dehydroascorbate, lipoic acid, ubiquinone, cytochrome C, or the cancer drugs motexafin gadolinium andalloxan [61,133].

Overexpression of TrxR1 has been observed in many tumors and cancer cells [2,136,138-141] rendering it an interesting target candidate for chemotherapy (discussed in chapter XIII). TrxR1 in cancer cells is essential for self-sufficiency of growth, progression into S phase [142], tumor progression, and metastasis [143]. However, knockout of TrxR1 in mice exposed to a liver carcinogen showed a significantly increased incidence for chemically induced liver cancer [144]. Thus, the dual role of TrxR in cancers, prevention/promotion, may depend on the stage of cancer development and on tissues as well.

4.5.**Thioredoxin Interacting Protein (TXNIP)**

Human Trx-binding protein-2 (TBP-2)/vitamin D_3 up-regulated protein 1(VDUP1)/Trx-interacting protein (TXNIP) is a negative regulator of Trx1 function because it interacts with the active center of Trx1, thereby inhibiting its reducing activity [145-148]. It is one of six members of the mammalian α-arrestin family. The α-arrestins are structurally related to the β-arrestins, which are well-characterized mediators of G protein-coupled

receptor signaling and endocytosis. TXNIP expression is ubiquitous and is induced by a variety of stresses, including UV light, γ-rays, heat shock, and H_2O_2, as well as glucose [149,150]. TXNIP overexpression renders cells more susceptible to oxidative stress and promotes apoptosis [151-153]. Furthermore, peroxisome proliferator-activated receptor gamma (PPARγ) activation stimulates apoptosis in human macrophages by altering the cellular redox balance *via* regulation of TXNIP [154]. In addition to its inhibitory role, TXNIP plays important roles in lipid and glucose metabolism [70,155-158], inflammation [159-161], cardiac function [162], and carcinogenesis [163]. Interestingly, the ability of TXNIP to inhibit glucose uptake was found to occur independent of Trx1 binding [164].

4.6.**Regulation and Post-Translational Modifications of Thioredoxin-1**

In spite of being cell cycle dependent, the expression of Trx1 can be upregulated by a variety of stress stimuli including hypoxia, *Staph. aureus* protein, lipopolysaccharide, O_2, H_2O_2, phorbol ester, viral infection, photochemical oxidative stress, X-radiation, and UV irradiation [113,165].

As mentioned earlier, Trx1 can undergo post-translational modifications. Glutathionylation of Trx1 leads to a reduction in the enzymatic activity of Trx1 under conditions of oxidative stress, which it seems to regain again, indicating the ability of Trx1 to degluthionylate itself by some means of autoactivation [112]. *S*-Nitrosylation is the covalent addition of a NO moiety onto a cysteine thiol. It is a dynamic post-translational modification for the regulation of protein functions. *S*-nitrosylation of protein thiols may occur on exposure of specific redox-active motifs to NO, or as a result of transnitrosation/transfer reactions from low-mass carrier S-nitrosothiols or transfer from other protein S-nitrosothiols [166]. Available evidences imply S-nitrosylation in cardiovascular, pulmonary, musculoskeletal and neurological (dys)functions, as well as in cancer (see [167] and [168] for more details). In contrast to other proteins such as caspases [169], peroxiredoxin 2 (PRX2) [170] or methionine adenosyl transferase [171], which are inhibited, the activity of Trx1is increased upon *S*-nitrosylation [114]; consistently, purified Trx1 is sensitive to *S*-nitrosylation. Stimulation of HEK-293 cells with S-nitrosoglutathione resulted in Trx1 *S*-nitrosylation and consequently ASK-1 activation [172], whereas others found that S-nitrosylation occurring at Cys69 enables Trx1 to scavenge ROS, to preserve its redox regulatory activity, and to act as an antiapoptotic protein in endothelial cells [114]. Interestingly, Trx system can act as a major S-nitrothiol–caspase-3 denitrosylating protein [173], because denitrosylation by Trx1 alone in the absence of TrxR1 was ineffective. Furthermore, Trx2 mediates Fas-induced denitrosylation of mitochondria-associated S-nitrothiol–caspase-3 and

promotes apoptotic signaling [168,173]. Cys73 of Trx1was shown to be responsible for the transnitrosylation of caspase 3 at Cys163 because mutation of Cys73 to Ser, yielded a mutant exhibiting normal disulfide reduction activity, which was resistant to nitrosylation, and which lacked transnitrosylation activity toward caspase 3 [117]. On the other hand, the redox states of the Cys32 and Cys35 seem to be crucial regulators determining the nitrosylation of Trx1 at Cys73 and its ability to transnitrosylate target proteins [119,138]. Therefore, under certain conditions, Trx1 plays a major role in protein S-denitrosylation and it can also trans-S-nitrosylate other proteins [138,168,174].

4.7. **General Functions of Trx**

Since 1964, the number of publications on thioredoxin is exponentially growing (Fugyre 9) representing a promising molecule. The Trx system is a crucial and essential system for the protection against oxidative stress, for the maintenance of the cellular redox balance, and for the regulation of differentiation and cell fate (recently reviewed [175]). This wide range of cellular functions of Trx system leads into its involvement in a variety of diseases (Figure 9) [176].

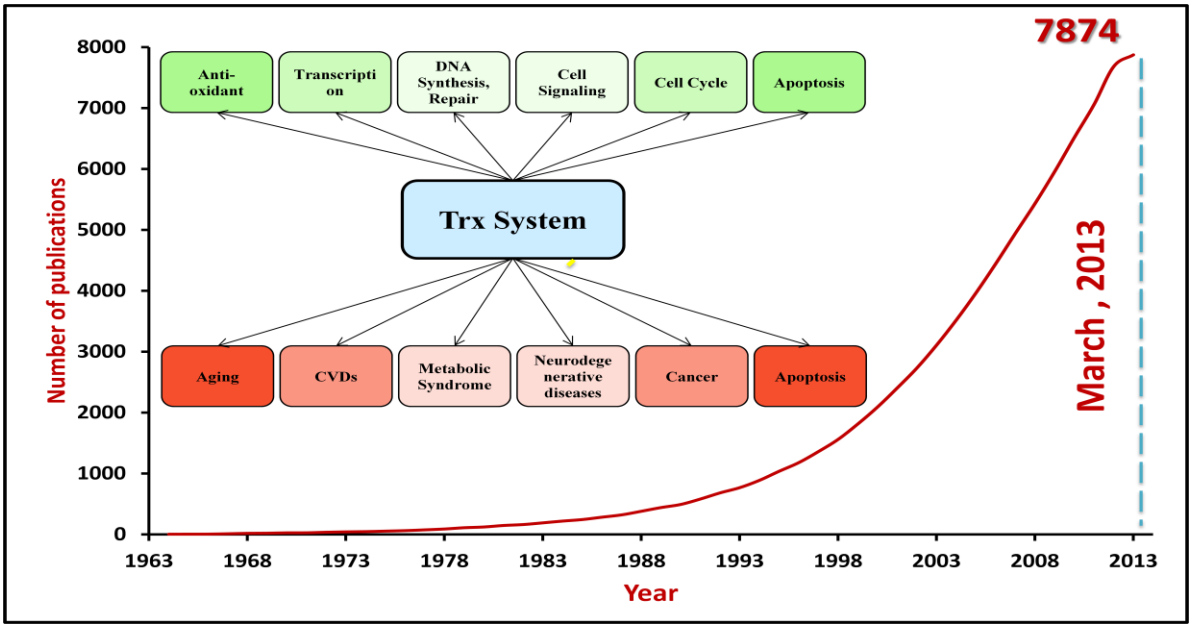


Figure 9: The regulatory role of the Trx system and exponential growing of number of publications.

Knockout of either Trx1 or Trx2 in mice is embryonically lethal [77,177]. The regulation of the cellular redox balance is critically determined by the activity of several antioxidant systems. Trx1 seems to exert most of its antioxidant properties through peroxiredoxin (PRX), which uses SH groups as reducing equivalents. Trx1 reduces the oxidized form of PRX, and

reduced PRX in turn scavenges ROS, such as H_2O_2 [178,179]. Trx1 acts as a hydrogen donor for enzymes involved in reducing reactions, for example, ribonucleotide reductase [180], PRX, and methionine sulfoxide reductases [181,182]. Trx1 reduces protein disulfide bonds and other cysteine oxidations in the respective cellular compartments [138].

As mentioned above, Trx1 and Trx2 expressions are essential for early differentiation and morphogenesis of the mice embryos since embryos of *TXN1* [177] and *TXN2*-null [77] mice are not viable, whereas heterozygous mice are fertile. The role of Trx system has been studied in transgenic animal models of different human diseases. Transgenic mice (Tg) overexpressing Trx1 showed increased resistance to oxidative stress [183,184] and a longer life span [183,185,186], suppressed UV-induced inflammation [187], a higher resistance against the teratogenic effects of hyperglycaemia [188], attenuated osteopenia [189] and nephropathy [190] associated with streptozotocin-induced diabetes, increased availability of glucose for fetal growth [191], attenuated apoptosis of cardiomyocyte and myocardial fibrosis after myocardial infarction [184], and a decreased local macrophage migration inhibitory factor resulting in a suppressed Th2-driven airway inflammation [192]. Furthermore, Tg mice with cardiac-specific overexpression of a dominant negative mutant (C32S/C35S) of Trx1 showed increased markers of oxidative stress and exhibited cardiac hypertrophy with maintained cardiac function at baseline [193]. The role of Trx2 in the endothelial cell function has been studied using endothelial cell-specific transgenesis and it was found that Trx2 improved endothelial cell function and reduced atherosclerotic lesions in the apolipoprotein E-deficient mouse model [80]. Interestingly, cardiomyocyte size enlargement and hypertension due to chronic angiotensin II infusion were significantly diminished in Tg mice that overexpress Trx2 comparing to wild type [194].

4.8. **Role of Trx in Apoptosis**

Apoptosis is a stringently controlled process of cell death that is fundamental for the normal development of organs. Evidence now suggests that the initiation and execution of apoptosis is triggered by changes in the redox environment [175,195]. Trx1 has been shown to be involved in apoptotic processes in at least two different ways (Figure 8). Firstly, the redox status of one of the key determinants of the apoptotic process, caspase-3, is maintained by Trx1 [117,174,196]. Secondly, ASK-1, an enzyme involved with regulating apoptotic events, is a substrate for Trx1, which binds to ASK-1 and inhibits its apoptotic activity [175]. This molecule, ASK-1, has since been identified as mitogen-activated protein (MAP) kinase kinase kinase and oxidation of Trx1 causes the release of this binding followed by the induction of apoptosis *via* the

activation of p38 MAP kinase and c-Jun-NH_2-terminal kinase [97]. Experiments with various lymphoid cells also confirmed the involvement of Trx1 in the apoptotic process. Sato *et al.* implicated the human Trx1, in the regulation of lymphocyte function, most likely *via* the oxidation of this protein [197]. This regulatory process may prove to be of significance in the redox-sensitive pathway of apoptosis induced by oxidative stress and the oxidation of sulfhydryl groups. Also when lymphoid cells were cultured in the absence of L-cystine and glutathione (GSH), the addition of recombinant Trx1 to these cultures was found to block partially the apoptotic process in a concentration-dependent manner [67]. Moreover, it has been shown that Trx2 plays an important role in the mitochondrial pathway of apoptosis since, compared to wild type mice, $TXN2^{+/-}$ mice displayed increased caspase-3 and caspase-9 activities as well as increased mitochondrial cytochrome c release [198].

4.9.**Trx Regulates Glucocorticoid and Estrogen Receptors**

Glucocorticoids, as major peripheral effector of the hypothalamic-pituitary-adrenal axis, play an essential role in reestablishing the homeostatic status in every peripheral tissue in human., Upon binding of glucocorticoids to their receptor, they promote the dissociation of heat shock proteins, and the ligand–receptor complex translocates to the nucleus, where it binds to palindromic DNA sequences, The redox status of cysteine residues of glucocrticoids maintain their structure and binding abilities [199,200]. Previously it has been revealed that Trx1 is required for generating the ligand-binding conformation of the glucocorticoid receptor. Antibody-mediated sequestration of either Trx or TrxR inhibited the ligand binding activity of the glucocorticoid receptor [201]. The same group conducted another study to show the direct effect of Trx1 on the DNA binding of the gluccocorticoid receptor. They have inhibited the binding ability of receptors by methyl methanethiosulfonate (MMTS), which can be reversed by adding dithiothreitol (DTT). Surprisingly, the promoting effect of partially purified Trx1 on DNA binding of MMTS-treated receptors in the presence of DTT was not affected by anti-Trx1 serum [202].

Additional studies on the role of Trx1 on the responsiveness to glucocorticoids revealed that the downregulation of Trx1 by antisense ODN or treatment with H_2O_2 negatively modulated the glucocorticoid receptor function and decreased the glucocorticoid-inducible gene expression. However, the cellular responsiveness to glucocorticoids was rescued by overexpression of Trx1 [103,203].

In vitro and *in vivo* results showed that the specific DNA binding activity of recombinant estrogen receptors was inhibited by sulfhydryl-modifying reagents and restored by the addition of recombinant Trx1 protein in an electrophoretic mobility shift assay revealing that the transcriptional activity of estrogen receptors is strongly influenced by their redox state, which is reversibly modulated by Trx1 [204]. Moreover, treatment of ovariectomized adult mice with a time course of estradiol resulted in a rapid decrease of the expression of TXNIP, but increased the levels of cytosolic Trx1 and TrxR1 as well as Trx2. This clearly suggests that rapid estradiol-mediated activation of the Trx1 pathway is an important step in the response of the mammalian uterus to estrogen [205]. Therefore, Trx and TrxR are members of an interconnected network of proteins, which collectively help to maintain the structural integrity and activity of estrogen receptor alpha, its associated coregulatory proteins, and other complex members [206].

The implication of Trx system in different pathophysiological conditions has extensively reviewed ([207]) but in this section its role in CVDs and aging is more highlighted.

4.10. **The Trx System and Aging**

Multicellular organisms generally undergo qualitative changes with time (aging) that are associated with progressive degeneration of biological functions, increased susceptibility to diseases, and increased probability of death within a given time period [208]. Age-specific mortality rates from heart disease and stroke increase exponentially with age throughout the later years of life, accounting for more than 40% of all deaths among people aged 65 - 74 years and almost for 60% of all fatalities at the age of 85 years and older [209].

Cellular aging is commonly associated with instability of the mitochondrial and nuclear genomes as well as oxidative protein damage in response to environmental and genetic stress [210]. Several theories have been proposed to explain the molecular mechanisms behind aging and aging-related diseases. The free-radical theory of aging was first contented by Harman in 1956 proposing that free oxygen radicals cause cumulative oxidative damage, which eventually results in aging and age-related diseases in humans and animals. Subsequently, he refined his theory and suggested that mitochondria play a key role in the aging process because these organelles are the major source and likewise a major target of free radicals [211]. Furthermore, the capacities of the cellular antioxidant systems will decline with age, leading to a gradual loss of the free radical/antioxidant balance and subsequently to the accumulation of oxidative damage during the aging process [212]. Aging is a major risk

factor for cardiovascular diseases. It has been demonstrated that endothelium-dependent vasodilatation and the basal release of NO are reduced during aging. Further studies showed that Trx1 can improve endothelial function and is able to rescue endothelial cells from age-induced disorders [213]. It has also been shown that TrxR in aging muscle cells plays a role in the sensitization of cells to oxidative challenge and for the increased susceptibility to the mitochondrial pathway of apoptosis [214]. It is important to note that overexpression of human Trx1 in Tg was shown to suppress oxidative stress damage and to elongate the lifespan [186]. Moreover, telomerase activity, another determinant of longevity, was found to be higher in Trx1-Tg mice in comparison to wild mice [186]. Nevertheless, Tg overexpressing Trx1 are indeed resistant to oxidative stress and survival studies demonstrated that male Tg(*TXN1*)$^{+/0}$ mice have a significantly reduced mortality in their earlier part of life span compared with wild-type littermates, whereas no significant changes were observed in females [185]. Moreover, neither male nor female Tg *TXN1*$^{+/0}$ mice showed changes in their maximum life span. These findings suggested that the increased levels of Trx1 in the Tg *TXN1*$^{+/0}$ mice were correlated to increased resistance to oxidative stress, which could be beneficial in the earlier part of the life span but would not extend the maximum life span in the C57BL/6 mice [185]. It has been reported that *TXN2*$^{+/-}$ mice show reduced mitochondrial function as indicated by reduced ATP production, reduced activity of electron transport chain complexes, and subsequently increased ROS generation. Accordingly, these mice have higher oxidative damage of cellular components [198,215] and no longer lifespan than their age-matched wild type counterparts [198]. Furthermore, Trx1 has been associated with a wide variety of diseases with oxidative imbalance, including cancer, human immunodeficiency virus infection, neurodegenerative diseases, and cardiovascular diseases [58].

4.11. **The Trx System in Cardiovascular Diseases**

Growing evidence indicates that overproduction of ROS under pathophysiological conditions is an integral component in the development of CVD such as atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy and congestive heart failure [54,55] because they activate various signaling pathways that underlie the vascular inflammation in atherogenesis (Figure 10). Therefore, Trx1 may play a beneficial role antagonizing CVD. In isolated perfused hearts adapted to ischemia and reperfusion by short cycles of ischemia and reperfusion (I/R), the protection is apparently afforded by the enhanced induction of Trx1, because ROS increases, when Trx1 is inhibited by a suitable inhibitor [216]. Further studies conducted in animal models *in vivo* suggest that Trx1 has many

beneficial functions in the heart [59,193]. Only recently, it has been demonstrated that impaired angiogenesis and myocardial dysfunction can be counteracted by adenoviral vector-based gene therapy encoding Trx1 [155]. Several studies indicate that Trx1 plays a role in the pathogenesis of atherosclerosis. NO and peroxynitrite contribute to the damage to smooth muscle and endothelial cells seen in atherosclerotic plaques [217] with increased Trx and TrxR mRNAs [218]. Trx1 prevents the NO-dependent inhibition of purified NO-synthase. Furthermore, endogenous levels of Trx1 are critical for restoring the NO-induced loss of eNOS activity because overexpression of Trx1 prevents the NO-induced loss of eNOS catalytic activity (Figure 10) [96,219]. It has been well documented that the mechanism(s) responsible for the improved angiogenesis as well as cardioprotection mediated by resveratrol, operates both *in vitro* and *in vivo* through the induction of vascular endothelial growth factor expression triggered by Trx1 and HO-1. Significant increase in the Trx1 expression was observed at baseline level in the myocardium of streptozotocin-induced diabetic rats after I/R. A protective role for Trx1 has also been implicated in cardiac hypertrophy. Several studies support the concept that Trx1, as antioxidant, inhibits and protects from cardiac hypertrophy in animal models. Transgenic mice with a cardiac-specific overexpression of a dominant negative Trx1 mutant showed a loss of the endogenous oxidoreductase activity of Trx1 [219-222]. In patients with coronary artery disease (CAD), high homocysteine levels may cause low Trx1 activity, which is closely correlated to the extent and severity of the CAD [119]. During I/R, Trx1 also inhibits expression of proinflammatory cytokines, chemotaxis, complement activation, and neutrophil adhesion. This is an important issue, because I/R stimulate cell adhesion molecules and the migration of neutrophils into myocardial tissue. Subsequent production of inflammatory cytokines and ROS enhances myocardial injury. Another potentially important mechanism is the effect of Trx1 upon ion channel remodeling. Downregulation of the K^+ channel by oxidative stress could cause lethal arrhythmia and cardiac contractile dysfunction. Upregulation of Trx1 may prevent downregulation of the Kv4 channel and the subsequent electrical instability during I/R [59]. Additionally, exogenous Trx1 exerts distinct cytoprotective effects on cerebral I/R injury in mice by means of its redox-regulating activity [223]. Furthermore, it has been demonstrated that *S*-nitrosation of Trx1 potentiates its protective activity against myocardial I/R [115]. It has been suggested that there is a possible association between Trx1 secretion and the severity of heart failure. Trx1 is increased in both inflammatory cells and myocytes during myocarditis [111]. Furthermore, increased Trx1 levels have been observed in several oxidative stress-associated CVDs, for instance, serum Trx1 levels were significantly increased in patients with abdominal

aortic aneurysm (AAA) relative to healthy subjects. Indeed, levels correlate well with the size and expansion of AAA, suggesting its potential role as a biomarker of AAA evolution [224]. Furthermore, recent analysis of *TXNIP*$^{-/-}$ mice revealed suppression of genes, which participate in mitochondrial metabolism [225]. Consequently, *TXNIP*$^{-/-}$ mitochondria were functionally and structurally altered, showing reduced oxygen consumption and ultrastructural derangements [225]. TXNIP deletion would therefore enhance I/R damage. Surprisingly, *TXNIP*$^{-/-}$ hearts had greater recovery of cardiac function after an I/R insult [225]. Similarly, cardiomyocyte-specific TXNIP deletion reduced infarct size after reversible coronary ligation. Thus, in addition to reduced mitochondrial function, deletion of TXNIP enhanced anaerobic glycolysis [225]. Whereas mitochondrial ATP synthesis was minimally decreased by TXNIP ablation, cellular ATP content and lactate formation were higher in *TXNIP*$^{-/-}$ hearts after I/R injury [225]. Inhibition of glycolytic metabolism abolished the protection of the heart by TXNIP deficiency under hypoxic conditions. Thus, although TXNIP deletion suppresses mitochondrial function, protection from myocardial ischemia is increased as a result from a coordinated shift to enhanced anaerobic metabolism, which supplies an energy source outside mitochondria [225].

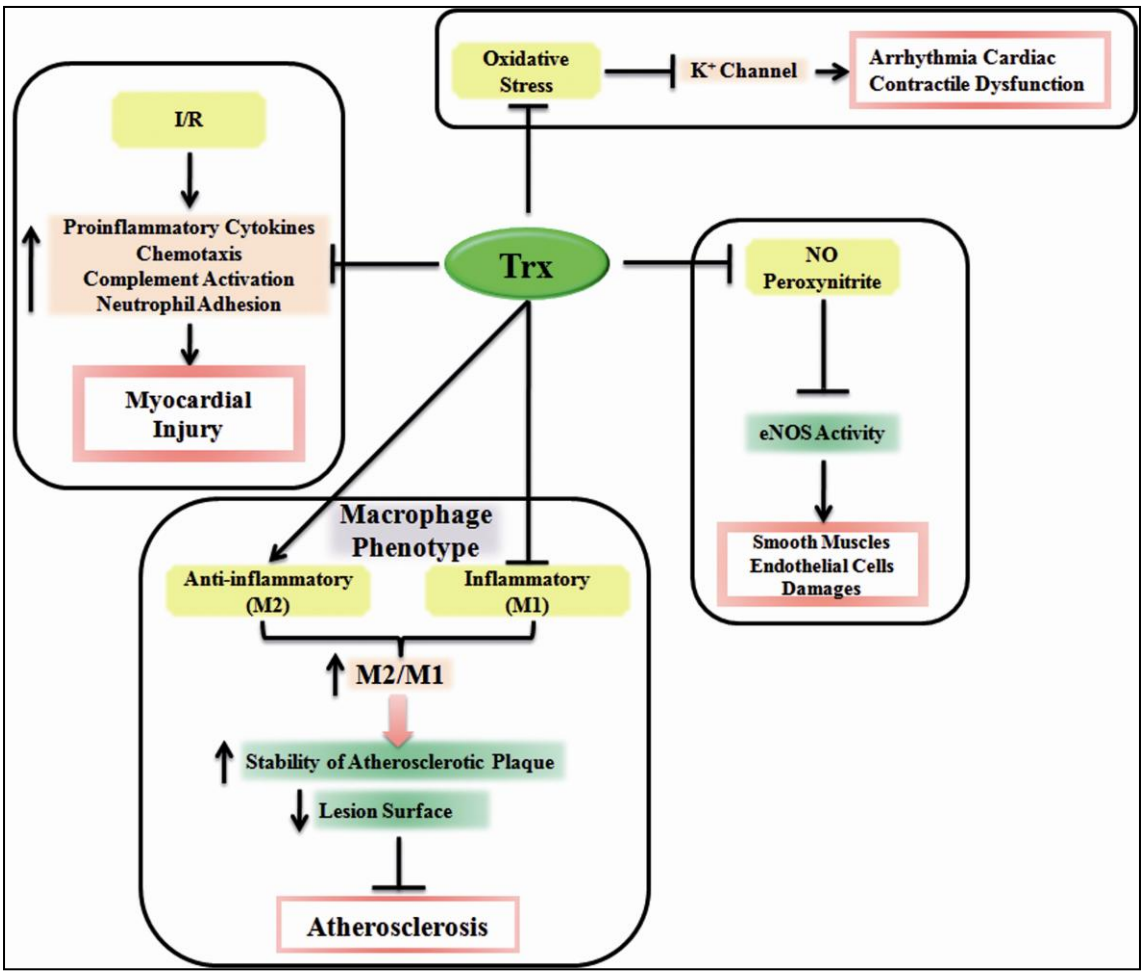


Figure 10: Protective roles of Trx in CVDs.

Trx can attenuate ischemia-reperfusion injuries by inhibiting the expression of proinflammatory cytokines, chemotaxis, complement activation, and neutrophil adhesion. Furthermore, Trx can prevent smooth muscle and endothelial cell damage by protecting eNOS against NO and peroxynitrite. As a powerful antioxidant, Trx can also prevent lethal arrhythmia and cardiac contractile dysfunction by protecting K^+ channel and by inhibiting electrical instability. Recently, it has been shown that Trx can antagonize atherosclerosis because it can potentiate the anti-inflammatory macrophage phenotype [48].

CONTEXT OF THE PROJECT

5. Context of the project

Cardiovascular diseases (CVDs) claim more lives worldwide than any other, accounting for over 4 million deaths each year only in Europe [2-4]. Etiologically, the dominant trajectory involves atherosclerosis, a chronic inflammatory process of lipid-rich lesion growth in the vascular wall that can cause life-threatening myocardial infarction [2].

Atherosclerosis involves both the innate and adaptive arms of the immune response that modulate lesion initiation, progression, and potentially devastating thrombotic complications [10,11]. In this scenario, macrophages play a central role [35] in lipid metabolism, immune responses [32,226,227] and the maintenance of inflammation [38]. Therefore, macrophage recruitment and plasticity are key components of atherosclerosis. Defective 'efferocytosis', phagocytic clearance of apoptotic cells in the vascular wall, impacts on plaque necrosis, stability and vulnerability to thromboembolism [228].

Interestingly, macrophages residing within atherosclerotic lesions represent a heterogeneous cell population whose activation and function are influenced by various cytokines and microbial products. Thus, interleukin-1β (IL-1β), interferon- γ, and endotoxin lipopolysaccharide (LPS) increase the classical or inflammatory activation profile yielding the so-called M1 macrophages [229], which produce proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-6, and IL-12 as well as reactive oxygen species (ROS) and nitrogen intermediates [37]. Consistently, M1 macrophages are associated with inflammation and tissue damage. In contrast, IL-4, IL-13 [19,37,230] peroxisome proliferator–activated receptor-γ (PPAR-γ) activators [19] and adiponectin [231,232] promote polarization of macrophages into the anti- inflammatory, alternative M2 type. M2 macrophages secrete the anti- inflammatory cytokine IL-10, transforming growth factor-β, and IL-1 receptor antagonist, and upregulate scavenger receptors, the mannose receptor CD206, and arginase-1. Accumulation of M2 macrophages leads to reduction of inflammation.

Growing evidence indicates that overproduction of ROS under pathophysiological conditions is an integral component in the development of CVDs such as atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy and congestive heart failure [54,55,233]. An imbalance favoring the cellular production of free radicals in amounts exceeding the cellular defense capacities is referred to as oxidative stress [52-54] which markedly contributes to arterial inflammation [41]. Therefore, cells have evolved several strategies (enzymatic and non-enzymatic) to overcome free radical-induced oxidative stress including

preventive and repairing mechanisms, physical barriers, and antioxidant defenses. Non-enzymatic antioxidants are ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, carotenoids, flavonoids, and other antioxidants. Enzymatic antioxidant defenses include superoxide dismutase, glutathione peroxidase, catalase, glutaredoxin, and thioredoxin system [55].

The thioredoxin system comprises thioredoxin (Trx), truncated Trx (Trx80), thioredoxin reductase (TrxR), and NADPH, besides a natural Trx inhibitor, the thioredoxin-interacting protein (TXNIP). This system is essential for maintaining the balance of the cellular redox status and it is involved in the regulation of redox signaling. It is also pivotal for growth promotion, neuroprotection, inflammatory modulation, anti-apoptosis, immune function, and atherosclerosis. As an ubiquitous and multifunctional protein, Trx is expressed in all forms of life executing its function through its antioxidative, protein reducing, signal transducing activities [207]. Several studies reported the beneficial role of thioredoxin in CVDs [96,111,175,234-236] but the detailed molecular mechanism is not addressed yet. Therefore, the present study aims to:

- Study the role of both Trx1 and Trx80 in the biology of atherosclerosis through the modulation of macrophage polarization
- Investigate the molecular mechanism of the Trx1 and Trx80 through which they control macrophage polarization.
- Explore the implicated signaling pathways used by both Trx1 and Trx80 to induce macrophage polarization
- Characterize and study *in vivo* effects of Trx1 and Trx80 on the atherosclerosis using transgenic mice that have been generated in our laboratory.

Thioredoxin-1 induces M2 polarization

6. Thioredoxin-1 induces M2 polarization

6.1. Study presentation

Macrophages are decisive in the chronic inflammatory processes that drive atherogenesis. Recent works indicate that these cells can alter their phenotypes and functions accordingly in response to changes in the microenvironment switching between inflammatory (M1) and anti-inflammatory phenotypes. Interestingly, the ratio of M2 to M1 determines the pathogenesis, evolution and complication of many diseases including the stability of the atherosclerotic plaque [4]. Therefore, agents that can invert infiltrated macrophages toward the M2 phenotype may exert an atheroprotective action.

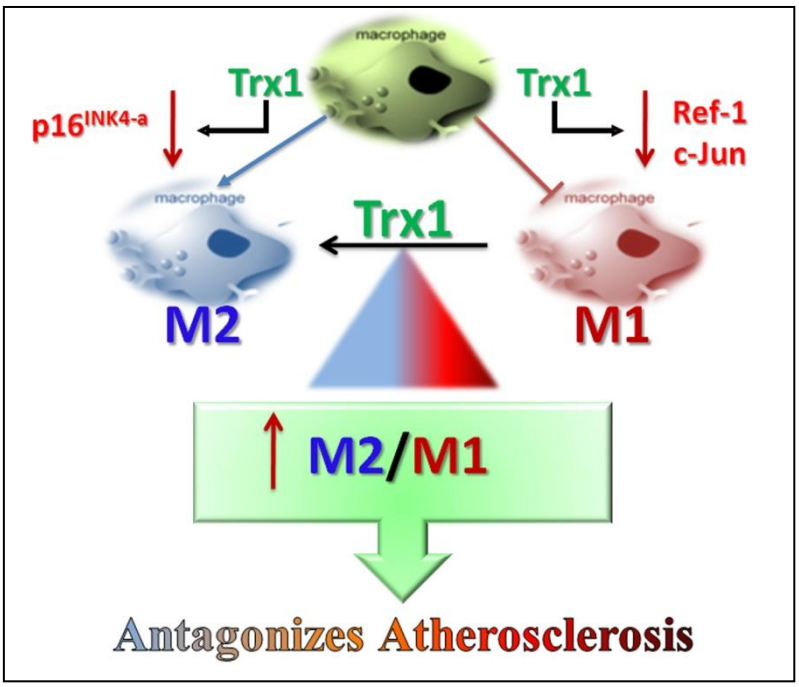
Trx1, a small (12-kDa) ubiquitous protein with a highly conserved redox-active dithiol/disulfide in the active site sequence Cys32-Gly-Pro-Cys35, plays a variety of redox-related roles in essentially all organisms. In addition to its direct antioxidant activity, Trx1 stimulates cell growth, inhibits apoptosis, activates numerous transcription factors and regulates immune function. Therefore, it is involved in a whole range of pathophysiological conditions representing a promising therapeutic target. Several *in vitro* and *in vivo* studies documented anti-inflammatory properties of Trx1 whereas the effects of Trx1 on the macrophage phenotype have not been addressed yet. Therefore, we hypothesized that Trx1 might promote polarization of human and murine macrophages toward an anti-inflammatory M2 phenotype.

To verify this hypothesis, both *in vitro* and *in vivo* approaches have been used. As an *in vivo* atherosclerosis model, we have chosen ApoE2.Ki mice expressing human ApoE2 (2/2), which virtually exhibits all characteristics of type III hyperlipoproteinemia in man. Accordingly, their plasma cholesterol and triglyceride levels are two to three times those of normolipidemic mice.[237]. These animals are markedly defective in clearing β-migrating VLDL particles, and spontaneously develop atherosclerotic plaques, even on a regular diet. In ApoE2.Ki mice on

an atherogenic diet [237] or exposed to LPS, an exacerbation of atherosclerosis is observed.[238,239].

In this study, we demonstrated that Trx1 can promote differentiation of macrophages into an alternative, anti-inflammatory phenotype that may explain its protective effects in CVDs [207]. Indeed, we have shown that Trx1 induced downregulation of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ and significantly promoted the polarization of anti-inflammatory M2 macrophages in cells exposed to IL-4 or IL-4/IL-13 *in vitro*, as evidenced by the expression of the CD206 also called mannose receptor and IL-10 markers.

In addition, Trx1 induced downregulation of nuclear translocation of AP-1 and Ref-1 and significantly reduced the LPS-induced differentiation of inflammatory M1 macrophages, as indicated by the decreased expression of the M1 cytokines TNF-α and MCP-1. Consistently, Trx1 administered to hyperlipoproteinemic ApoE2.Ki mice challenged either with LPS or IL-4 significantly induced the M2 phenotype while inhibiting differentiation of macrophages into the M1 phenotype in liver and thymus. ApoE2.Ki mice challenged with LPS for 5 weeks developed severe atherosclerotic lesions enriched with macrophages expressing predominantly M1 over M2 markers. In contrast, however, daily injections of Trx1 shifted the phenotype pattern of lesional macrophages in these animals to predominantly M2 over M1, and the aortic lesion area was significantly reduced.



1.1.**Article I**

El Hadri K, Mahmood DF, Couchie D, Jguirim-Souissi I, Genze F, Diderot V, Syrovets T, Lunov O, Simmet T, and Rouis M. Thioredoxin-1 promotes anti-inflammatory macrophages of the M2 phenotype and antagonizes atherosclerosis. *Arterioscler Thromb Vasc Biol* 32: 1445-1452, 2012.

ARTICLE I

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Thioredoxin-1 Promotes Anti-Inflammatory Macrophages of the M2 Phenotype and Antagonizes Atherosclerosis

Khadija El Hadri, Dler Faieeq Darweesh Mahmood, Dominique Couchie, Imene Jguirim-Souissi, Felicitas Genze, Vimala Diderot, Tatiana Syrovets, Oleg Lunov, Thomas Simmet and Mustapha Rouis

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Thioredoxin-1 Promotes Anti-Inflammatory Macrophages of the M2 Phenotype and Antagonizes Atherosclerosis

Khadija El Hadri,* Dler Faieeq Darweesh Mahmood,* Dominique Couchie, Imene Jguirim-Souissi, Felicitas Genze, Vimala Diderot, Tatiana Syrovets, Oleg Lunov, Thomas Simmet, Mustapha Rouis

Objective—Oxidative stress is believed to play a key role in cardiovascular disorders. Thioredoxin (Trx) is an oxidative stress-limiting protein with anti-inflammatory and antiapoptotic properties. Here, we analyzed whether Trx-1 might exert atheroprotective effects by promoting macrophage differentiation into the M2 anti-inflammatory phenotype.

Methods and Results—Trx-1 at 1 μg/mL induced downregulation of $p16^{INK4a}$ and significantly promoted the polarization of anti-inflammatory M2 macrophages in macrophages exposed to interleukin (IL)-4 at 15 ng/mL or IL-4/IL-13 (10 ng/mL each) in vitro, as evidenced by the expression of the CD206 and IL-10 markers. In addition, Trx-1 induced downregulation of nuclear translocation of activator protein-1 and Ref-1, and significantly reduced the lipopolysaccharide-induced differentiation of inflammatory M1 macrophages, as indicated by the decreased expression of the M1 cytokines, tumor necrosis factor-α and monocyte chemoattractant protein-1. Consistently, Trx-1 administered to hyperlipoproteinemic ApoE2.Ki mice at 30 μg/30 g body weight challenged either with lipopolysaccharide at 30 μg/30 g body weight or with IL-4 at 500 ng/30 g body weight significantly induced the M2 phenotype while inhibiting differentiation of macrophages into the M1 phenotype in liver and thymus. ApoE2.Ki mice challenged once weekly with lipopolysaccharide for 5 weeks developed severe atherosclerotic lesions enriched with macrophages expressing predominantly M1 over M2 markers. In contrast, however, daily injections of Trx-1 shifted the phenotype pattern of lesional macrophages in these animals to predominantly M2 over M1, and the aortic lesion area was significantly reduced (from 100%±18% to 62.8%±9.8%; n=8; $P<0.01$). Consistently, Trx-1 colocalized with M2 but not with M1 macrophage markers in human atherosclerotic vessel specimens.

Conclusion—The ability of Trx-1 to promote differentiation of macrophages into an alternative, anti-inflammatory phenotype may explain its protective effects in cardiovascular diseases. These data provide novel insight into the link between oxidative stress and cardiovascular diseases. **(*Arterioscler Thromb Vasc Biol*. 2012;32:1445-1452.)**

Key Words: atherosclerosis ■ inflammation ■ macrophage ■ thioredoxin-1

A crucial step in atherogenesis/atherosclerosis is the infiltration of monocytes into the subendothelial space of large arteries and their subsequent differentiation into tissue macrophages.[1] Macrophages residing within atherosclerotic lesions represent a heterogeneous cell population whose activation and function are influenced by various cytokines and microbial products. Thus, interleukin-1β (IL-1β), interferon-γ, and endotoxin lipopolysaccharide (LPS) increase the classical or inflammatory activation profile yielding the so-called M1 macrophages,[2] which produce proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-6, and IL-12 as well as reactive oxygen species (ROS) and nitrogen intermediates.[3,4] Consistently, M1 macrophages are associated with inflammation and tissue damage. In contrast, IL-4, IL-13,[3,5,6] peroxisome proliferator–activated receptor-γ (PPAR-γ) activators,[7] and adiponectin[8,9] promote polarization of macrophages into the anti-inflammatory, alternative M2 type. M2 macrophages secrete the anti-inflammatory cytokine IL-10, transforming growth factor-β, and IL-1 receptor antagonist, and upregulate scavenger receptors, the mannose receptor CD206, and arginase-1. Accumulation of M2 macrophages leads to reduction of inflammation.

Increasing evidence suggests that risk factors for cardiovascular disease can lead to dramatic increases in the concentration of ROS in the vascular wall. When the rate of ROS formation exceeds the capacity of the antioxidant defense system, oxidative stress occurs, which markedly contributes to arterial inflammation.[10] Various antioxidants, including dietary antioxidants protect against oxidative stress. However, their protective mechanisms are still not well defined.[11]

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Thioredoxin-1 (Trx-1), a 12-kDa highly conserved protein, has recently been recognized as a critical protection system against oxidative stress.[10,12] Trx-1 is important for keeping intracellular cysteine residues in the reduced state.[13–15] It functions by the reversible oxidation of 2 Trx-specific redox-active cysteine residues (Cys-32 and Cys-35) to form a disulfide bond that, in turn, can be reduced by the action of Trx reductase and nicotinamide adenine dinucleotidephosphate. Trx-1 exerts most of its antioxidant ROS scavenging properties through the activity of Trx peroxidase.[16] Trx-1 reduces the oxidized form of Trx peroxidase, and the reduced Trx peroxidase scavenges ROS[17] not only in the cytosol but also in the nucleus. Because ROS can modify the activity of transcription factors, Trx-1 by limiting nuclear ROS levels also affects gene transcription.[15,18–22] In addition, Trx-1 interacts with and inhibits the activity of the apoptosis signal–regulating kinase 1[13,14,18,23] as well as the tumor suppressor gene phosphatase and tensin homolog deleted on chromosome ten (PTEN).[24] Through these mechanisms, Trx-1 inhibits apoptosis.

Trx-1 is negatively regulated by interaction with a specific inhibitor called thioredoxin-interacting protein (TXNIP),[25,26] which exerts its activity in the cytosol as well as in the nucleus.[27] Trx-1 is ubiquitously distributed and, despite its lack of a signal peptide, it can be secreted by several cell types through an unknown pathway.[28,29] Indeed, Trx-1 is actively secreted by a variety of normal and transformed cells and neither brefeldin A nor dinitrophenol, 2 drugs that block the exocytic pathway and inhibit secretion of Trx, indicating that the latter does not follow the classical ER-Golgi route.[28] It appears that the secretory mechanism for Trx-1 shares several features with the alternative pathway described for IL-1β.[28] Nevertheless, translocation of Trx-1 to the membrane requires TXNIP and Trx-1 binding,[30] indicating that this association might be instrumental for the secretion process. Because it is a ubiquitous protein, the precise origin of Trx-1 found in plasma is not known, but every tissue could contribute to its plasma level. The mechanism of cellular uptake remains undefined as well. However, in preliminary experiments with fluorescent-labeled Trx-1 we observed a specific and saturable binding on human macrophages, suggesting the presence of specific cellular receptors or binding sites for Trx-1 (Oleg Lunov, PhD, unpublished data, 2012). Apart from its intracellular function, Trx-1 may be released by cells and can act as a cytokine.[29,31–34] We have previously shown in vitro that human recombinant Trx-1 downregulates the expression of a number of inflammatory genes such as IL-1β, TNF-α, IL-6, and IL-8 in human macrophages.[35] It has also been shown that homocysteine (Hcy) can induce Trx-1 expression in human monocytes,[36] and secreted Trx-1 was found to inhibit Hcy-induced ROS and monocyte chemoattractant protein-1 (MCP-1) production in human monocytes.[37] In addition, Trx-1 specifically cross-desensitizes monocytes to MCP-1.[38] Moreover, a direct association of Trx-1 with macrophage migration inhibitory factor was reported, suggesting that Trx-1 on the cell surface serves as 1 of the migration inhibitory factor binding molecules or migration inhibitory factor receptor components, and it inhibits migration inhibitory factor–mediated inflammatory signals.[39] Furthermore, overexpression of human Trx-1 in transgenic mice attenuated focal ischemic brain damage[40] and increased the resistance to various oxidative stresses leading to longer survival compared with that of control mice.[41]

Although these in vitro and in vivo studies document anti-inflammatory properties of Trx-1, the effects of Trx-1 on the macrophage phenotype have not been addressed yet. We hypothesized that Trx-1 might promote polarization of human and murine macrophages toward an anti-inflammatory M2 phenotype. As an in vivo atherosclerosis model, we have chosen ApoE2.Ki mice expressing human ApoE2 (2/2), which virtually exhibits all characteristics of type III hyperlipoproteinemia in humans. Accordingly, their plasma cholesterol and triglyceride levels are 2× to 3× those of normolipidemic mice.[42] These animals are markedly defective in clearing β-migrating very-low-density lipoprotein particles, and spontaneously develop atherosclerotic plaques, even on a regular diet. In apoE2.Ki mice on an atherogenic diet[42] or exposed to LPS, an exacerbation of atherosclerosis is observed.[43,44]

Methods

For in vitro studies, both murine peritoneal and human macrophages were used. Cells were left untreated or treated with LPS, IL-4 or IL-4 + IL-13, Trx-1, or a combination thereof. In some samples, a specific antibody against full-length human Trx-1 was added. To study the binding of Trx-1, recombinant Trx-1 was fluorescently labeled and incubated with macrophages, and fluorescence images were taken using Zeiss LSM710 confocal scanning microscope. The expression of macrophage phenotype markers, CD206, MCP-1, IL-10, and TNF-α, and other involved genes, $p16^{INK4a}$, activator protein (AP)-1 and Ref-1, were investigated at both transcription and protein levels using real-time polymerase chain reaction and immunoblotting or ELISA, respectively. In vivo study was performed on C57Bl/6. ApoE2.ki mice. Heart, liver, spleen, thymus, pancreas, and kidneys were excised, fixed, and immunohistochemical studies were performed. Serial paraffin-embedded sections on proximal aorta sections were stained and the mean lesion area per animal was quantified. Human atherosclerotic vessel specimens from patients undergoing vascular surgery for atherosclerotic complications were fixed and immunohistochemical analysis was performed. Statistical significance was calculated with the Newman-Keuls test (given in detail in the online-only Data Supplement).

Results

Trx-1 Binds to Macrophages

Extracellular Trx-1 binds rapidly to the surface of macrophages (Figure I and Video S1I in the online-only Data Supplement), which internalize Trx-1. After 24 hours, Trx-1 was found predominantly within the lysosomal compartments of macrophages (Figure II and Video II in the online-only Data Supplement).

Trx-1 Induces M2 Markers in Murine Peritoneal Macrophages

The structure of Trx-1 is highly conserved, human and murine Trx-1 being 90% homologous. Therefore, we analyzed whether human recombinant Trx-1 would affect polarization of murine and human macrophages. In line with reported data,[5,7] IL-4 significantly induced mRNA expression of the M2 macrophage marker CD206, 2.8±0.6 a.u. versus control cells 1.0±0.4 a.u., $P<0.01$ (Figure 1A) as well as protein expression, 2.8±1.2 a.u. versus control cells 1.0±0.2 a.u., $P<0.05$ (Figure 1B) in peritoneal macrophages from C57BL/6 mice. In addition, the expression of CD206 induced by IL-4 was amplified by Trx-1.

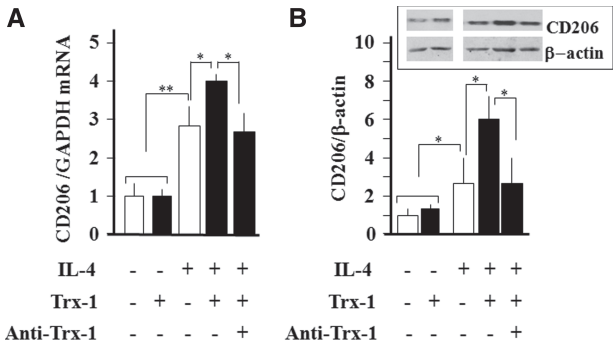

Figure 1. Thioredoxin (Trx)-1 promotes an anti-inflammatory phenotype in freshly isolated murine peritoneal macrophages. **A**, mRNA levels of CD206 after treatment with recombinant Trx-1 (1 μg/mL), interleukin (IL)-4 (15 ng/mL), and specific antibody against full-length human Trx-1 (5 μg/mL) for 24 hours. mRNA levels of CD206 were quantified with real-time polymerase chain reaction (qPCR) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **B**, Quantification of Western blots of CD206 and β-actin after treatment with recombinant Trx-1 (1 μg/mL), IL-4 (15 ng/mL), and specific antibody against full-length human Trx-1 (5 μg/mL) for 24 hours. Data are presented as mean±SE of 3 independent experiments performed in triplicate. *$P<0.05$, **$P<0.01$ as indicated. One representative Western immunoblot was inserted.

Thus, in IL-4-treated cells, Trx-1 significantly increased the expression of CD206 mRNA from 2.8±0.6 a.u. to 4±0.2 a.u., $P<0.05$ (Figure 1A) and CD206 protein levels from 2.8±1.2 a.u. to 6±1.2 a.u., $P<0.05$ (Figure 1B). The effect of Trx-1 on macrophage polarization was specific, because it could be virtually abolished by an anti-Trx-1 antibody (Figure 1A and 1B).

Similarly, IL-4 induced the expression of IL-10 mRNA, 3.2±0.3 a.u. versus control cells 1.0±0.2 a.u., $P<0.01$ (Figure 2A) as well as protein, 33±7 pg/mL versus control cells 6.5±3.4 pg/mL, $P<0.01$ (Figure 2B), and this expression was amplified by Trx-1 from 3.2±0.3 a.u. to 4.8±0.2 a.u., $P<0.01$ (for mRNA) and from 33±7 pg/mL to 70±5 pg/mL, $P<0.05$ (for protein) (Figure 2A and 2B). The effect of Trx-1 on macrophage polarization was specific, because again it could be neutralized by an anti-Trx-1 antibody (Figure 2A and 2B). Analogous upregulation of CD206 and IL-10 expression by Trx-1 was observed in macrophages treated with IL-13 alone or in combination with IL-4 (data not shown). Moreover, a similar increase in M2 markers was also observed in human monocyte–derived macrophages treated with Trx-1 (Figure III in the online-only Data Supplement). Of note, we used Trx-1 at 1 μg/mL, because in pilot experiments this concentration exerted the maximum effect on macrophage polarization in vitro (data not shown).

Trx-1 Attenuates the Expression of Murine M1 Macrophages Markers

To determine whether Trx-1 affects the expression of the M1 macrophage markers, peritoneal macrophages were treated with LPS in the absence or presence of recombinant human Trx-1. LPS significantly induced the mRNA of the M1 macrophage marker TNF-α, 5.7±0.5 a.u. versus control cells 1.0±0.2 a.u., $P<0.01$ (Figure 3A) as well as protein, 3.0±0.5 ng/mL versus control cells 0.6±0.2 ng/mL, $P<0.01$ (Figure 3B). Trx-1 significantly reduced the expression of TNF-α in LPS-treated cells. Thus, Trx-1 reduced the LPS-induced expression of TNF-α mRNA from 5.7±0.5 a.u. to 3.0±0.4 a.u., $P<0.01$ as well as its protein from 3.0±0.5 ng/mL to 1.3±0.5 ng/mL, $P<0.01$ (Figure 3A and 3B). Moreover, this effect was also almost completely abolished by an anti-Trx-1 antibody (Figure 3A and 3B).

LPS also induced significant expression of another M1 marker, MCP-1. LPS increased MCP-1 mRNA from 1.0±0.3 a.u. (controls) to 7.6±0.6 a.u., $P<0.001$ (Figure 4A) and for

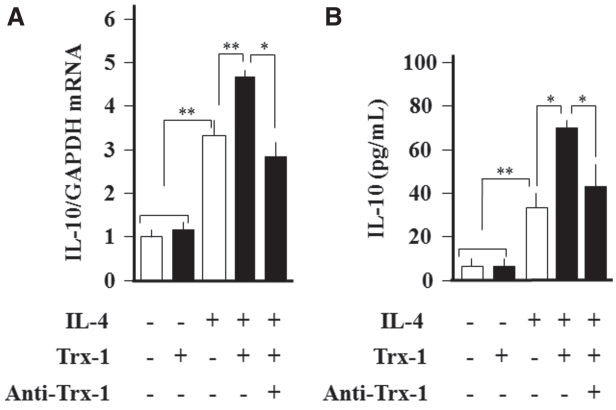

Figure 2. Thioredoxin (Trx)-1 promotes the M2 anti-inflammatory phenotype in freshly isolated murine peritoneal macrophages. **A**, mRNA levels of IL-10 after treatment with recombinant Trx-1 (1 μg/mL), interleukin (IL)-4 (15 ng/mL), and specific antibody against full-length human Trx-1 (5 μg/mL) for 24 hours. mRNA levels of IL-10 were quantified with real-time polymerase chain reaction (qPCR) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **B**, Levels of IL-10 in cell supernatants after treatment with recombinant Trx-1 (1 μg/mL), IL-4 (15 ng/mL), and specific antibody against full-length human Trx-1 (5 μg/mL) for 24 hours. Data are presented as mean±SE of 3 independent experiments performed in triplicate. *$P<0.05$, **$P<0.01$ as indicated.

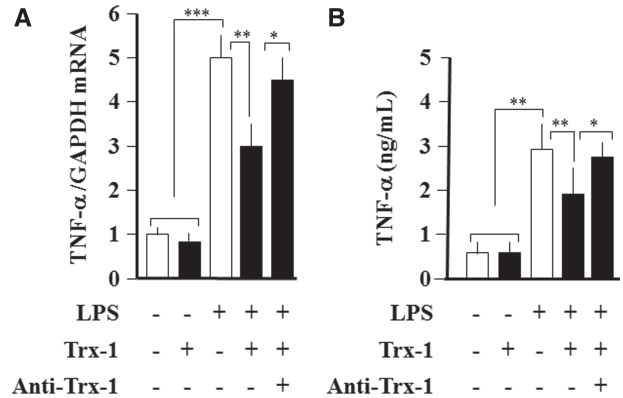

Figure 3. Thioredoxin (Trx)-1 attenuates the M1 proinflammatory phenotype in freshly isolated murine peritoneal macrophages. **A**, mRNA levels of tumor necrosis factor-α (TNF-α) after treatment with recombinant Trx-1 (1 μg/mL), lipopolysaccharide (LPS) (100 ng/mL), and specific antibody against full-length human Trx-1 (5 μg/mL) for 24 hours. mRNA levels of TNF-α were quantified with real-time chain reaction (qPCR) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **B**, Levels of TNF-α in cell supernatants after treatment with recombinant Trx-1 (1 μg/mL), IL-4 (15 ng/mL), and specific antibody against full-length human Trx-1 (5 μg/mL) for 24 hours. Data are presented as mean±SE of 3 independent experiments performed in triplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ as indicated.

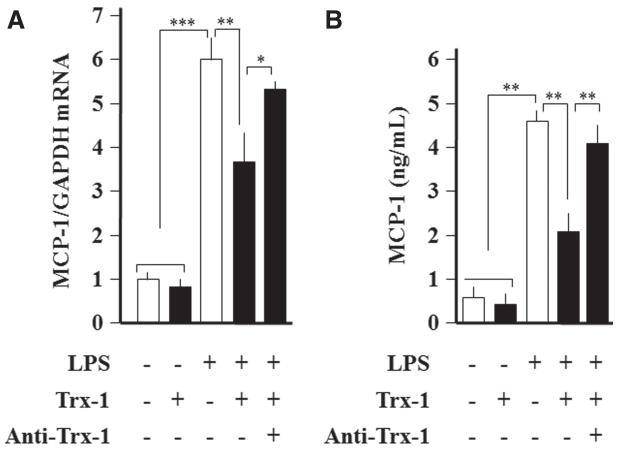

Figure 4. Thioredoxin (Trx)-1 attenuates the M1 proinflammatory phenotype in freshly isolated murine peritoneal macrophages. **A**, mRNA levels of monocyte chemoattractant protein-1 (MCP-1) after treatment with recombinant Trx-1 (1 μg/mL), lipopolysaccharide (LPS) (100 ng/mL), and specific antibody against full-length human Trx-1 (5 μg/mL) for 24 hours. mRNA levels of MCP-1 were quantified with real-time polymerase chain reaction (qPCR) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **B**, Levels of MCP-1 in cell supernatants after treatment with recombinant Trx-1 (1 μg/mL), IL-4 (15 ng/mL), and specific antibody against full-length human Trx-1 (5 μg/mL) for 24 hours. Data are presented as mean±SE of 3 independent experiments performed in triplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ as indicated.

the protein level from 0.6±0.4 ng/mL (controls) to 4.9±0.7 ng/mL, $P<0.01$ (Figure 4B). In addition, the expression of MCP-1 was significantly reduced by Trx-1 in LPS-treated cells. Thus, Trx-1 reduced the LPS-induced expression of MCP-1 mRNA from 7.6±0.6 a.u. to 3.6±0.9 a.u., $P<0.01$ (Figure 4A) and protein from 4.9±0.7 ng/mL to 2.1±0.4 ng/mL, $P<0.01$ (Figure 4B). In analogy to previous experiments, the inhibitory effect of Trx-1 on LPS-induced expression of MCP-1 marker was almost totally prevented by a specific anti-Trx-1 antibody (Figure 4A and 4B). Similar to murine peritoneal macrophages, Trx-1 decreased the expression of M1 markers in human monocyte–derived macrophages (Figure IV in the online-only Data Supplement).

Trx-1 Induces M2 Macrophage Polarization Through Downregulation of p16^{INK4a} and Reduces M1 Polarization Through Downregulation of AP-1 and Ref-1

To determine the mechanism of macrophage polarization into the M2/M1 phenotype, we analyzed the effect of Trx-1 on the expression of the cyclin-dependent kinase inhibitor p16^{INK4a}. Only recently, it has been shown that p16^{INK4a} deficiency (p16$^{-/-}$) modulates the macrophage phenotype.[45] Thus, p16$^{-/-}$ bone marrow–derived macrophages display a decreased response to classical polarizing by interferon-γ and LPS but an increased sensitivity to alternative polarization by IL-4.[45] Moreover, Trx-1 downregulates MCP-1 expression and secretion in oxidized low-density lipoprotein–stimulated human endothelial cells by suppressing nuclear translocation of AP-1 and redox factor-1.[46] Therefore, we hypothesized that, on the one hand, Trx-1 might induce macrophage polarization into the M2 phenotype through downregulation of p16^{INK4a} and on the other hand, it might reduce macrophage polarization into the M1 phenotype through downregulation of AP-1 and Ref-1 expression. Indeed, our results confirmed that Trx-1 downregulated the expression of p16^{INK4a} at the protein level in IL-4-stimulated macrophages (Figure 5A) and the expression of AP-1 and Ref-1 in LPS-induced macrophages (Figure 5B and 5C).

Short-Term Effects of Trx-1 on M1/M2 Macrophages in Tissues of ApoE2.Ki Mice

Macrophages are highly plastic cells that enter target tissues and gain the phenotypic and functional attributes of their tissue of residence.[47] To address such phenotypic changes, we analyzed the effects of Trx-1 administration on the expression of M1/M2 markers in murine tissues. Basal plasma level of Trx-1 in 7 mice (C57BL/6) is approximately 9 ng/mL (Mahmood et al, personal communication). For the in vivo study, we injected recombinant human Trx-1 at 30 μg/g of mouse body weight because in a pilot study, mice injected with various doses of Trx-1 showed the maximum effect at 30 μg/g of mouse body weight. The results showed no changes in the expression of M1 and M2 macrophage markers in spleen, pancreas, and kidneys (data not shown). However, in agreement

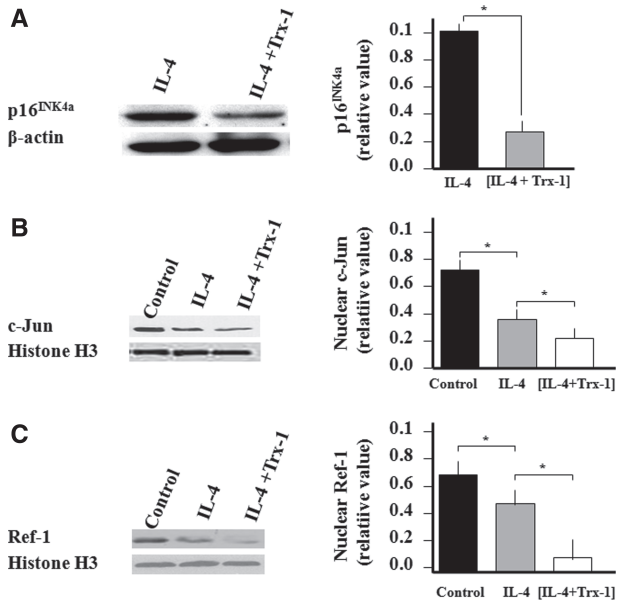

Figure 5. Thioredoxin (Trx)-1 attenuates expression of p16^{INK4a}, activator protein (AP)-1, and Ref-1 in macrophages. **A, left**, Cells were treated with interleukin (IL)-4 (15 ng/mL) or with IL-4 (15 ng/mL) and Trx-1 (1 μg/mL) for 24 hours. Aliquots of proteins from whole cell lysates were electrophoresed and transferred to nitrocellulose membranes. Immunoblot analysis was performed with p16^{INK4a} primary polyclonal antibody. β-actin was used for normalization. **Right**, Relative p16^{INK4a} protein content in IL-4 or in IL-4 plus Trx-1–treated macrophages based on 3 independent immunoblot analyses. **B, left**, Immunoblot analysis for nuclear c-Jun protein content in control or in stimulated macrophages. Histone 3 was stained for normalization. **Right**, Relative c-Jun content based on 3 independent immunoblot analyses. **C, left**, Immunoblot analysis for nuclear Ref-1 protein content in control or in stimulated macrophages. Histone H3 staining for normalization. **Right**, Relative Ref-1 content based on 3 independent immunoblot analyses. Data are mean±SEM, *$P<0.05$.

with the data obtained in vitro with mouse peritoneal and human macrophages, Trx-1 increased the number of CD206$^+$ M2 and decreased the TNF-α$^+$ M1 macrophages in thymus (Figure VA in the online-only Data Supplement). Moreover, it decreased the LPS-induced M1 marker TNF-α to an even greater extent than IL-4 and antagonized the LPS-induced decrease of CD206$^+$ thymus macrophages (Figure VA in the online-only Data Supplement).

Trx-1 exhibited comparable effects on liver macrophages, where it potentiated the IL-4-induced expression of CD206 (Figure VA in the online-only Data Supplement) and inhibited the LPS-induced expression of TNF-α (Figure VB in the online-only Data Supplement). Trx-1 also revered the LPS-induced decrease of CD206 (Figure VA in the online-only Data Supplement). Thus, Trx-1 promotes M2 macrophage markers and inhibits the M1 phenotype in tissue macrophages.

Chronic Treatment With Trx-1 Decreases the Number of Lesional Macrophages and Reduces the Size of Atherosclerotic Lesions

ApoE2.Ki hyperlipoproteinemic mice challenged repeatedly with LPS exhibit a significant increase in lesion size compared with control animals, as we have shown previously.[43] Lesional macrophages in these mice express high levels of TNF-α and low levels of CD206 exhibiting predominantly the proinflammatory M1 phenotype. However, when LPS-injected ApoE2.Ki mice were additionally treated with human recombinant Trx-1, the amount of TNF-α$^+$ M1 macrophages was significantly reduced, and the number of CD206$^+$ M2 macrophages was significantly increased (Figure 6A). Moreover, Trx-1 significantly reduced the size of aortic atherosclerotic lesions in ApoE2.Ki hyperlipoproteinemic mice challenged with LPS, 100±18% (LPS-treated) versus 62.8%±9.8 % (Trx-1-treated LPS-challenged mice), n=8, $P<0.01$ (Figure 6B). Similarly, liver tissue of mice chronically treated with Trx-1 exhibited a reduced expression of M1 macrophage marker TNF-α but an enhanced expression of the M2 marker CD206 (Figure VB in the online-only Data Supplement).

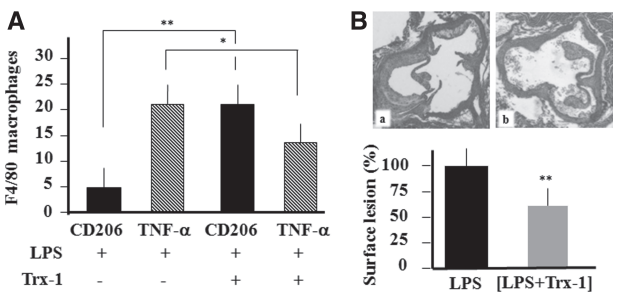

Figure 6. Thioredoxin (Trx)-1 induces the M2 macrophage polarization in murine atherosclerotic lesions. C57Bl/6.ApoE2.Ki mice were intravenously injected with lipopolysaccharide (LPS) (10 μg/30 g body weight) once weekly and daily with either Trx-1 (30 μg/30 g body weight) or PBS for 5 weeks, when the mice were euthanized. **A**, Frozen serial sections (7 μm) of proximal aortas were analyzed by immunohistochemistry using double immunostaining with antibodies against either the macrophage marker F4/80 and the M1 marker tumor necrosis factor-α (TNF-α) or the M2 marker CD206, respectively. Furthermore, Trx-1 reduces the atherosclerotic lesion size. C57Bl/6.ApoE2.Ki mice were intravenously injected with LPS (10 μg/30 g body weight) once weekly and in addition daily with either Trx-1 (30 μg/30 g body weight) or PBS for 5 weeks. Serial paraffin-embedded sections (5 μm) of proximal aortas were prepared and stained with hematoxylin, eosin, and saffron for lesion area measurement. **B, lower right**, Average of lesion area staining in 3 sections of 5 μm each separated by 100 μm from individual animal. Eight mice in total were analyzed. **Upper right**, Representative photomicrographs of atherosclerotic lesions in the aortic sinus of LPS-treated mice versus LPS- and Trx-1–treated mice. Data are mean±SEM of 8 mice, *$P<0.05$, **$P<0.01$ as indicated.

Trx-1 Colocalizes With M2 Macrophages in Human Atherosclerotic Lesions

Previous studies have shown the presence of M1 and M2 macrophages in atherosclerotic lesions.[1,7] However, it was unknown whether Trx-1 would colocalize with M1 or M2 markers in complicated atherosclerotic lesions. Not unexpected, the staining pattern of CD206 in serial sections of human atherosclerotic plaques confirmed that Trx-1-expressing cells colocalize with the M2 macrophage marker CD206. In contrast, Trx-1 does not colocalize with TNF-α, a marker of inflammatory M1 macrophages (Figure VI in the online-only Data Supplement), indicating that Trx-1 could promote the M2 differentiation in human atherosclerotic vessels as well.

Discussion

Macrophages possess a remarkable diversity, which depends on the type, duration, and concentration or intensity of environmental signals such as bacterial products and cytokines. Macrophages are heterogeneous, and subpopulations exhibit differential gene expression, release different cytokines and secondary mediators, and express different cell surface antigens. According to their phenotype, macrophages can be divided into 2 major populations: those which promote inflammation and those which limit inflammation and support tissue remodeling and angiogenesis.[48] At variance to lymphocytes, which cannot be reprogrammed, macrophages retain plasticity, meaning that alteration of environmental signals could tip the balance between M1 and M2 populations. Changing the composition of macrophage population in tissues would affect the pathogenesis, evolution, and complication of many diseases including atherosclerosis.[49] Therefore, identification of agents that might favorably modulate the balance of macrophage polarization is of great importance.

In the present study, we show that human Trx-1, an oxidative stress-limiting protein, potentiates the expression of anti-inflammatory M2 macrophages as evidenced by the characteristic markers CD206 and IL-10 through downregulation of p16^{INK4a} expression. In the same time, Trx-1 blunted the expression of proinflammatory M1 markers such as TNF-α and MCP-1 in human monocyte–derived macrophages and in murine peritoneal macrophages through downregulation of AP-1 and Ref-1 transcription factor expression. The exact correlation between human macrophage subpopulations and their murine counterparts, particularly in atheroma, is still unknown. Nevertheless, we found similar effects in human as well as in murine macrophages. In addition, we complemented our in vitro experiments with in vivo studies using a hyperlipoproteinemic transgenic mouse model.

Our in vitro data showing promotion of the M2 over the M1 phenotype by Trx-1 were supported by in vivo data in this model. Trx-1, when administered intravenously to ApoE2. Ki mice for 5 days or for 5 weeks, increased the expression of the M2 anti-inflammatory macrophage marker CD206 in thymus and liver, as well as in the atherosclerotic lesions. In contrast, the high expression of the macrophage M1 marker TNF-α observed in mice injected with LPS was significantly decreased by the Trx-1 treatment. Accordingly, the surface area of atherosclerotic lesions was significantly reduced in mice treated with Trx-1 compared with mice treated with LPS alone. Moreover, in human atherosclerotic plaques, we observed colocalization of Trx-1 with CD206, a marker of anti-inflammatory M2 macrophages, but not with TNF-α, a marker of inflammatory M1 macrophages.

Taken together, these studies indicate that Trx-1 functions as a regulator of macrophage phenotype differentiation tipping the balance toward the anti-inflammatory M2 state. As a consequence, atherosclerotic plaques would become smaller and more stable. Of note, plasma cholesterol and triglycerides levels in LPS-treated mice versus LPS- and Trx-1-treated mice were not significantly different (Table II in the online-only Data Supplement). However, whether the stability of atheroma plaques attributed to M2 phenotype could be explained by a difference in lipid metabolism and lipid accumulation in M1 in comparison with M2 macrophages was unknown. Therefore, we conducted additional experiments on murine and human macrophages to determine the level of ATP-binding cassette transporter A1 expression in the M1 or M2 phenotype. These results showed a decrease of ATP-binding cassette transporter A1 expression in M2 macrophages in comparison with proinflammatory M1 macrophages (data not shown), suggesting that M2 macrophages may have reduced reverse cholesterol efflux capacities. Of note, similar results have just been published by Chinetti-Gbaguidi et al.[50] This group also observed that, indeed, ApoA-I- and HDL_3-mediated cholesterol efflux was significantly lower in [^{3}H]cholesterol-AcLDL–loaded M2 macrophages compared with M1 macrophages. The consequence of a defective cholesterol efflux in M2 macrophages is an increase of cholesterol esterification by inducing lysosomal acid lipase and acetyl-CoA acetyltransferase-1 gene expression. This is a protective mechanism against the toxicity of excess free cholesterol.[50] Moreover, expression of both scavenger receptors SR-A and CD36 was similar in M2 and M1 macrophages.[50] In contrast, M2 macrophages displayed a lower gene expression level of LOX-1 and caveolin-1 compared with M1 macrophages.[50] M2 macrophages accumulate less oxidized and native LDL, an effect that is expected to lower the chance of foam cell formation.[50] In our experimental conditions, Trx-1 enhances macrophage polarization into M2 phenotype, which might reduce the number of foam cells.

One well-established pathway by which Trx-1 might control the inflammatory response is by changing NF-κB and Nrf2 redox-sensitive signaling pathways.[35,51] In addition, TXNIP, the endogenous inhibitor of Trx-1, can activate the Nlrp3 inflammasome leading to caspase-1-dependent IL-1β maturation.[52] It is known that through autocrine and paracrine action secreted IL-1β could mediate β cell death and dysfunction[53] as well as arterial inflammation.[54] Thus, enhanced concentrations of Trx-1 could neutralize the TXNIP and therefore prevent the Nlrp3 inflammasome activation and IL-1β production.

Moreover, in a recent publication, PPAR-γ, a ligand-activated nuclear receptor with potent anti-inflammatory properties, was found to prime human monocytes into alternative M2 macrophages with anti-inflammatory properties.[7] However, at variance to Trx-1, PPAR-γ activation does not influence the expression of M2 markers in resting or M1 macrophages, nor does treatment with a PPAR-γ agonist influence the expression of M2 markers in atherosclerotic lesions, indicating that only native monocytes can be primed by PPAR-γ activation shifting them toward the M2 phenotype.[7] Because we have previously shown that PPAR-γ activation significantly downregulates the expression of the Trx-1 gene and upregulates the expression of Trx-1 inhibitor TXNIP in human macrophages,[55] PPAR-γ could not account for the effects of Trx-1 and vice versa. In addition, different to PPAR-γ, Trx-1 induces M2 differentiation in atherosclerotic lesions and in tissue macrophages in vivo, and it is also able of reverting the LPS-induced macrophage M1 polarization. Therefore, Trx-1 seems to be a more versatile inducer of M2 differentiation compared with PPAR-γ.

Indeed, a number of reports indicate that Trx-1 has beneficial effects on ischemic reperfusion injury, LPS-induced neutrophil chemotaxis in the mouse air pouch model,[56] as well as on focal ischemic brain damage[40] and the resistance to oxidative stress.[41] These pathological conditions all involve the activation of an acute or chronic inflammatory response. Thus, the ability of Trx-1 to change the balance between M1 and M2 macrophages, to promote an alternative, anti-inflammatory macrophage phenotype provides an attractive mechanism explaining its protective effects under these diverse pathological conditions.

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Disclosures

None.

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Supplemental Material

Thioredoxin-1 Promotes Anti-inflammatory Macrophages of the M2 Phenotype and Antagonizes Atherosclerosis

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Supplemental Material

Methods

Isolation of Human Monocyte-Derived Macrophages

Mononuclear cells were isolated from buffy-coats of healthy normolipidemic donors using Ficoll gradient centrifugation and were subsequently cultured in RPMI 1640 medium containing gentamycin (40 µg/mL), glutamine (0.05%) (Sigma) and 10% pooled human serum (Promocell) at a density of 6×10^6 cells/well in 60 mm Primaria culture dishes (Polylabo). Monocytes were permitted to differentiate into macrophages by adhesion to the culture dish and subsequent maturation for 8 days in culture.

Isolation and treatment of Mouse Peritoneal Macrophages

Peritoneal macrophages were collected from 8-10 week-old female C57Bl/6 mice by peritoneal lavage with 10 mL of phosphate-buffered saline, centrifuged at 400 *g* for 10 min, and cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum (FBS), 2 mmol L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from BioWhittaker). Before each experiment, cells were plated in RPMI 1640 with 2% FBS for 16 h. Cells were left untreated or were treated with LPS (100 ng/mL, *E. coli*, serotype 055:B5, Sigma-Aldrich), IL-4 (15 ng/mL) or IL-4 (10 ng/mL) + IL-13 (10 ng/mL), Trx-1 (1 µg/mL) (all from R&D Systems) or a combination thereof. In some samples, a specific antibody against full length human Trx-1 was added (5 µg/mL).

RNA Extraction and Analysis

Total cellular RNA extracted from cells using Trizol (Invitrogen SARL) was reverse transcribed. cDNAs were quantified by quantitative polymerase chain reaction (qPCR) on a MX4000 apparatus (Stratagene) using specific primers (Supplemental Table I). PCR amplification was performed in a volume of 25 µL containing 100 nmol/L of each paired primer, 4 mmol/L $MgCl_2$, and the Brilliant Quantitative PCR Core reagent Kit mix as recommended by the manufacturer (Stratagene). The thermal cycling conditions were 95°C

for 10 min, followed by 40 cycles of 30 sec each at 95, 55, and 72 °C. The relative gene expression was determined by normalizing to GAPDH or cyclophilin mRNA using the ΔC_T method.

Western Blot Analysis

Proteins from whole cell lysates of macrophages were separated on 4-12% Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen). Immunoblot analysis was performed with CD206 primary polyclonal antibody (R&D Systems) or with $p16^{INK4a}$ primary antibody followed by incubation with appropriate horseradish peroxidase-associated secondary antibody before the signal was visualized by enhanced chemiluminescence (Amersham Bioscience)**.** Beta-actin staining served as loading control.

Nuclear Protein extraction and immunoblotting

Cells were lysed in lysis buffer (20 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L h-glycerolphosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leuptin, and 1 mmol/L phenylmethylsulphonyl fluoride, pH 7.5) on ice for 15 min. Cell debris was removed by centrifugation at 18,000 *g* at 4°C for 15 min, and the nuclear protein extracts were obtained from the cell lysates with NE-PER extraction reagents (Thermo Scientific). Protein contents of the nuclear extracts were determined using a Bio-Rad protein assay kit. Equal protein contents were loaded and separated by 12% SDS-PAGE. The protein bands were electrotransferred onto nitrocellulose membrane and blocked with 5% nonfat milk in TBS/T buffet (20 mmol/L Tris base, 135 mmol/L NaCl, 0.1% Tween 20, pH 7.6) for 1 h. After incubation of the membrane with rabbit polyclonal c-Jun antibody and mouse monoclonal Ref-1 antibody (Santa Cruz), and rabbit polyclonal Histone H3 antibody (Abcam) for 4 h. Specific protein bands were visualized with SuperSignal West Pico Chemiluminescent Substrate.

ELISA

Release of IL-10, TNFα and MCP-1 by mouse peritoneal macrophages was analyzed by ELISA (R&D Systems) 24 h after treatment with recombinant Trx-1 (1 µg/mL), IL-4 (15 ng/mL) and specific antibody against full length human Trx-1 (5 µg/mL).

In vivo Studies

C57Bl/6.ApoE2.ki mice bearing the human *APOE2* allele were obtained from Dr. Nobuyo Maeda (Department of Pathology and Laboratory Medicine, University of North Carolina). Female C57Bl/6.ApoE2.Ki mice (body weight 20 to 23 g; 8 weeks of age) were intravenously injected into the tail vein with PBS (control), LPS, IL-4, or Trx-1 alone, or in combination at day 0 and day 3 as indicated. At day 5, mice were sacrificed and tissues of different organs were collected for further immunohistochemical analysis.

Alternatively, C57Bl/6.ApoE2.Ki mice were randomly divided into two groups (n=8 per group). The first group was challenged intravenously with LPS (10 µg/30 g body weight) once every week and with daily PBS for 5 weeks. The second group was injected with LPS (10 µg/30 g body weight) once every week and daily with human recombinant Trx-1 (30 µg/30 g body weight) for 5 weeks. At the end of the study, the animals were killed by exsanguination, perfused transcardially with PBS, and the heart, liver, spleen, thymus, pancreas and kidneys were excised. Hearts and proximal aortas were fixed in 10% buffered formalin and then either embedded in paraffin for lesion quantification or frozen for immunohistochemical studies. Serial paraffin-embedded sections (5 µm) were stained with hematoxylin, eosin, and saffron for lesion area measurement and the mean lesion area per animal was quantified (three 5 µm aortic sections, each separated by 100 µm).

All procedures involving animal handling and their care were in accordance with the University of Pierre et Marie Curie guidelines for Husbandry of laboratory mice.

Immunohistochemical Analysis

Frozen serial sections (7 μm) of proximal aortas were treated with 0.3% H_2O_2 in PBS to block endogenous peroxidase activity, followed by blocking in 4% BSA (Sigma). Slides were incubated (2 h at 20°C) with either purified rat monoclonal antibody against mouse F4/80 macrophages (AbDSerotec), followed by peroxidase-conjugated secondary antibody and diaminobenzidine (Merck) as a substrate. For fluorescent microscopy, liver and thymus were fixed, paraffin-imbedded, and 5 μm sections were stained with anti-TNF-α and CD206 antibodies, and secondary fluorescence-labeled $F(ab')_2$ (Dianova). TNF-α and CD206 positive cells were quantified using ImageJ software (NIH).

Human atherosclerotic vessel specimens from 5 patients undergoing vascular surgery for atherosclerotic complications were formalin-fixed, paraffin-embedded, and prepared for immunohistochemical analysis. The 5 μm sections were double-stained either with TNF-α or CD206 primary antibodies and with AP-conjugated secondary antibody and Fast-Red chromogenic substrate, and Trx-1 monoclonal mouse antibody that recognizes exclusively the full length protein but not its truncated form (BD Biosciences). The Trx-1 antibody was visualized with HRP-conjugated secondary antibody and diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate (Picture™ Double Staining Kit, Invitrogen). In control samples, the primary antibodies were substituted with control IgG. Sections were counterstained with hematoxylin. The images were digitally recorded with an Axiophot microscope (Carl Zeiss) and a Sony MC-3249 CCD camera using Visupac 22.1 software (Carl Zeiss). Original magnifications, 200x and 400x.

Confocal Laser Scanning Microscopy

Recombinant Trx-1 (R&D Systems) was fluorescently labeled with Alexa 488 succinimidyl ester (green dye) according to the manufacturer's instruction (Invitrogen). Macrophages seeded on IBIDI slides (Munich, Germany) were treated with fluorescently labeled Trx-1-1 (1

μg/mL). Cells were stained with the membrane stain CellMask™ Deep Red or with a lysosomal marker LysoTracker® Red DND-99 (75 nmol/L, 30 min) (Invitrogen). Fluorescence images were taken with the acquisition software ZEN 2009 using Zeiss LSM710 confocal scanning microscope with a 63×1.4NA oil immersion objective. ImageJ software (NIH) was used for imaging processing, 3D and movie reconstruction.

Statistical Analysis

Statistical significance was calculated with the Newman-Keuls test. Differences were considered significant for $P< 0.05$.

Supplemental Material

Supplemental Data

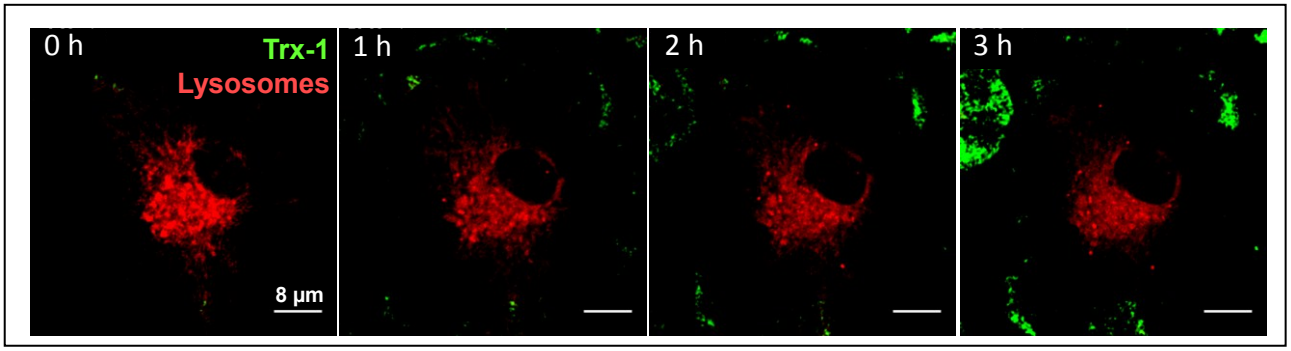
Trx-1 Promotes Differentiation of Human Monocytes into M2 Macrophages

To determine whether Trx-1 plays a role in M2 differentiation, primary human monocytes were differentiated *in vitro* into M2 macrophages with recombinant IL-4 in the absence or presence of recombinant human Trx-1. As reported, IL-4 significantly induced the M2 macrophage markers CD206 (also called mannose receptor, 8.5 ± 0.7 a.u. *vs*. control cells 0.7 ± 0.6 a.u., $P<0.001$) and IL-10 (6.9 ± 1.4 a.u. *vs*. control cells 0.7± 0.6 a.u., $P<0.001$) (Supplemental Figure III). As expected, the expression of both markers was amplified by Trx-1. Thus, in IL-4-treated cells, Trx-1 significantly increased the expression of CD206 from 8.5 ± 0.7 a.u. to 16 ± 1.0 a.u. ($P<0.01$). Similarly, Trx-1 increased the expression of IL-10 from 6.9 ± 1.4 a.u. to 18 ± 2 a.u. ($P<0.01$) (Supplemental Figure III). Of note, we used Trx-1 at 1 µg/mL in all *in vitro* experiments reported in this manuscript, because in pilot concentration-response experiments, 1 µg/mL Trx-1 exerted the maximum effect on macrophage polarization *in vitro* (data not shown). The effect of Trx-1 on macrophage polarization was specific, because it could be virtually neutralized by an anti-Trx-1 antibody (Supplemental Figure III). A similar regulation of CD206 and IL-10 expression by Trx-1 was observed in M2 macrophages differentiated from monocytes in the presence of IL-13 alone or in combination with IL-4 (data not shown).

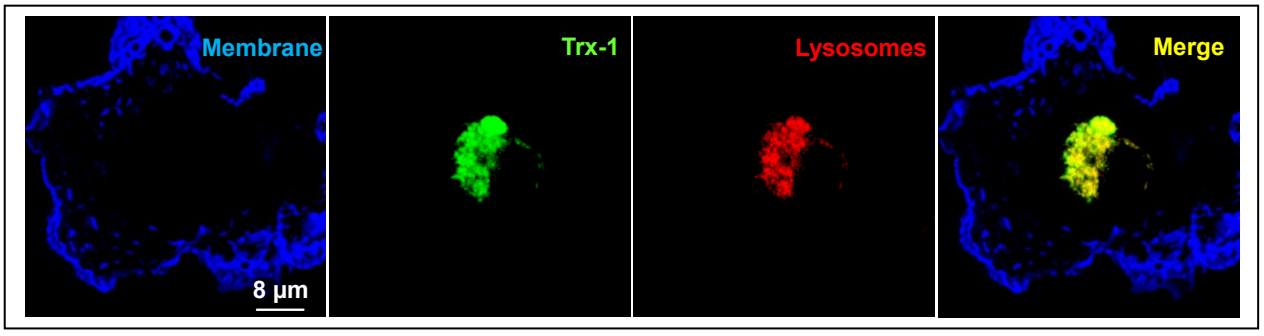
Trx-1 Attenuates Differentiation of Human Monocytes into M1 Macrophages

To determine whether Trx-1 plays a role in M1 differentiation, primary human monocytes were differentiated *in vitro* into the M1 phenotype with LPS in the absence or presence of recombinant human Trx-1. LPS significantly induced M1 macrophage markers such as TNF-α (6.05 ± 0.9 *vs*. control cells 0.9 ± 0.8 a.u., $P<0.01$) and MCP-1 (3.5 ± 1.4 *vs*. control cells 0.9± 0.4 a.u., $P<0.01$) (Supplemental Figure IV). The expression of both markers was significantly

reduced by Trx-1 at 1 µg/mL. Thus, Trx-1 reduced the LPS-induced expression of TNF-α from 6.05 ± 0.9 a.u. to 3.5 ± 0.3 (P<0.01) and that of MCP-1 from 3.5 ± 0.3 a.u. to 1.9 ± 1.0 a.u. (P<0.01) (Supplemental Figure IV). In analogy to the observation on M2 differentiation markers, the inhibitory effect of Trx-1 on the LPS-induced expression of M1 markers was almost totally abolished in the presence of specific anti-Trx-1 antibody (Supplemental Figure IV).

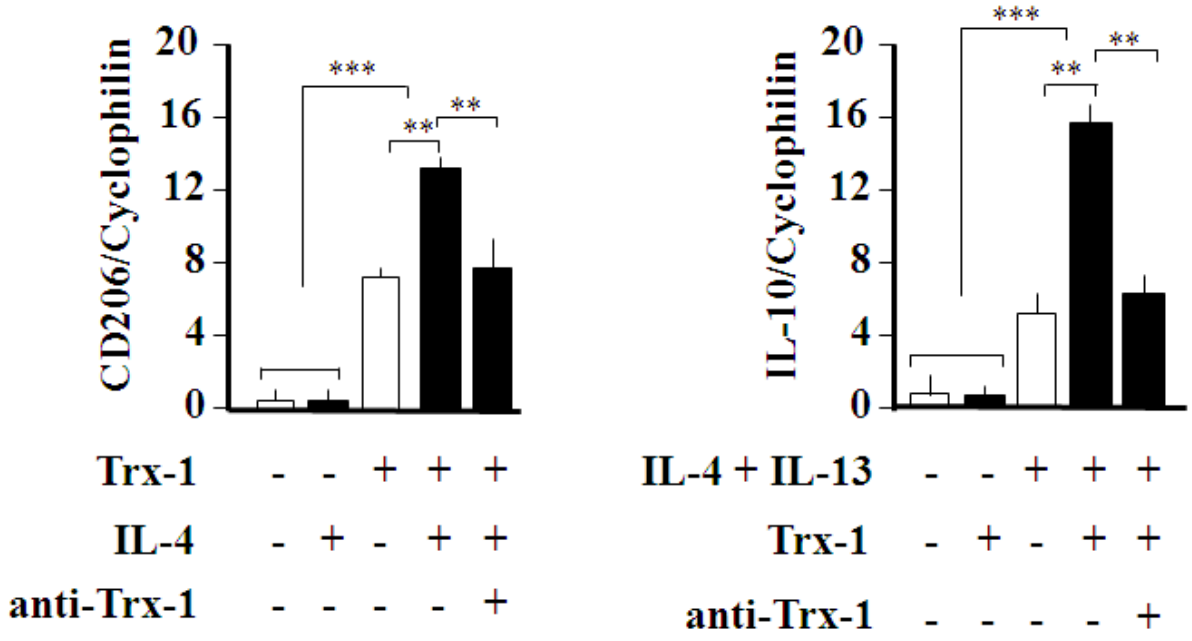


Supplemental Figure I. Binding of Trx-1 to macrophages. Thioredoxin-1 (1 µg/mL) labeled with Alexa488 (green) was added to macrophages. The macrophages were stained with the lysosomal marker LysoTracker® Red DND-99 (75 nmol/L, 30 min, red dye). Fluorescence lifetime imaging was done during the next 3 h with a Zeiss LSM710 confocal scanning microscope with a 63×1.4NA oil immersion objective, original magnification 630x, scale bar 8 µm. Images at time 0, 1, 2 and 3 h are shown.

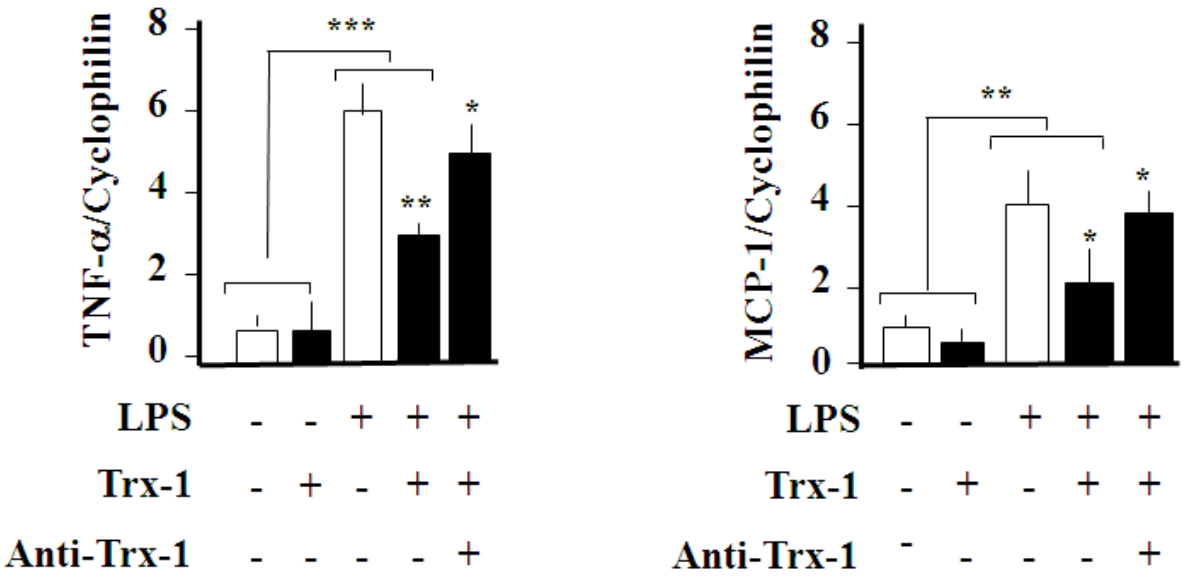


Supplemental Figure II. Accumulation of Trx-1 in lysosomes of human macrophages. Macrophages were exposed to Trx-1 (1 µg/mL) labeled with Alexa488 (green) for 24 h; the cells were then stained with the membrane stain CellMask™ Deep Red (blue) and with a

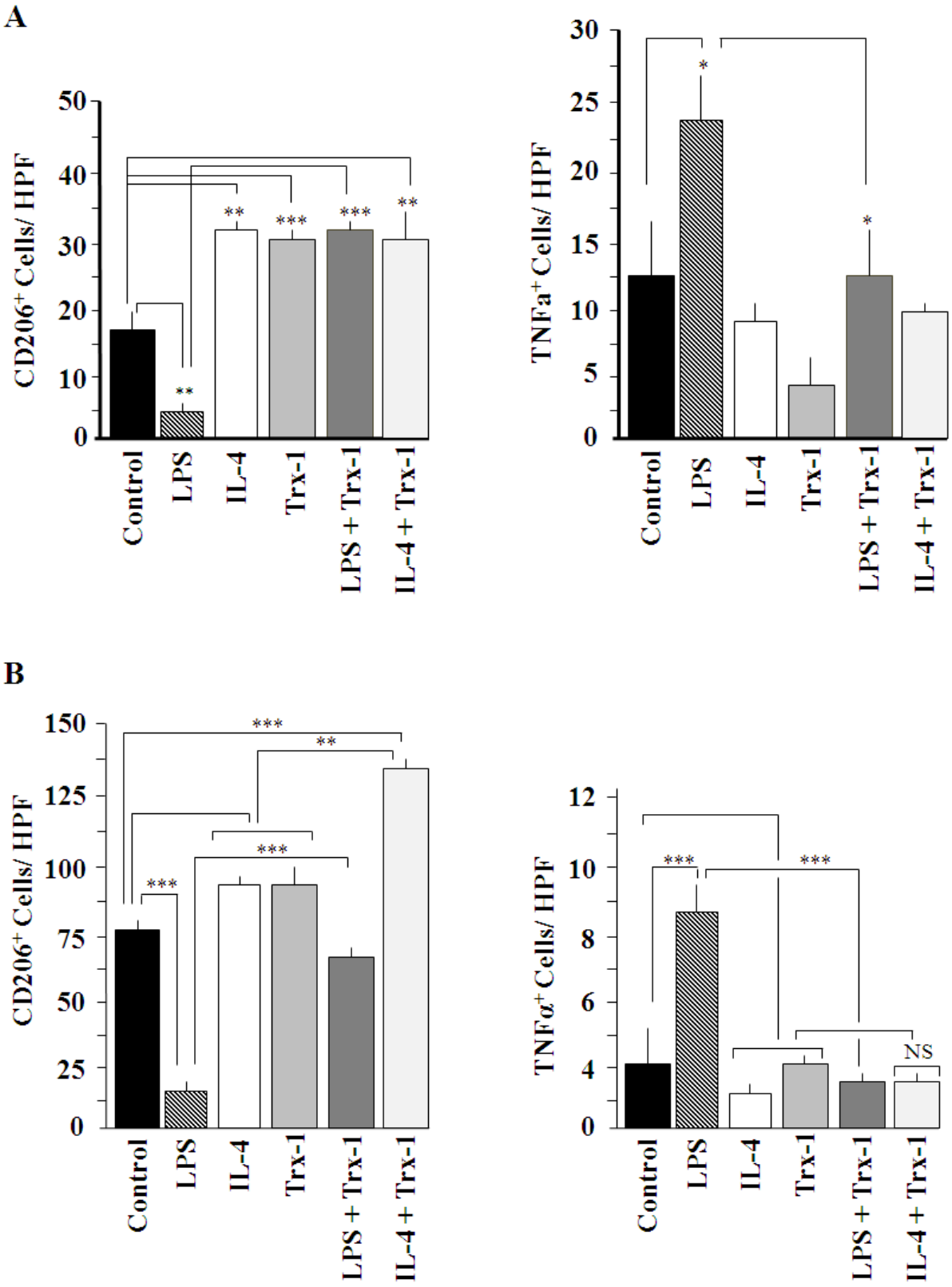
lysosomal marker LysoTracker® Red DND-99 (red); the overlay is shown (yellow). Labeled cells were imaged using a Zeiss LSM710 confocal scanning microscope with a 63×1.4NA oil immersion objective. Original magnification 630x.



Supplemental Figure III. Trx-1 promotes differentiation of monocytes into M2 macrophages. Human monocytes maintained in culture for 8 days were differentiated into macrophages by adhesion. At day 0, the cells were either left untreated or were treated with IL-4 (15 ng/mL) or IL-4 (10 ng/mL) + IL-13 (10 ng/mL), Trx-1 (1 µg/mL), or a combination thereof. In some samples, specific antibody against full length Trx-1 was added (5 µg/mL). Fresh medium containing the activating agents was changed at days 3 and 5. mRNA levels of CD206 and IL-10 were quantified with qPCR and normalized to cyclophilin mRNA. Bars represent the mean ± SEM of three independent experiments performed in triplicate. ***P*<0.01, ****P*<0.001 as indicated.

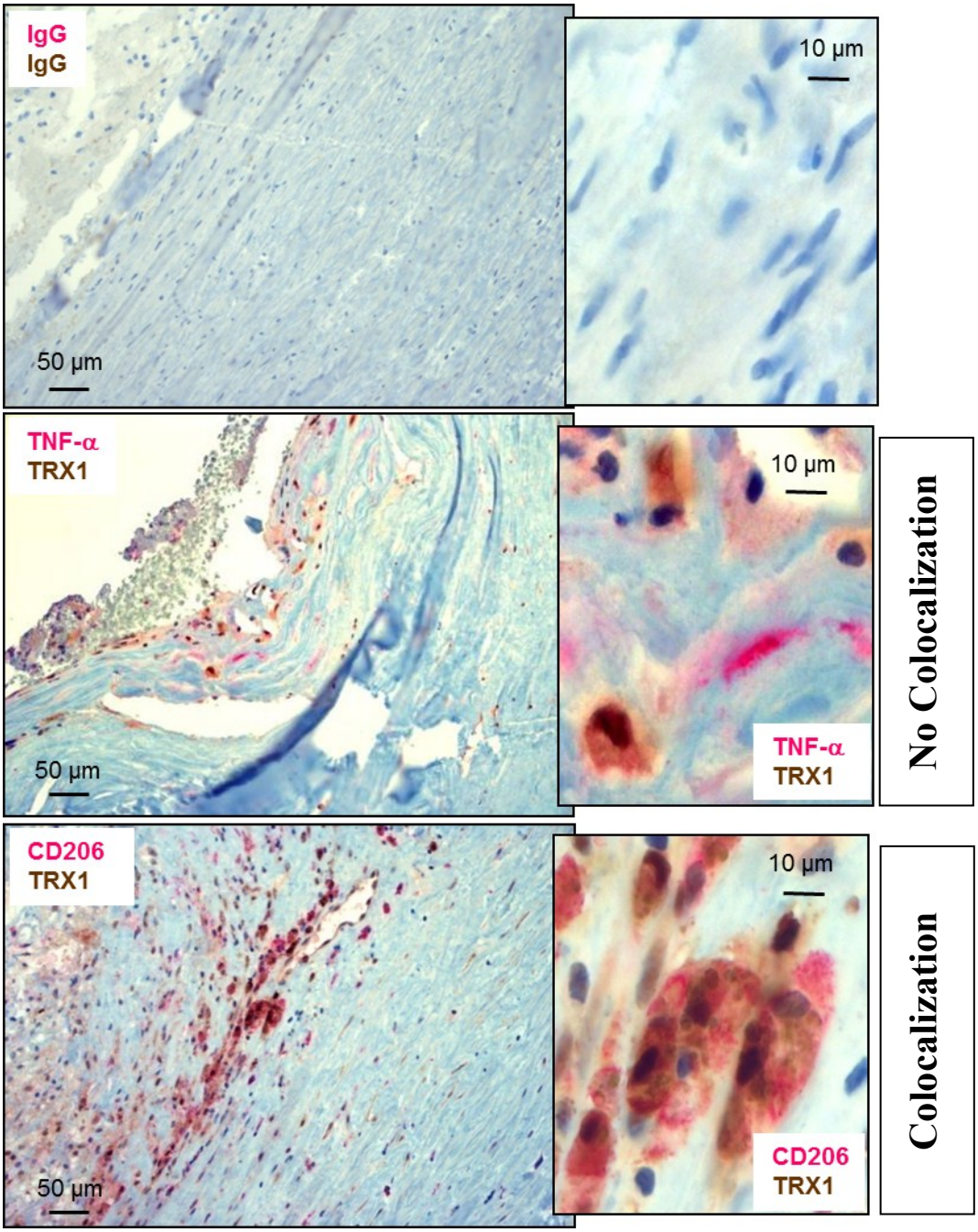


Supplemental Figure IV. Trx-1 attenuates differentiation of monocytes into M1 macrophages. Human monocytes maintained in culture for 8 days were differentiated into macrophages by adhesion. At day 0, the cells were either left untreated or were treated with LPS (100 ng/mL), Trx-1 (1 µg/mL), or a combination of both. In some culture dishes, specific antibody against full length Trx-1 was added (5 µg/mL). Fresh medium containing the indicated activating agents was changed at days 3 and 5. TNF-α and MCP-1 mRNA were quantified with qPCR and normalized to cyclophilin mRNA. Bars represent the mean ± SEM of three independent experiments performed in triplicate. *P<0.05, LPS+Trx-1+Anti-Trx-1 treated *vs*. LPS+Trx-1 treated macrophages; #p<0.01, LPS+Trx-1 treated *vs*. LPS treated cells; **P<0.01 as indicated.



Supplemental Figure V. Trx-1 induces M2 polarization of murine thymus and liver macrophages. C57Bl/6.ApoE2.Ki mice were intravenously injected with LPS (10 µg/30 g

body weight), IL-4 (500 ng/30 g b.w.), and Trx-1 (30 µg/30 g b.w.) alone or in combination at days 0 and 3. At day 5, the mice were sacrificed. Paraffin embedded serial sections (5 µm) of thymus (A) and liver (B) were immunohistochemically analyzed with antibodies either against the M1 marker TNF-α or the M2 marker CD206, and fluorescently labeled secondary $F(ab')_2$; nuclei were stained with DAPI. TNF-α and CD206 positive cells were quantified using ImageJ software (NIH). The data represent the number of positive cells/HPF (magnification 200x), mean ± SEM, n = 8. **P*<0.05, ***P*<0.01, ***P<0.001 as indicated.



Supplemental Figure VI. Trx-1 colocalizes with M2 macrophages in human atherosclerotic lesions. Serial sections of paraffin-embedded human atherosclerotic vessel specimens from 5 patients undergoing vascular surgery for atherosclerotic complications were double stained with Trx-1 and TNF-α or CD206 primary antibodies, respectively. TNF-α and CD206 were visualized by AP-conjugated secondary antibody and Fast-Red chromogenic substrate (pink). Trx-1 monoclonal mouse antibody that reacts exclusively with the full length

protein was stained with HRP-conjugated secondary antibody and diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate (brown). In control samples, the primary antibodies were substituted with control IgG. Sections were counterstained with hematoxylin. Original magnifications 200x and 400x. Representative images are shown.

Online Video S1. Binding of human Trx-1-1 to macrophages. Thioredoxin-1 (1 μg/mL) labeled with Alexa488 (green) was added to the macrophages. The macrophages were stained with the lysosomal marker LysoTracker® Red DND-99 (75 nmol/L, 30 min, red dye). Fluorescence images were taken with a Zeiss LSM710 confocal scanning microscope with a 63×1.4NA oil immersion objective, original magnification 630x, scale bar 8 μm.

Online Video S2. 3D reconstruction of localization of fluorescently labeled Trx-1 in human macrophages. Trx-1 (1 μg/mL) labeled with Alexa488 (green) was added to the macrophages for 24 h. Cells were stained with the membrane stain CellMask™ Deep Red (blue) and with a lysosomal marker LysoTracker® Red DND-99 (red). Fluorescence images were taken with the acquisition software ZEN 2009 using Zeiss LSM710 confocal scanning microscope with a 63×1.4NA oil immersion objective, original magnification 630x. ImageJ software (NIH) was used for image processing.

Supplemental Table I. Sequences of primers used for qPCR analysis

Species	Genes	Primers	
Human	IL-10	Forwards	5'-GATCCAGTTTTACCTGGAGGAG-3'
		Reverse	5'-CCTGAGGGTCTTCAGGTTCTC-3'
	CD206	Forwards	5'-CGAGGAAGAGGTTCGGTTCACC-3'
		Reverse	5'-GCAATCCCGGTTCTCATGGC-3'
	TNF-α	Forwards	5'-CAGAGGGCCTGTACCTCATC-3'
		Reverse	5'-GGAAGACCCCTCCCAGATAG-3'
	MCP-1	Forwards	5'-CCCCAGTCACCTGCTGTTAT-3'
		Reverse	5'-TGGAATCCTGAACCCACTTC-3'
	Cyclophilin	Forwards	5'-GCATACGGGTCCTGGCATCTTGTC-3'
		Reverse	5'-ATGGTGATCTTCTTGCTGGTCTTGC-3'
Mouse	IL-10	Forwards	5'-AACATACTGCTAACCGACTCCT-3'
		Reverse	5'-CTGCCTTGCTCTTATTTTCACA-3'
	CD206	Forwards	5'-CAGCGGTTGGCAGTGGA-3'
		Reverse	5'-CAGCTGATGGACTTCCTGGTAAG-3'
	TNF-α	Forwards	5'-TCACCCACACCGTCAGCCGATTT-3'
		Reverse	5'-CACCCATTCCCTTCACAGAGCAA-3'
	MCP-1	Forwards	5'-CGACATCCTGGAACTGCCCTACC-3'
		Reverse	5'-CACTGTGCCGCTCTCGTTCAC-3'
	GAPDH	Forwards	5'-TCAACGGCACAGTCAAGG-3'
		Reverse	5'-ACTCCACGACATACTCAGC-3

Supplemental Table II. Plasma lipids in apoE2.ki mice

Treatment	Total Cholesterol (mg/dL)	Triglycerides (mg/dL)
PBS (Control)	238 ± 48	184 ± 46
LPS	276 ± 38	280 ± 57
LPS+Trx-1	255 ± 35	235 ± 37

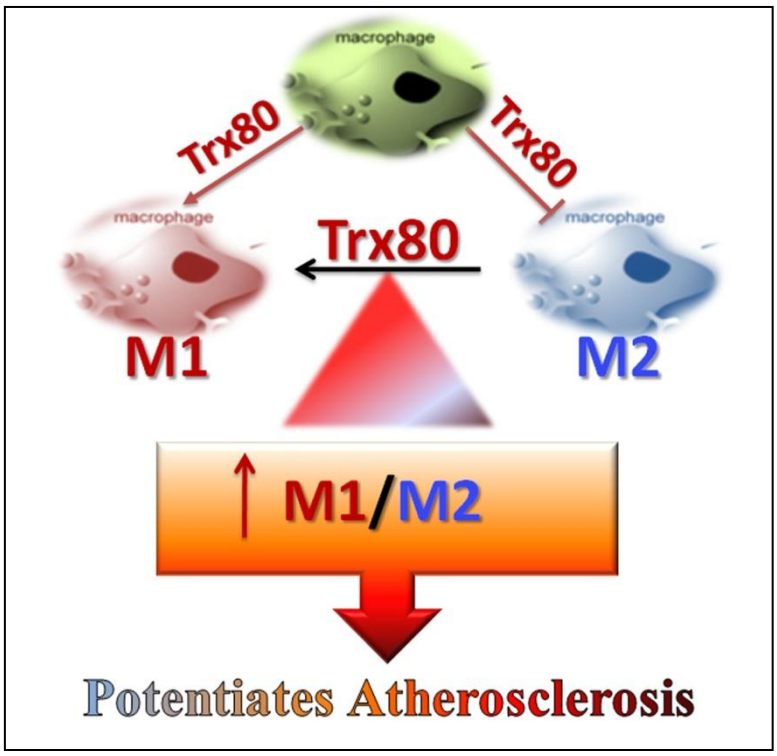
Three groups of C57Bl/6.ApoE2.Ki mice (8 mice in each group) were used to determine the effect of Trx-1 on the level of plasma lipids. The first group was intravenously injected with LPS (10 µg/30 g b.w.) once weekly for 5 weeks. The second group was intravenously injected with LPS (10 µg/30 g b.w.) once weekly for 5 weeks and daily with Trx-1 (30 µg/30 g b.w.). The third group was intravenously injected daily with PBS (control) for 5 weeks. Plasma, blood was drawn from the retro-orbital plexus of each mouse for assay of total cholesterol and triglycerides.

Truncated thioredoxin induces M1 polarization

7. Truncated Thioredoxin induces M1 polarization

7.1. Study presentation

Truncated thioredoxin, Trx80, is a natural cleavage product of Trx1with 10 kD sharing the 80 or 84 N-terminal amino acids with Trx1 [124,125]. It has been suggested that the enzyme responsible for its cleavage would be an inducible protease [126].Very recent findings, indicated that the disintegrins and metalloproteinases (ADAM10 and 17), two α-secretases processing the amyloid β precursor protein, are responsible for Trx80 generation in brain. Up to date, there are very limited works on Trx80. Previously, it has been shown that macrophages can cleave Trx1 producing Trx80 [128] which can activates monocytes and induces up-regulation of cell surface pathogen recognition receptors, molecules essential for T-cell activation and function [126] as well as release of proinflammatory cytokines evoking inflammation [90]. Since Trx80 has very different properties from the full-length protein, lacking the disulfide reductase activity of the full-length species, and instead having pro-inflammatory cytokine-like effects on immune cells, [124,131] we further investigated the vascular proinflammatory mechanism to clarify its atherogenic implication [48]. In his study, we report that Trx80 on one hand potentiates the expression of M1 macrophages markers, through the AP-1 and Ref-1 transcription factors-independent pathway and on other and it blunts the expression of anti-inflammatory cytokines. Taken together, these studies indicate that Trx80 functions as regulator of macrophage phenotype tipping the balance toward the pro-inflammatory M1 state. As a consequence, atherosclerotic plaques become larger and probably more unstable.



1.1.**Article II**

Mahmood DF, Abderrazak A, Couchie D, Lunov O, Diderot V, Syrovets T, Slimane MN, Gosselet F, Simmet T, Rouis M, and Hadri KE. Truncated thioredoxin (Trx80) promotes pro-inflammatory macrophages of the M1 phenotype and enhances atherosclerosis. *J Cell Physiol*, 2013.

ARTICLE II

Journal of Cellular Physiology

Truncated Thioredoxin (Trx-80) Promotes Pro-Inflammatory Macrophages of the M1 Phenotype and Enhances Atherosclerosis

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Vascular cells are particularly susceptible to oxidative stress that is believed to play a key role in the pathogenesis of cardiovascular disorders. Thioredoxin-1 (Trx-1) is an oxidative stress-limiting protein with anti-inflammatory and anti-apoptotic properties. In contrast, its truncated form (Trx-80) exerts pro-inflammatory effects. Here we analyzed whether Trx-80 might exert atherogenic effects by promoting macrophage differentiation into the M1 pro-inflammatory phenotype. Trx-80 at 1 μg/ml significantly attenuated the polarization of anti-inflammatory M2 macrophages induced by exposure to either IL-4 at 15 ng/ml or IL-4/IL-13 (10 ng/ml each) in vitro, as evidenced by the expression of the characteristic markers, CD206 and IL-10. By contrast, in LPS-challenged macrophages, Trx-80 significantly potentiated the differentiation into inflammatory M1 macrophages as indicated by the expression of the M1 cytokines, TNF-α and MCP-1. When Trx-80 was administered to hyperlipoproteinemic ApoE2.Ki mice at 30 μg/g body weight (b.w.) challenged either with LPS at 30 μg/30 g (b.w.) or IL-4 at 500 ng/30 g (b.w.), it significantly induced the M1 phenotype but inhibited differentiation of M2 macrophages in thymus and liver. When ApoE2.Ki mice were challenged once weekly with LPS for 5 weeks, they showed severe atherosclerotic lesions enriched with macrophages expressing predominantly M1 over M2 markers. Such effect was potentiated when mice received daily, in addition to LPS, the Trx-80. Moreover, the Trx-80 treatment led to a significantly increased aortic lesion area. The ability of Trx-80 to promote differentiation of macrophages into the classical proinflammatory phenotype may explain its atherogenic effects in cardiovascular diseases.

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Atherosclerosis is a complex inflammatory disease that involves interaction of a variety of cells including monocytes/macrophages within the vessel wall. A crucial step in this process is the infiltration of monocytes into the subendothelial space of large arteries and their subsequent differentiation into tissue macrophages (Ross, 1999) residing within the atherosclerotic lesions. These macrophages represent a heterogeneous cell population whose activation and function is influenced by various cytokines and microbial products. Thus, interleukin-1β (IL-1β), interferon γ (IFNγ), and lipopolysaccharide (LPS) increase the "classical" or inflammatory activation profile yielding the so-called M1 macrophages (Lumeng et al., 2007), which produce pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), IL-6, and IL-12 as well as reactive oxygen species (ROS) and nitrogen intermediates (Gordon, 2003, 2007). Consistently, M1 macrophages are associated with inflammation and tissue damage. In contrast, IL-4, IL-13 (Stein et al., 1992; Savill et al., 2002; Gordon, 2003), peroxisome proliferator-activated receptor γ (PPARγ) activation (Bouhlel et al., 2007), and adiponectin (Lovren et al., 2010; Ohashi et al., 2010) promote polarization of macrophages into the anti-inflammatory, alternative M2 type. M2 macrophages secrete the anti-inflammatory cytokine IL-10, transforming growth factor β, IL-1 receptor antagonist, and upregulate mannose receptor (CD206), and arginase-1. Accumulation of M2 macrophages leads to reduction of inflammation. In addition, anti-inflammatory macrophages promote angiogenesis, tissue

Conflict of interest: None.

Additional supporting information can be found in the online version of this article.

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remodeling and repair (Mantovani et al., 2001; Gordon, 2003; Odegaard and Chawla, 2008).

Increasing evidence indicates that risk factors for cardiovascular disease can lead to dramatic increases in the concentration of ROS in the vascular wall. When the rate of ROS formation exceeds the capacity of the antioxidant defense system, oxidative stress occurs, which markedly contributes to arterial inflammation (see review Forstermann, 2008). Thioredoxin-1 (Trx-1), a 12-kDa highly conserved protein that has recently been recognized as a critical protective system against oxidative stress (Yamawaki et al., 2003; Forstermann, 2008). Nevertheless, posttranslational modifications of Trx-1 have been described. Thus, a C-terminal truncated form, composed of 1–80 or 1–84 N-terminal amino acids (Trx-80), which is either secreted from cells already truncated or cleaved by an unknown enzyme at the cell surface, possesses a cytokine-like activity (Pekkari and Holmgren, 2004). Macrophages have been reported to cleave full-length Trx-1 to yield Trx-80 (Bizzarri et al., 2005). Trx-80 is expressed also by U937 monocytes, cytotrophoblasts and $CD4^+$ T cells (Silberstein et al., 1989; Di Trapani et al., 1998).

The levels of Trx-80 varies from 2 to 175 ng/ml, those of Trx-1 varies from 16 to 55 ng/ml without any correlation to Trx-80, which is highly increased under inflammatory conditions (Pekkari et al., 2000; Cortes-Bratti et al., 2011). Trx-80 activates monocytes and induces up-regulation of cell surface pathogen recognition receptors, molecules essential for T-cell activation and function (Lemarechal et al., 2007) as well as release of the proinflammatory cytokines evoking inflammation (Bertini et al., 1999).

We have previously shown in vitro that human recombinant Trx-1 down-regulates the expression of a number of inflammatory genes such as IL-1β, TNF-α, IL-6, and IL-8 (Billiet et al., 2005). In addition, we have recently shown that intravenous injection of recombinant human Trx-1 promotes anti-inflammatory macrophages of the M2 phenotype and antagonizes atherosclerosis in mice (El Hadri et al., 2012).

Since Trx80 has very different properties from the full-length protein, lacking the disulfide reductase activity of the full-length species, and instead having pro-inflammatory cytokine-like effects on immune cells (Pekkari et al., 2000, 2001), we further investigated the vascular proinflammatory mechanism to clarify its atherogenic implication. We found that recombinant human Trx-80 promotes mouse peritoneal and human macrophages toward a "classical" pro-inflammatory M1 phenotype. As an in vivo model of atherosclerosis, we have chosen ApoE2.Ki mice expressing human ApoE2 (2/2), which virtually exhibits all characteristics of type III hyperlipoproteinemia in man. Accordingly, their plasma cholesterol and triglyceride levels are two to three times of that of normolipidemic mice (Sullivan et al., 1998). These animals are markedly defective in clearing β-migrating VLDL particles, and spontaneously develop atherosclerotic plaques, even on a regular diet. In apoE2.Ki mice on an atherogenic diet (Sullivan et al., 1998) or exposed to LPS, an exacerbation of atherosclerosis is observed (Ostos et al., 2002; Cuaz-Perolin et al., 2008). We demonstrated that Trx-80 treatment led to a significantly increased aortic lesion surface area in ApoE2.Ki mice challenged with LPS.

Methods

For in vitro studies, both murine peritoneal and human macrophages were used. Cells were left untreated or treated with LPS, IL-4, IL-4/IL-13, Trx-80, or a combination thereof. Recombinant Trx-80 containing by the LAL method less than <1.0 EU of endotoxin per 1 μg of protein was obtained from R&D systems. To exclude any potential endotoxin interference, we have added in all in vitro experiments (except when LPS was added) polymyxin B that is known to neutralize endotoxin. In some samples, a specific antibody against truncated human Trx-80 was added. To study the binding of Trx-80, recombinant Trx-80 was fluorescently labeled and incubated with macrophages, and fluorescence images were taken using Zeiss LSM710 confocal scanning microscope. The expression of macrophage phenotype markers, CD206, MCP-1, IL-10, and TNF-α, and other involved genes, activator protein (AP)-1 and Ref-1, were investigated at both transcription and protein levels using real-time polymerase chain reaction and immunoblotting or ELISA, respectively. In vivo study was performed on C57Bl/6.ApoE2.ki mice. Heart, liver, spleen, thymus, pancreas, and kidneys were excised, fixed, and immunohistochemical studies were performed. Serial paraffin-embedded sections on proximal aorta sections were stained and the mean lesion area per animal was quantified. Human atherosclerotic vessel specimens from patients undergoing vascular surgery for atherosclerotic complications were fixed and immunohistochemical analysis was performed. Statistical significance was calculated with the Newman–Keuls test (given in detail in the online-only Data Supplement).

Results

Trx-80 Reduces the Expression of M2 Markers in Murine Peritoneal Macrophages

Macrophages rapidly bind Trx-80, which is later taken up by the cells and which can be detected primarily in the cytosol after extended periods of time. Interestingly, Trx-80 does not colocalize with acidic compartments such as lysosomes, and is barely degraded even after 24 h (Figs. 1A and 1B in the online-only Data Supplement).

We further analyzed whether human recombinant Trx-80 would reduce an M2 polarization of murine macrophages. Indeed, peritoneal macrophages from C57BL/6 mice were incubated with recombinant IL-4 in the absence or in the presence of recombinant human Trx-80. As reported (Stein et al., 1992; Bouhlel et al., 2007), IL-4 induced significantly the M2 macrophage marker CD206 (also called mannose receptor); 3.1 ± 0.9 a.u. versus control cells 1.0 ± 0.2 a.u., $P < 0.001$ (for mRNA; Fig. 1A) and 3 ± 0.2 a.u. versus control

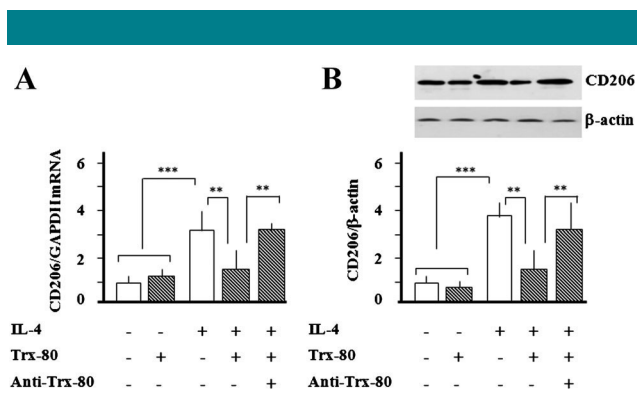

Fig. 1. Trx-80 attenuates the M2 anti-inflammatory phenotype in freshly isolated murine peritoneal macrophages. A: Left part, shows mRNA levels of CD206 after treatment with recombinant Trx-80 (1 μg/ml), IL-4 (15 ng/ml) and specific antibody against human Trx-80 (5 μg/ml) for 24 h. mRNA levels of CD206 were quantified with qPCR and normalized to GAPDH. B: Right part, shows quantification of Western blots of CD206 and β-actin after treatment with recombinant Trx-80 (1 μg/ml), IL-4 (15 ng/ml), and specific antibody against human Trx-80 (5 μg/ml) for 24 h. Data are presented as mean ± SE of three independent experiments performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$, as indicated. One representative Western immunoblot was inserted.

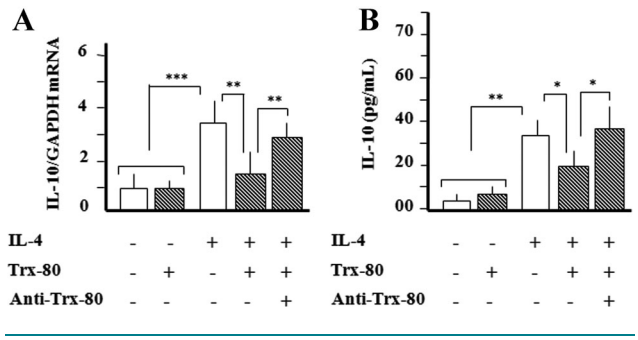

Fig. 2. Trx-80 attenuates the M2 anti-inflammatory phenotype in freshly isolated murine peritoneal macrophages. A: Left part, shows mRNA levels of IL-10 after treatment with recombinant Trx-80 (1 μg/ml), IL-4 (15 ng/ml), and specific antibody against human Trx-80 (5 μg/ml) for 24 h. mRNA levels of IL-10 were quantified with qPCR and normalized to GAPDH. B, right part, shows the levels of IL-10 in cell supernatants after treatment with recombinant Trx-80 (1 μg/ml), IL-4 (15 ng/ml) and specific antibody against human Trx-80 (5 μg/ml) for 24 h. Data are presented as mean ± SE of three independent experiments performed in triplicate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.01$, as indicated.

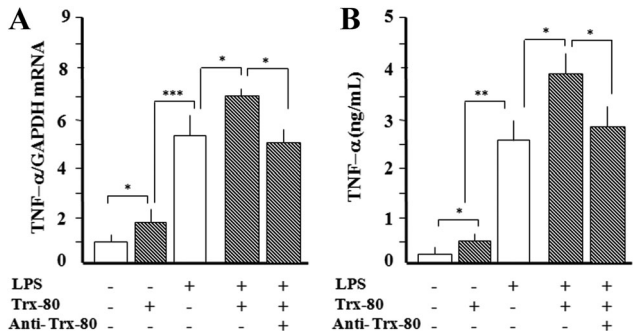

Fig. 3. Trx-80 promotes the M1 pro-inflammatory phenotype in freshly isolated murine peritoneal macrophages. A: Left part, shows mRNA levels of TNF-α after treatment with recombinant Trx-80 (1 μg/ml), LPS (100 ng/ml), and specific antibody against human Trx-80 (5 μg/ml) for 24 h. mRNA levels of TNF-α were quantified with qPCR and normalized to GAPDH. B: Right part, shows the levels of TNF-α in cell supernatants after treatment with recombinant Trx-80 (1 μg/ml), LPS (100 ng/ml), and specific antibody against human Trx-80 (5 μg/ml) for 24 h. Data are presented as mean ± SE of three independent experiments performed in triplicate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, as indicated.

cells 1.0 ± 0.2 a.u., $P < 0.001$ (for protein; Fig. 1B). In contrast, the expression of the marker CD206 marker was reduced by Trx-80. Thus, in IL-4-treated cells, Trx-80 significantly reduced the expression of CD206 mRNA from 3.1 ± 0.9 to 1.5 ± 0.9 a.u. $P < 0.01$ (Fig. 1A) and CD206 protein level from 3.0 ± 0.2 to 1.5 ± 0.9 a.u. ($P < 0.01$; Fig. 1B). The effect of Trx-80 on macrophage polarization was specific, because it could be virtually abolished by an anti-Trx-80 antibody (Fig. 1A,B). Of note, we used Trx-80 at 1 μg/ml in all in vitro experiments reported in this manuscript, because in pilot concentration-response experiments, 1 μg/ml Trx-80 exerted the maximum effect on macrophage polarization in vitro (data not shown). Similarly, IL-4 induces the expression of IL-10 (3.5 ± 0.7 a.u vs. control cells 1.0 ± 0.4 a.u., $P < 0.001$ (for mRNA; Fig. 2A) and 32 ± 8 pg/ml versus control cells 4.5 ± 1.4 pg/ml, $P < 0.01$ (for protein; Fig. 2B) and concomitant administration of Trx-80 reduced such expression (from 3.5 ± 0.7 to 1.4 ± 0.2 a.u., $P < 0.01$ (for mRNA) and 32 ± 8 pg/ml to 20 ± 6 pg/L, $P < 0.05$ (for protein; Fig. 2A,B), respectively. Again, the effect of Trx-80 on macrophage polarization was specific, because the effect could be neutralized by an anti-Trx-80 antibody (Fig. 2A,B). A similar regulation of CD206 and IL-10 expression by Trx-80 was observed in macrophages treated with IL-13 alone or in combination with IL-4 (not shown). In all experiments polymyxin B was added in order to prevent any effect from a potential endotoxin contamination. Moreover, similar results were also observed in human monocyte-derived macrophages (Figs. II and III in the online-only Data Supplement).

Trx-80 Induces the Expression of Murine M1 Macrophages Markers

To determine whether Trx-80 affects the expression of the M1 macrophage markers, peritoneal macrophages were treated with LPS in the absence or presence of recombinant human Trx-80. LPS significantly induced M1 macrophage markers such as TNF-α, 5.2 ± 1.0 a.u. vs. control cells 1.0 ± 0.2 a.u., $P < 0.001$ (for mRNA; Fig. 3A) and 2.5 ± 0.5 ng/ml versus control cells 0.5 ± 0.2 ng/ml, $P < 0.01$ (for protein; Fig. 3B). In addition, the expression of TNF-α was significantly induced by Trx-80 at 1 μg/ml in LPS-treated cells. Thus, Trx-80 enhanced the LPS-induced expression of TNF-α from 5.2 ± 1.0 to 6.8 ± 0.2 a.u.; $P < 0.05$ (for mRNA) and from 2.5 ± 0.5 to 3.8 ± 0.6 ng/ml; $P < 0.05$ (for protein; Fig. 3A,B, respectively). Moreover, the effect was also almost completely neutralized by an anti-Trx-80 antibody (Fig. 3A,B). Similarly, LPS significantly induces the expression of MCP-1 (another M1 marker). Thus, in the presence of LPS, the MCP-1 mRNA increased from 1.0 ± 0.2 (control cells) to 7.8 ± 0.6 a.u. $P < 0.001$ (Fig. 4A) and for the

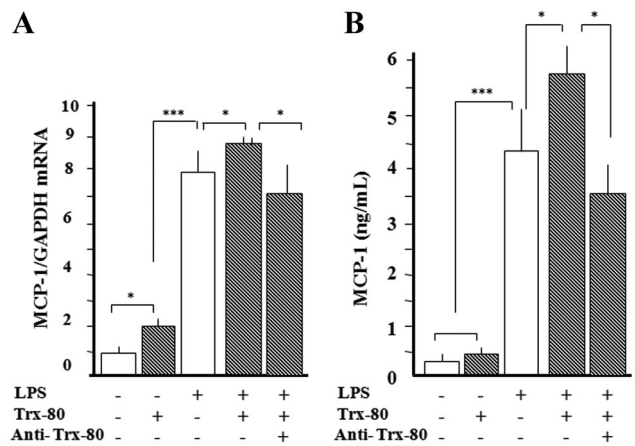

Fig. 4. Trx-80 promotes the M1 pro-inflammatory phenotype in freshly isolated murine peritoneal macrophages. A: Left part, shows mRNA levels of MCP-1 after treatment with recombinant Trx-80 (1 μg/ml), LPS (100 ng/ml), and specific antibody against human Trx-80 (5 μg/ml) for 24 h. mRNA levels of MCP-1 were quantified with qPCR and normalized to GAPDH. B: Right part, shows the levels of MCP-1 in cell supernatants after treatment with recombinant Trx-80 (1 μg/ml), LPS (100 ng/ml) and specific antibody against human Trx-80 (5 μg/ml) for 24 h. Data are presented as mean ± SE of three independent experiments performed in triplicate. *$P < 0.05$, ***$P < 0.001$, as indicated.

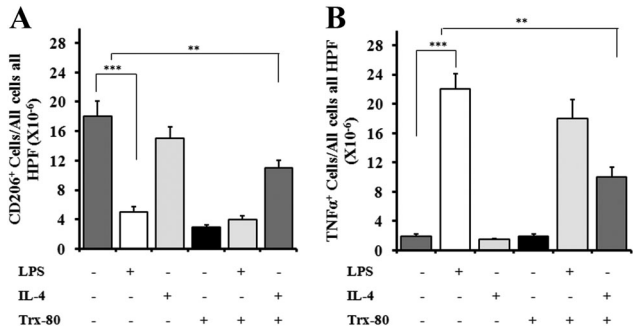

Fig. 5. Trx-80 attenuates M2 polarization of murine thymus-macrophages. C57Bl/6.ApoE2.Ki mice were intravenously injected with LPS (10 μg/30 g body weight (b.w.)), IL-4 (500 ng/30 g b.w.) and/or Trx-80 (30 μg/30 g b.w.) at Days 0 and 3. At Day 5, the mice were sacrificed. Paraffin embedded serial sections (5 μm) of the thymus were immunohistochemically analyzed with antibodies either against the M2 marker CD206 (A) or M1 marker TNF-α (B), and fluorescently labeled secondary F(ab′)$_2$; nuclei were stained with DAPI. TNF-α and CD206 positive cells were quantified using ImageJ software (NIH). The data represent the number of positive cells/HPF (magnification 200×), mean ± SEM, n = 8. $^{**}P < 0.01$, $^{***}P < 0.001$ as indicated.

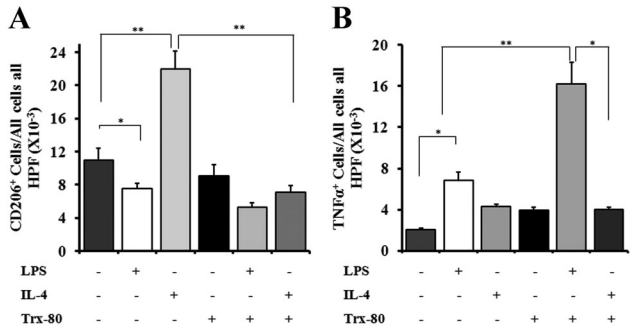

Fig. 6. Trx-80 attenuates M2 polarization of murine liver-macrophages. C57Bl/6.ApoE2.Ki mice were intravenously injected with LPS (10 μg/30 g body weight (b.w.)), IL-4 (500 ng/30 g b.w.) and/or Trx-80 (30 μg/30 g b.w.) at Days 0 and 3. At Day 5, the mice were sacrificed. Paraffin embedded serial sections (5 μm) of liver macrophages were immunohistochemically analyzed with antibodies either against the M2 marker CD206 (A) or the M1 marker TNF-α (B), and fluorescently labeled secondary F(ab′)$_2$; nuclei were stained with DAPI. TNF-α and CD206 positive cells were quantified using ImageJ software (NIH). The data represent the number of positive cells/HPF (magnification 200×), mean ± SEM, n = 8. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as indicated.

protein level from 0.5 ± 0.2 (control cells) to 4.8 ± 1.2 ng/ml; $P < 0.05$ (Fig. 4B). In addition, the expression of MCP-1 was further increased by Trx-80 at 1 μg/ml in LPS-treated cells. Thus, Trx-80 increases the LPS-induced expression of MCP-1 from 7.8 ± 0.6 to 9.0 ± 0.2 a.u.; $P < 0.05$ (for mRNA; Fig. 4A) and from 4.8 ± 1.2 to 6.2 ± 0.9 ng/ml; $P < 0.05$ (for protein; Fig. 4B). In analogy to the previous experiments, the stimulation effect of Trx-80 on LPS-induced expression of MCP-1 marker was totally abolished in the presence of the specific anti-Trx-80 antibody (Fig. 4A,B). Similar to murine peritoneal macrophages, Trx-80 inhibited the expression of M2 macrophage markers and increased the expression of M1 markers in human monocyte-derived macrophages (Figs. IV and V in the online-only Data Supplement).

Short Term Effects of Trx-80 on M1/M2 Macrophages in Tissues of ApoE2.Ki Mice

Macrophages are highly plastic cells that enter target tissues and gain the phenotypic and functional attributes of their tissue of residence (Mantovani et al., 2005). To address such phenotypic changes, we analyzed the effect of Trx-80 administration on the expression of M1/M2 markers in murine tissues. For the in vivo study, we injected mice with recombinant human Trx-80 at 30 μg/g b.w. because in a pilot study, mice injected with various doses of Trx-80, showed the maximum effect at 30 μg/g b.w. There were no changes in the expression of M1 and M2 macrophage markers in spleen, pancreas and kidneys (data not shown). However, in agreement with the data obtained in vitro with murine peritoneal and human macrophages, Trx-80 decreased the number of CD206$^+$ M2 and given together with IL-4 increased the TNF-α$^+$ M1 macrophages in the thymus (Fig. 5). Moreover, it antagonized the IL-4- induced expression of CD206 in thymus macrophages (Fig. 5).

Trx-80 exhibited comparable effects on hepatic macrophages, where it antagonized the IL-4-induced expression of CD206 and potentiated the LPS-induced expression of TNF-α (Fig. 6). Trx-80 also enhanced the LPS-induced decrease of CD206 (Fig. 6). Thus, Trx-80 promotes M1 macrophage markers and inhibits the M2 phenotype in tissue macrophages.

Trx-80 Colocalizes With M1 Macrophages in Human Atherosclerotic Lesions

Previous studies have shown the presence of M1 (Ross, 1999) and M2 (Bouhlel et al., 2007) macrophages in atherosclerotic lesions. However, it was unknown whether Trx-80 would colocalize with M1 or M2 markers in complicated atherosclerotic lesions. Not unexpected, the staining pattern of CD206 in serial sections of human atherosclerotic plaques confirmed that Trx-80-expressing cells colocalize with the M1 macrophage marker TNF-α. In contrast, Trx-80 does not colocalize with CD206, a marker of inflammatory M2 macrophages (Fig. 7), indicating that Trx-80 could promote the M1 differentiation in human atherosclerotic vessels as well.

Chronic Treatment With Trx-80 Increases the Number of Lesional M1 Macrophages and Enhances the Size of Atherosclerotic Lesions

ApoE2.Ki hyperlipoproteinemic mice challenged repeatedly with LPS exhibit a significant increase in lesion size compared to control animals, as we have previously shown (Cuaz-Perolin et al., 2008). Lesional macrophages in these mice express high levels of TNF-α and low levels of CD206 exhibiting predominantly the proinflammatory M1 phenotype. When LPS-injected ApoE2.Ki mice were additionally treated with human recombinant Trx-80, the amount of TNF-α$^+$ M1 macrophages was significantly potentiated, and the number of CD206$^+$ M2 macrophages was significantly reduced (Fig. 8A). Similarly, liver tissue of mice chronically treated with Trx-80 exhibited increased expression of M1 macrophage marker TNF-α, but reduced expression of the M2 marker CD206 (data not shown). Moreover, Trx-80 significantly increased the size of aortic atherosclerotic lesions in ApoE2.Ki hyperlipoproteinemic mice challenged with LPS (100 ± 18% (LPS-challenged) vs. 136% ± 21% (Trx-80-treated LPS-challenged mice; n = 8); $P < 0.01$ (Fig. 8B)).

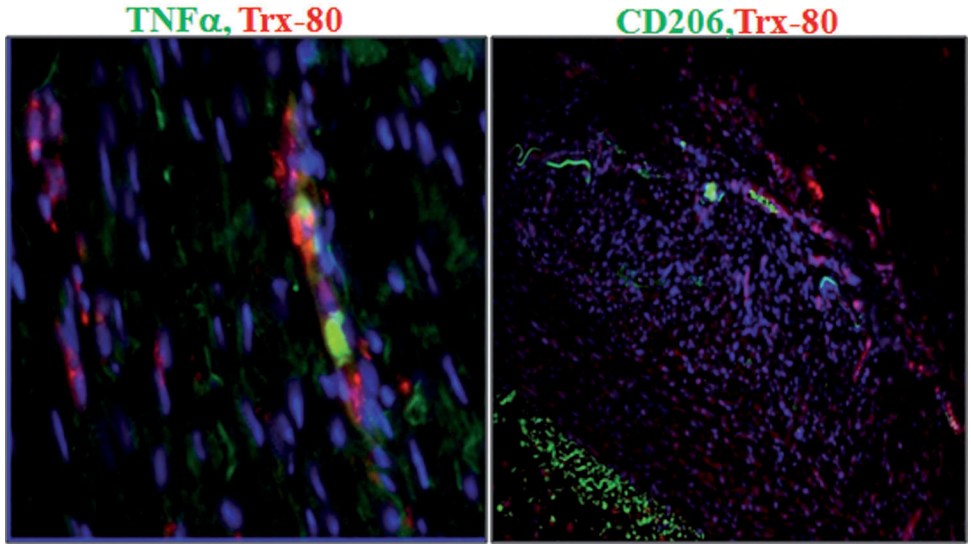

Fig. 7. Trx-80 colocalizes with M1, but not with M2 macrophages in human atherosclerotic lesions. Serial sections of paraffin-embedded human atherosclerotic vessel specimens from five patients undergoing vascular surgery for atherosclerotic complications were double stained with Trx-80 and TNF-α or CD206 primary antibodies, respectively. TNF-α and CD206 were visualized by Cy2-conjugated secondary antibody (green). Trx-80monoclonal mouse antibody that reacts exclusively with the truncated protein was stained withCy-5-conjugated secondary antibody (red). In control samples, the primary antibodies were substituted with control IgG. Sections were counterstained with hematoxylin. Original magnifications 400×. Representative images are shown.

Treatment of macrophages With Trx-80 Enhances the ABCA1 Expression in M1 Macrophages But Does Not Affect the Reverse Cholesterol Transport

To determine the role of Trx-80 in lipid metabolism and its accumulation in the M1 macrophages, the expression of ABCA1, scavenger receptors SR-A, CD36, and LOX-1 were studied. It was found that Trx-80 (1 μg/ml) significantly enhanced the expression of ABCA1 in M1 macrophages (LPS-treated cells) compared to resting macrophages (RM; Fig. 9A), whereas the expression of SR-A, CD36 and LOX-1 (data not shown) as well as the reverse cholesterol transport were not affected (Fig. 9B).

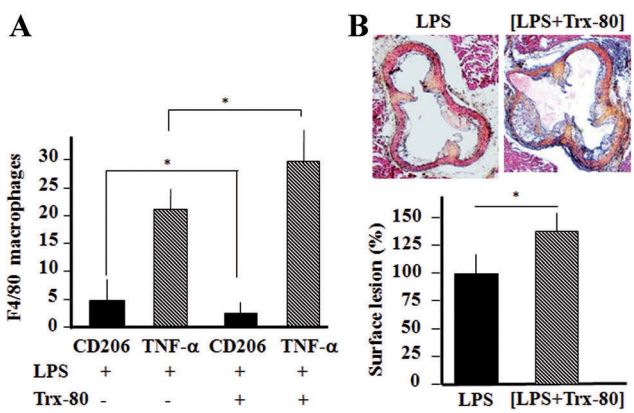

Fig. 8. Trx-80 induces M1 macrophage polarization in murine atherosclerotic lesions. C57Bl/6.ApoE2.Ki mice were intravenously injected with LPS (10 μg/30 g b.w.) once weekly and daily with either Trx-80 (30 μg/30 g b.w.) or PBS for 5 weeks, when the mice were sacrificed. Frozen serial sections (7 μm) of proximal aortas were analyzed by immunohistochemistry using double immunostaining with antibodies either against the macrophage marker F4/80 and the M1 marker TNF-α or the M2 marker CD206, respectively (A). Furthermore, serial paraffin-embedded sections (5 μm) of proximal aortas were prepared, stained with hematoxylin, eosin, and saffron for lesion area measurement. Three aortic sections, each separated by 100 μm, from eight mice were analyzed (B). Data are mean ± SEM of eight mice, $^{*}P < 0.05$.

Discussion

Macrophages possess a remarkable diversity, which depends on the type, duration and concentration of environmental signals, including bacterial products and cytokines. Macrophage subpopulations exhibit differential gene expression, release different cytokines and secondary mediators, and express different cell surface antigens. According to their phenotype, macrophages are divided into two major populations, those which promote inflammation, and those which limit inflammation and support tissue remodeling and angiogenesis (Mantovani et al., 2009). In contrast to lymphocytes, which cannot be reprogrammed, macrophages retain plasticity, meaning that alteration of environmental signals could change the balance between M1 and M2 populations. Changing the composition of macrophage population in tissues would affect pathogenesis, evolution and complication of many diseases including atherosclerosis (Murray and Wynn, 2011). Therefore, identification of agents that might modulate the balance of macrophage polarization is of great importance.

In this manuscript we show that macrophages bind rapidly Trx-80, which is later taken up by the cells and which can be detected primarily in the cytosol after extended periods of time. Interestingly, Trx-80 does not colocalize with acidic compartments such as lysosomes, and is barely degraded even after 24 h (Figs. IA and IB in the online-only Data Supplement). Extracellular full length Trx-1also binds rapidly to the surface of macrophages, yet after 24 h, Trx-1 is localized predominantly within the lysosomal compartment (El Hadri et al., 2012). The

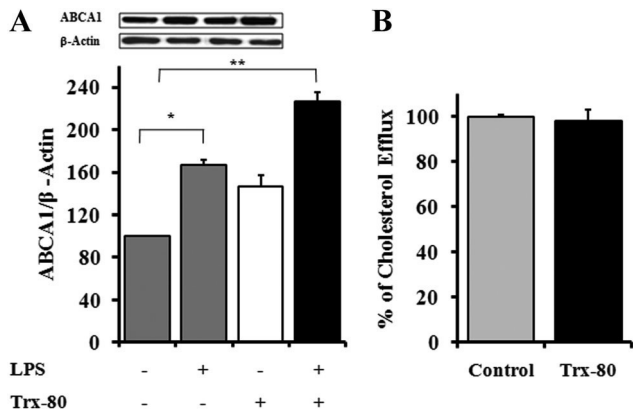

Fig. 9. Trx-80 induces ABCA1 expression in the M1 macrophages but without effect on the reverse cholesterol efflux. A, left part, shows quantification of Western blots of ABCA1 and β-actin (for normalization) after treatment with recombinant Trx-80 (1 μg/ml), LPS (100 ng/ml) and their combination for 24 h. B: Right part, shows the effect of Trx-80 (1 μg/ml) on the reverse cholesterol efflux. Data are presented as mean ± SE of three independent experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, as indicated. One representative Western immunoblot was inserted.

raison of such different localization is currently not known. Nevertheless, it is well documented that the lysosomal pathway of proteolysis is selective for cellular proteins containing a peptide sequence related to the Lys-Phe-Glu-Arg-Gln (KFERQ) motif. A heat shock protein of 73 kDa binds to such peptide regions in proteins and mediates their transfer to lysosomes for degradation. Therefore, we hypothesized that the lysosomal localization of full length Trx-1 but not of its truncated form, Trx-80, might be explained by a potential presence of the KFERQ motif in the C-terminal domain, which might be lost after cleavage of the protein. Unfortunately, we could not find such a KFERQ motif. Therefore, we considered another explanation. Thus, Cys-proteases such as cathepsin B are constitutively expressed in the lysosomes of human B cells (Rodriguez and Diment, 1995) and their activity requires active SH generating systems that protect them from oxidative inactivation. They play an important role in antigen processing, producing peptides, which can be recognized in the context of MHC class II (Matsunaga et al., 1993; Deussing et al., 1998). The enzymatic activity of cathepsin B requires the presence of Trx. Accordingly, Trx is thought to represent a good SH donor candidate for the processing of disulfide-bonded multimeric antigens. Trx could therefore be considered as a molecular chaperonin involved in antigen processing (Kerblat et al., 1999), which could explain its presence within the lysosome. In contrast, Trx-80 lacks redox activity (Pekkari et al., 2000) and its presence within the lysosome might not be necessary. Another hypothesis could be considered as well. Thus, Trx-1 is known to inhibit oxidative stress-induced apoptosis in different cell types (Andoh et al., 2002; Haendeler et al., 2002; Shioji et al., 2002). To overcome such apoptosis inhibition, cathepsin D (CatD), a lysosomal asparatic proteinase, induces degradation of Trx-1 (Haendeler et al., 2005). Hence, the lysosomal localization of Trx-1 might represent a mechanism used by macrophages to control the induction of apoptosis.

In addition, we show in the present study, that human Trx-80 potentiates the expression of pro-inflammatory M1 macrophages through AP-1 and Ref-1 transcription factor-independent pathways (not shown), as evidenced by the increase of the markers TNF-α and MCP-1, characteristic of the M1 polarization. In parallel, Trx-80 blunts the expression of anti-inflammatory M2 markers such as CD206 and IL-10 through unknown mechanism. The correlation between the human macrophage subpopulations and their murine counterparts, particularly in atheroma, are still unknown. Therefore, it is important that we found similar effects in human as well as in murine macrophages. In addition, we complemented our in vitro experiments with in vivo studies using a hyperlipoproteinemic mouse model.

Our in vitro data showing promotion of M1 over M2 phenotype by Trx-80 are supported by the in vivo data. In ApoE2.Ki mice treated intravenously with human recombinant Trx-80 for 2–5 days or for 5 weeks, Trx-80 increases expression of M1 pro-inflammatory macrophage marker in thymus and in liver, as well as in the atherosclerotic lesions. In contrast, the high expression of macrophage M1 markers observed in mice injected with LPS is significantly potentiated by the Trx-80 treatment. As a consequence, lesion surface area is significantly increased in mice treated with Trx-80 compared to mice treated with LPS alone. In addition, in human atherosclerotic plaques, we observed colocalization of Trx-80 with TNF-α, a marker of proinflammatory M1 macrophages, but not with CD206, a marker of anti-inflammatory M2 macrophages. This may be due to the Trx-80-induced activation of pro-inflammatory and pro-survival PI3K/Akt and MAP/ERK signaling pathways, a hypothesis that is currently under further investigations.

Taken together, these studies indicate that Trx-80 functions as regulator of macrophage phenotype tipping the balance towards the pro-inflammatory M1 state. As a consequence, atherosclerotic plaques become larger and probably more unstable.

Of note, in a previous publication, Trx-80 was shown to induce differentiation of human $CD14^{+}$ monocyte into a new cell type designate as Trx-activated monocytes (TAMs) (Bizzarri et al., 2005). TAMs resemble immature dendritic cells (iDCs) generated in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (Bizzarri et al., 2005). Functional assays revealed that TAMs release significantly higher amounts of the pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 and of the anti-inflammatory cytokine IL-10 (Bizzarri et al., 2005). In our present study, we also show that Trx-80, either alone or in combination with LPS, increases the TNF-α and MCP-1 expression and secretion by murine peritoneal macrophages as well as by HMDM. However, Trx-80 alone did not affect the expression of CD206 or IL-10 in these cells. In contrast, in IL-4-treated macrophages, Trx-80 decreased CD206 and IL-10 expression. The discrepancy with the data reported by Pekkari et al. (Bizzarri et al., 2005) who show an increase of IL-10 with Trx-80 might be due to the cell type and/or the cell purification procedure and culture condition.

Furthermore, it has been shown recently that the expression of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ modulates the macrophage phenotype (Cudejko et al., 2011; El Hadri et al., 2012). Thus, $p16^{-/-}$ bone marrow-derived macrophages display a decreased response to classical polarizing by IFNγ and LPS but an increased sensitivity to alternative polarization by IL-4 (Cudejko et al., 2011). Therefore, one could assume that Trx-80 might reduce macrophage polarization into the M2 phenotype through upregulation of $p16^{INK4a}$. However, such a mechanism does not seem to be relevant because we did not observe any effect of Trx-80 on $p16^{INK4}$ expression in macrophages (data not shown).

Of note, plasma cholesterol and triglycerides levels in LPS-treated mice versus LPS and Trx-80-treated mice were not significantly different (Table II in the online-only Data Supplement). However, whether the progression and

unstability of atheroma plaques attributed to M1 phenotype could be explained by a difference in lipid metabolism and lipid accumulation in M1 in comparison to M2 macrophages was unknown. Therefore, we conducted additional experiments on cultured macrophages to determine the level of ATP-binding cassette A1 (ABCA1) expression in the M1, M2, and in RM as well as the influence of Trx-80 on the ABCA1 gene expression. The results showed an increase of ABCA1 expression in M1 macrophages in comparison to M2 and RM and that Trx-80 enhanced such expression suggesting that M1 macrophages may have a high reverse cholesterol efflux activity which can be potentiated by Trx-80 (Fig. 9A).

In addition, the expression of the scavenger receptors SR-A, CD36, and LOX-1 were similar in RM, M2, and M1 macrophages and Trx-80 did not influence the expression of any of these genes (data not shown). Moreover, Trx-80 does not affect the reverse cholesterol transport (Fig. 9B). The reason for this apparent contradiction is currently not known. One might speculate that Trx-80 could activate macrophage pinocytosis through protein kinase C, which increases cholesterol accumulation. This cholesterol influx is counterbalanced by the increase of ABCA1 expression. Nevertheless, further studies are needed to clarify this point.

To date, the biological roles of Trx-80 are still largely unknown. Previously, Trx-80 was termed eosinophil cytotoxicity-enhancing factor (ECEF) due to its eosinophil cytotoxicity and it has been first detected in the plasma of patients suffering from severe schistosomiasis (Dessein et al., 1984; Lenzi et al., 1985; Silberstein et al., 1989). Of note, these patients also developed atherosclerosis. It may be rewarding to identify other pathologies associated with Trx-80 generation and to study in greater detail the molecular mechanisms leading to its generation.

Acknowledgments

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Possible signaling pathway involved in macrophage polarization

8. Possible signaling pathway involved in macrophage polarization

8.1. Study presentation

The importance of redox signaling is increasingly recognized, despite the highly transient and volatile nature of the redox modifications that makes them very difficult to access for broad in depth investigations. The various redox modifications are highly target- and site specific, and the Trx, Grx, and Prx systems are pivotal players in redox signal transduction both as transducers and as regulators of second messenger levels. As in previous two studies, we have shown that Trx1 and Trx80 play different role in macrophage polarization and the development of atherosclerosis. Therefore, it is thought that each may use different signaling pathway or regulate the same signaling pathway but in different way. Among the candidate signaling pathways, Akt pathway is most likely to be used by Trx1 and/or Trx80.

Akt (also known as PKB) is a family of three serine/threonine protein kinases (Akt1, Akt2, and Akt3) that regulate a host of cellular functions, including cell survival, proliferation, differentiation, and intermediary metabolism [240]. In the vascular wall, Akt has been shown to play an important role in the proliferation and migration of endothelial cells, regulation of vascular permeability, and angiogenesis [241-243]. Recent studies of *Akt* knockout mice have shown that despite significant sequence homology, the three Akt isoforms have some nonredundant functions. Although *Akt1*-deficient mice exhibit overall growth impairment [244], *Akt2* knockout mice have impaired glucose tolerance and insulin resistance [245], and *Akt3* nulls display a selective reduction in brain size [246].

Additionally, it has been reported that a global absence of Akt1 *in vivo* enhances atherosclerotic lesion burden and promotes coronary atherosclerosis in a mouse model of atherosclerosis suggesting that Akt1 exerts vascular protection against atherogenesis [240] interestingly it has been demonstrated that Akt2 ablation results in the M2 polarization of macrophages whereas Akt1 ablation promotes their M1 polarization [247].Therefore, the

implication of Akt pathway has been studied to explore the signaling pathway used by Trx1 and/or Trx80 to exert their effects.

8.2. Regulation of Cell Signaling by Trx1

Depending on its subcellular localization, Trx1 exerts different roles. It can be found in the extracellular environment, on the cell-surface and intracellularly.Trx1shows redox regulatory functions in signal transduction and transcriptional mechanisms.

8.2.1. Extracellular Trx1

In spite of lacking a signal peptide, Trx1 can be secreted by cells through an unknown mechanism [248,249]. Extracellular Trx1, either secreted or exogenously added, triggers a variety of physiologic or pathologic functions. Until now, no binding of Trx1 to a specific cell-surface receptor has been characterized. It seems that Trx1 acts as an autocrine growth factor and in synergy with other cytokines as a potent costimulatory molecule from the outside of cells [250]. Although it has not yet been extensively studied, Trx1 has been reported as a lipid raft (LR)-associated protein. LR are specialized domains of the plasma membrane that cluster specific proteins and provide a dynamic scaffold for organizing cellular processes such as signal transduction (Figure 11) [251]. There is convincing evidence that LRs redox signaling platforms may mediate the actions of Trx1 on leukocyte–endothelial cell interaction related to redox regulation during inflammation [252]. On the other hand, LR clusters get endocytosed and form redoxosomes that mainly produce ROS as signaling molecules to evoke intracellular downstream responses. Trx1 can be internalized into the cells through LR-mediated endocytosis [253]. Increasing evidence suggests that LR redox signaling may contribute to infection, host defense, and to the development of different diseases such as atherosclerosis, obesity or metabolic syndrome and tumor progression [254]. Further studies are needed to

investigate the molecular mechanisms involved in the formation of this membrane signaling complex highlighting the possible role of Trx1 through these platforms.

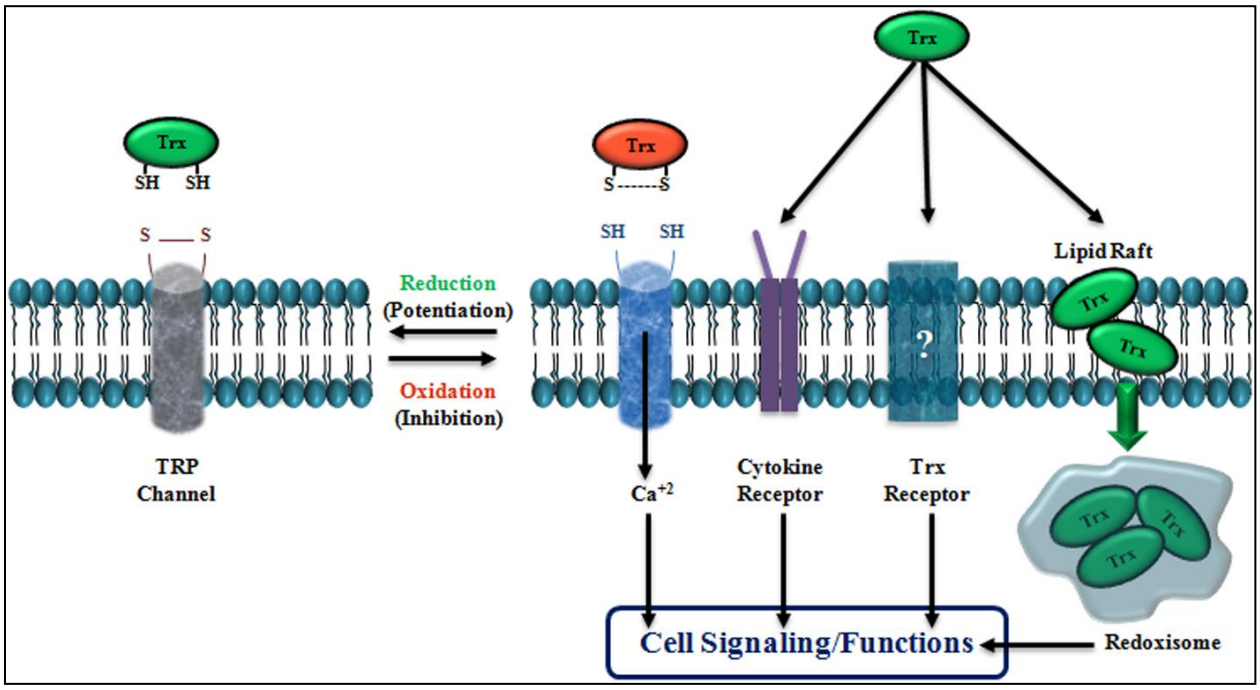

Figure 11 Possible signaling pathway of extracellular Trx1.

Trx1 has been reported as a lipid raft (LR)-associated protein. Trx1 may enter the cell through lipid rafts subsequently forming the redoxisome, endosomes responsible for early receptor-mediated signaling in nonphagocytic cells. Reduced Trx1 can also break a disulfide bond in the extracellular loop of transient receptor potential-C5 (TRPC5) channel, thereby increasing the Ca^{2+} ion influx of the channel [48].

8.2.2. Cytoplasmic Trx1

Disulfide reducing action is extremely important in regenerating oxidized proteins, which may render many signaling proteins inactive. Trx-dependent redox regulation of cellular signaling and stress response occurs through several mechanisms such as redox balance regulation or protein/protein interaction (Figure 12). Here we highlight the involvement of Trx1 in MAPKs and Ca^{+2} signaling.

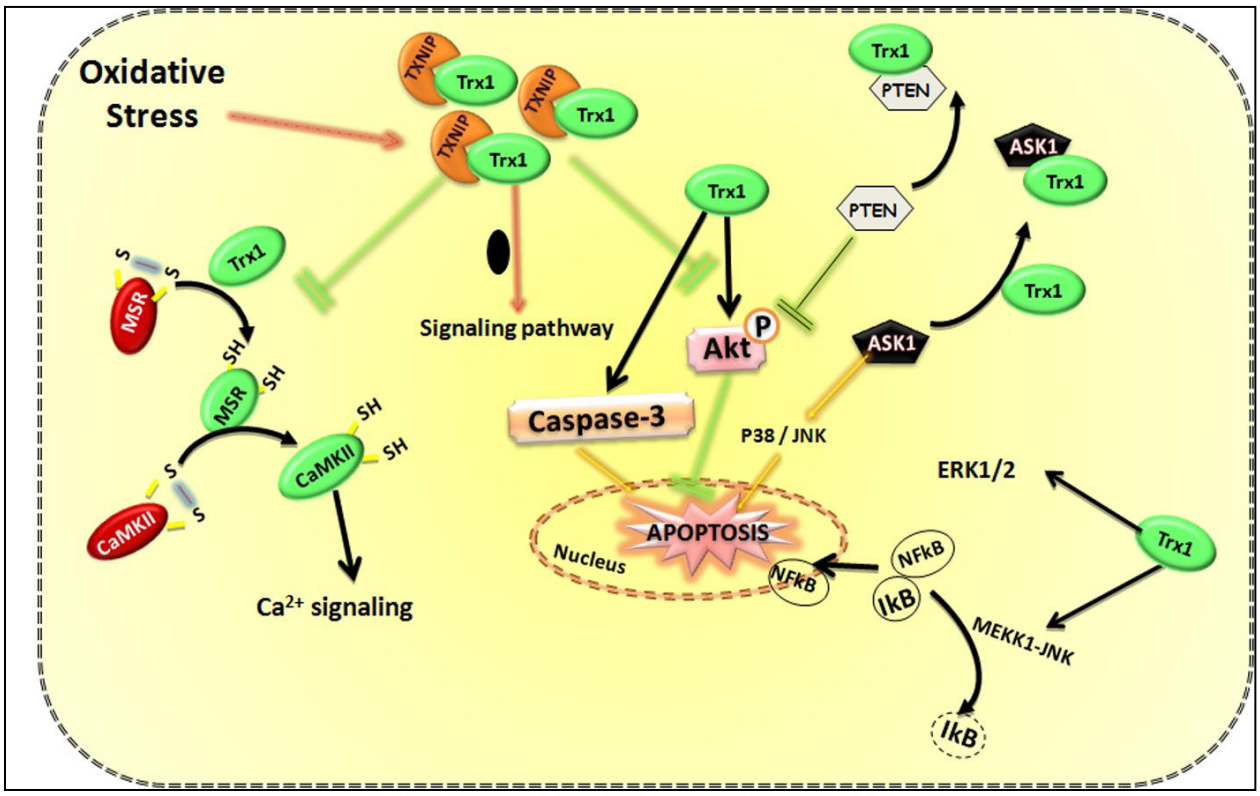

Figure 12: Cytoplasmic Trx1 and cell signaling.

Reduced Trx1 can inhibit apoptosis through binding to ASK-1. Furthermore, Trx1 activates the Akt pathway either directly or indirectly, by interacting with and thereby inhibiting PTEN activation. Trx1 can activate also certain components of the MEKK1-JNK signaling pathway, resulting in IκB degradation and NF-κB activation. The TXNIP inhibits Trx1 activities. ASK-1, Apoptosis Signal-Regulated Kinase-1 MEKK1,MAP/ERK Kinase Kinase-1; PTEN, Phosphatase And Tensin Homology; TXNIP,Thioredoxin Interacting Protein; CaMKII, Calcium/Calmodulin-dependent Protein Kinase II; MSR, Methionine Sulfoxide Reductase [48].

8.2.2.1 . Mitogen-Activated Protein Kinases (MAPKs)

Apoptosis signal-regulated kinase 1 (ASK-1) is an upstream MAP kinase kinase kinase that regulates the c-Jun N-terminal kinases (JNK) and p38 MAPK leading to stress-induced apoptosis and inflammation. ROS-activated ASK-1 mediates p38 signaling that may induce non-apoptotic outcomes, such as differentiation [255] and TLR4-mediated innate immunity [256]. Importantly, reduced Trx1 can bind to the amino-terminal portion of ASK-1, thereby inhibiting the kinase activity and ultimately leading to ASK-1 ubiquitination and degradation (Figure 12)[92,97]. The final actor in this triad is TXNIP, which binds to the catalytic cysteines

of Trx1, and thus inhibits Trx1 activity and its ability to bind to ASK-1.Trx1 has also been shown to activate certain components of the MEKK1-JNK signaling pathway, resulting in IκB degradation and NF-κB activation [257].

On the other hand, Trx1 knockdown suppressed epidermal growth factor-induced ERK1/2 activation, suggesting that Trx1 plays a positive regulatory role in ERK1/2 signaling [258].

In response to extracellular growth or survival factors, the Akt signaling pathway becomes activated to coordinate various cellular events and eventually controls physiological processes such as cell proliferation, cell growth, cell migration and survival [259]. On the other hand, the phosphatase and tensin homology (PTEN) phosphatase exerts the function of terminating Akt signaling [260]. Therefore, PTEN inactivation by oxidative stress is a physiological mechanism by which Akt becomes activated [261]. It has been demonstrated that Trx1 activates the Akt pathway, either directly or indirectly, by interacting with or inhibiting PTEN [262-264].

8.2.2.2 . Calcium Signaling

Oxidation of thiol systems has long been known to cause cell death due to dysregulation of Ca^{2+} homeostasis. Transient receptor potential (TRP) channels are cation channels that mainly mediate the influx of Na^{+} and Ca^{2+} across the plasma membrane into the cytoplasm. TRPC5 homotetrameric channels and TRPC5/TRPC1 heterotetrameric channels, expressed in HEK293 cells, are activated by extracellular Trx1. Reduced Trx1 breaks a disulfide bond in the extracellular loop adjacent to the ion selectivity filter of TRPC5 and increase the Ca^{2+} influx of the channel [265]. On the other hand, in mammalian tissues, activities of the phosphatase calcineurin and Ca^{2+}-calmodulin-dependent kinase II (CaMKII) have been demonstrated to be reversibly altered through oxidation of specific methionines within their calmodulin (CaM) binding and autoinhibitory sites, respectively. Trx-dependent reduction by

methionine sulfoxide reductase limits these methionine oxidations and promotes Ca^{2+} signaling pathways which are important to stress responses [266].

8.2.3. Nuclear Trx1

Oxidants in the cytoplasm induce oxidative stress, which triggers transcriptional activation of defense systems protecting the cells from uncontrolled oxidative reactions. It seems that a fine-tuned balance between antioxidative and oxidative systems likewise exists in the nucleus (Figure 13). Trx1was found to be translocated into the nucleus in response to oxidative stress, despite the lack of a nuclear localization signal (NLS)[66]. Schroeder *et al*. clearly demonstrated that Trx1 is imported into the nucleus in a karyopherin-α-dependent manner, and this nuclear import of Trx1 is required for inhibition of apoptosis [71]. In addition, TrxC32S/C35S can still be imported into the nucleus. However, Cys32 and Cys35 are required for the ability of Trx1 to bind to transcription factors and thereby modulating their activity. Because the nuclear import of Trx1 is greatly impaired when Cys69 is mutated to serine, one may speculate that modification of Trx1 has to occur in the cytoplasm to allow Trx1 to be imported into the nucleus. Trx1 then promotes antiapoptotic effects by binding to several transcription factors and increasing transcription-factor binding to genes through the ARE [71].

Trx1 reduces redox factor-1 (Ref-1), a bifunctional protein with redox activity and a separate DNA repair (APE-1, endonuclease) activity. Ref-1 is exclusively localized in nuclei [267] and its redox activity maintains conserved Cys residues of redox-dependent transcription factors including hypoxia-inducible factor-1 α (HIF-1α), NF-κB, p53, activator protein-1 (AP-1), Nrf-2, glucocorticoid receptor, and estrogen receptor [65-70]. Furthermore, inducible activation of HIF1-α protein in response to hypoxia is produced, at least in part, by thiol redox regulation of the C-terminal transactivation domain activity of the Trx1 and Ref-1 system leading to the interaction with CREB binding protein (CBP)/p300 [102]. Ref-1 was recognized

to reduce c-Fos and c-Jun members of AP-1 [268]. This redox modulation occurred at a conserved Cys residue (Lys-Arg) present in both c-Fos and c-Jun. Reductions of c-Fos and c-Jun by Ref-1 stimulate AP-1 binding to DNA, and this stimulatory effect is significantly elevated by reduced Trx1 [268]**.** Additionally, an important role of Trx1 in AP-1 activity is mediated by interaction with Jab1(Jun activation domain binding protein) [93].

NF-κB is a p50/p65 heterodimer and theCys62 residue must be reduced for the p50 subunit to facilitate DNA binding indicating that the redox state of the nuclear compartment is critical for NF-κB activation [269]. The Trx1 system modulates NF-κB transcriptional activity through Cys62 of the p50 subunit [66,270].

The p53 transcription factor controls the expression of a wide range of genes in response to DNA damage, chemotherapeutic drugs, growth factors, and oxidants [256]. Ten Cys residues are located within the DNA-binding domain of p53, and all are susceptible to oxidation, resulting in alteration of the DNA-binding properties of p53. Trx1 enhances the p53 DNA-binding activity directly or through Ref-1 mediation [91].

Nrf-2 is a prototypic redox signaling pathway [271]. Nrf-2 is bound to the actin-associated binding protein (Keap-1) in the cytoplasm. Oxidation or alkylation of Cys residues in Keap-1 provides a signal to release Nrf-2, which translocates to the nucleus and activates transcription *via* ARE within the control regions for antioxidant and detoxification genes [272]. Hansen *et al.* showed that oxidant-induced Nrf-2/Keap-1 dissociation in the cytoplasm is regulated by the GSH system, whereas the DNA-binding activity of Nrf-2 in the nucleus is controlled by nuclear Trx1 indicating a dual regulation [273].

Trx1 also mediates the interplay between cellular redox signaling and glucocorticoid receptor signaling *via* direct association with its conserved DNA-binding domain [103]. The function of the estrogen receptor (ER), another nuclear receptor, is strongly influenced by its redox state

and is modulated by endogenous Trx1 [204]. Indeed, Trx1 inhibits the binding of PPAR-α to the PPAR-response element by modulating its AF-1 transactivation domain [274].

TXNIP inhibits the intracellular Trx1 activity by forming a mixed disulfide bond between the Trx1 active site Cys32 and TXNIPCys247 [146]. In addition, TXNIP can also mediate the nuclear localization of Trx1 [101] because it can interact with importin alpha 1, a component of the nuclear import machinery [275].

Interestingly, TXNIP null hepatocytes exhibit reduced glucose production that can be rescued by reintroducing TXNIP-WT. Overexpression of the TXNIP mutant C247S failed to restore the liver glucose production, although the Trx1 activity was not affected [276]. In addition, the TXNIP/Trx1 complex has been shown to mediate activation of plasma membrane signaling thereby promoting cell survival and migration [72]. The TXNIP/Trx1 effects could be related to TXNIP as a scaffold protein, as an α-arrestin protein. These data strongly suggest that the TXNIP/Trx1 complex can have major signaling properties in a specific subcellular localization, independent of the inhibition of the Trx1 activity. It is clear that phosphorylation of Trx1 at T100 plays an important role in its cytoprotective activity in cancer cells [277].

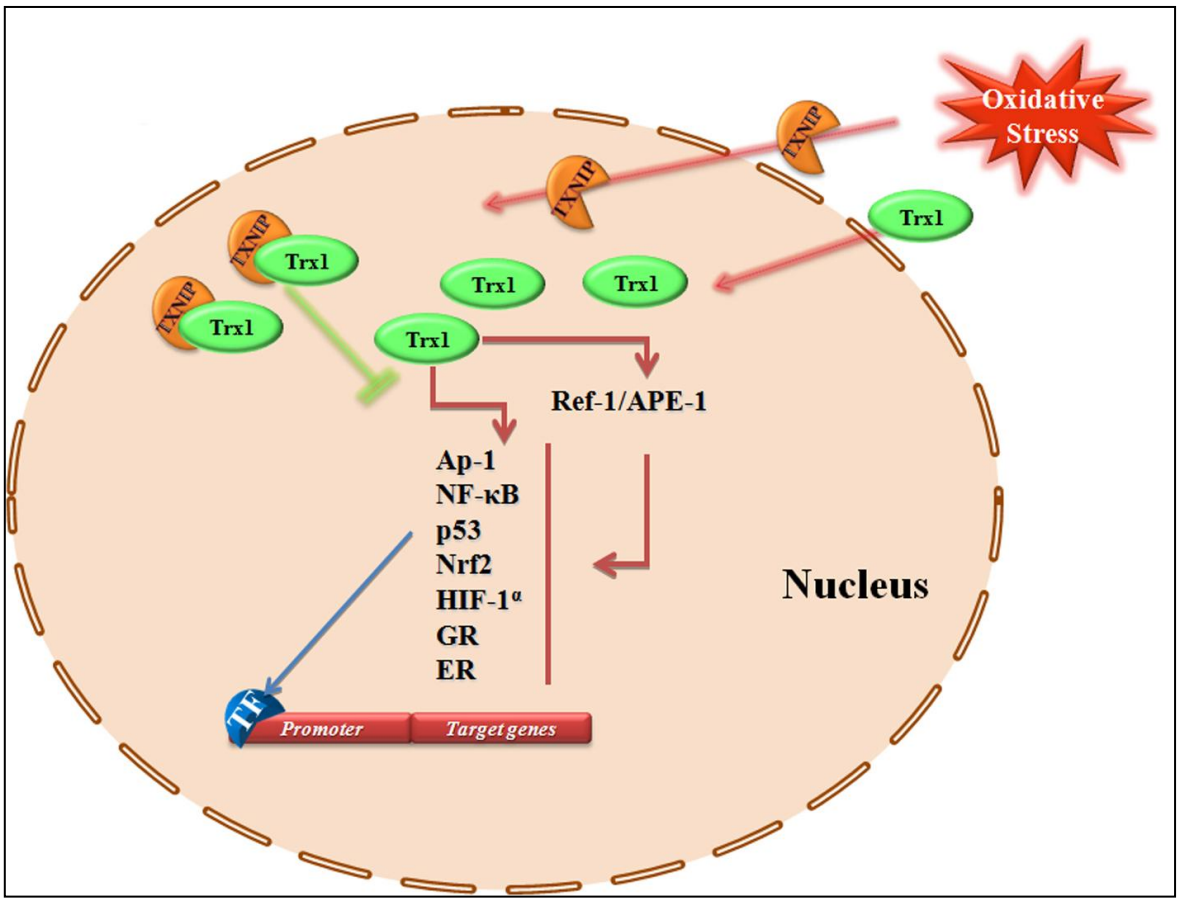

Figure 13: Nuclear Trx1 regulates the nuclear redox state and transcriptional activity. Despite the lack of a nuclear localization signal, Trx1 can translocate into the nucleus in response to oxidative stress, where it regulates different transcription factors.Ref-1, Redox Factor-1; HIF-1α, Hypoxia-Inducible Factor-1 α; AP-1, Activator Protein-1; Nrf-2, Nuclear Factor Erythroid-2 Related Factor 2; ER, Estrogen Receptor; GR, Glucocorticoid Receptor [48].

8.3. The mTOR signaling pathway

Eukaryotic TORs are large proteins (~280 kDa) that exhibit 40-60% sequence homology at the amino acid level and are members of the PI3K-related protein kinase family that function as serine/threonine protein kinases [278]. It has been shown that TOR knockout resulted in early embryonic death in *C. elegans* [279], *D. melanogaster* [280] and mice [281,282] implying its central role in primordial development. In the early 1990s, yeast genetic screens led to the identification of TOR as one mediator of the toxic effect of rapamycin in yeast. Shortly after,

the mammalian TOR (mTOR), now officially known as the mechanistic TOR, was identified as the physical target of rapamycin [283].

By binding with other proteins, mTOR forms two different large complexes, mTORC1 and mTORC2 (Figure 14), which have different serine/threonine kinase activities [284]. The mTOR signaling has been shown to regulate numerous biological processes, including transcription, translation, cell size, mRNA turnover, protein stability, ribosomal biogenesis, autophagy, long-term neural potentiation, vesicular trafficking and cytoskeletal organization (Figure 14) [278,285].

The TOR deregulation is associated with several human diseases, such as type 2 diabetes, cancer, obesity and neurodegeneration, highlights its importance in the maintenance of cellular homeostasis [286]. The mTOR regulates protein translation through two parallel pathways: activation of ribosomal S6 kinase-1 (S6K1) and suppression of the elongation factor 4E binding protein 1 (4EBP1). Phosphorylation of S6K1 on Thr389 and Ser371 residues is required for its activation and both sites are phosphorylated by mTOR *in vitro*. Active S6K1 then phosphorylates the 40S ribosomal protein S6 [278]. TOR inhibitors have been approved or are under investigation for treatment of several diseases, including various cancers. These inhibitors include the immunosuppressant rapamycin, which is widely used to combat kidney rejection after transplantation. In model organisms ranging from yeast to mice, inhibition of TOR signaling increases life span [287].

Recently, mTOR function in the cardiovascular system and aging has been studied indicating that mTORC1 is required for; embryonic cardiovascular development; postnatal maintenance of cardiac structure and function; reduction of pathological hypertrophy and heart failure; and reduction of myocardial damage after acute and chronic myocardial infarction [288]. However, genetic inhibition of mTORC1 in *C. elegans* was shown to extend life span through the SKN-1/Nrf, and DAF-16/FoxO which activate protective genes [289].

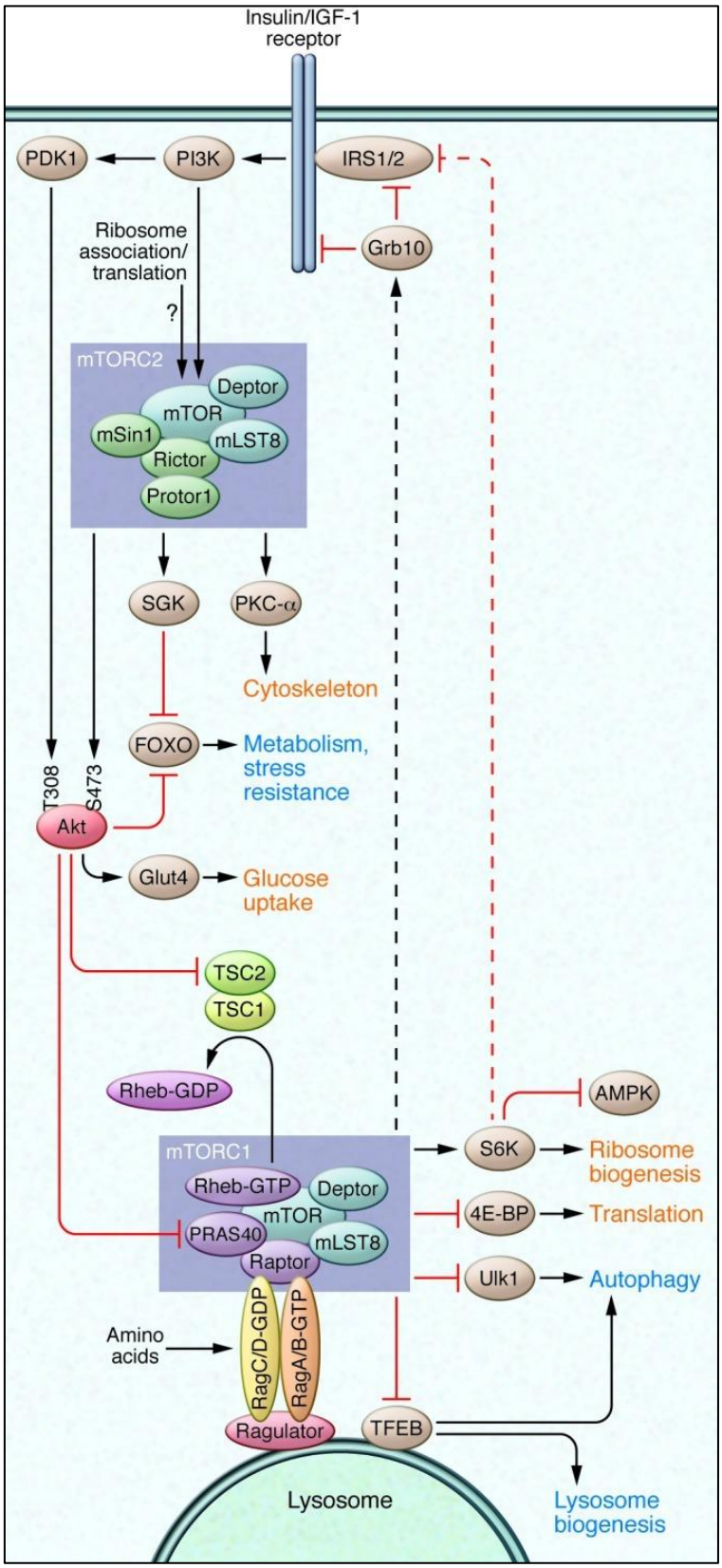

Figure 14: The mTOR signaling.

mTOR is found in two complexes, mTORC1 and mTORC2. mTORC1 is regulated in part via the TSC complex, which normally act as a GTPase-activating protein for Rheb to suppress mTORC1 signaling. mTORC1 is also regulated by amino acids via the Ras-related GTP binding (Rag) family of small GTPases. The Rag proteins activate mTORC1 by localizing mTORC1 to the lysosome via interaction with the ragulator complex. mTORC1 promotes growth by enhancing ribosomal biogenesis, translation, and other anabolic processes, while inhibiting autophagy. mTORC1 suppresses insulin/IGF-1 signaling via direct regulation of Grb10 and S6K, which subsequently reduces signaling to mTORC2. AKT, an inhibitor of TSC1/2, is one of several direct substrates of mTORC2. Processes that are upregulated by mTOR signaling are shown in red; those that are downregulated by mTOR signaling are shown in blue (from [287]).

The mTORC1 positively regulates cell growth and proliferation by promoting many anabolic processes, including biosynthesis of proteins, lipids and organelles, and by limiting catabolic processes such as autophagy [290]. Most recently, mTORC2 in particular has emerged as a key controller of lipid metabolism, regulating lipogenesis in the liver, regulating lipolysis in white adipose tissue, and controlling adipogenesis [291].

8.4 Materials and Methods

8.4.1. Isolation and treatment of mouse peritoneal macrophages

Peritoneal macrophages collected as described in Article 1-Supplementarory Data. Cells were plated in RPMI 1640. with 2% FBS for 16 h. and they were left untreated or were primed with LPS (100 ng/mL, *E. coli*, serotype 055:B5, Sigma-Aldrich), IL-4 (15 ng/mL) for 4-6 h. Then, cells were treated for 1 hr with an inhibitor: LY294002, Akt1, or Akt2. Subsequently, cells were treated either with Trx1 (1 μg/mL) or Trx80 (1 μg/mL) for 15 min. (all the products are from R&D Systems). Total proteins were extracted for western blotting.

8.4.2. Western Blot Analysis

Proteins from whole cell lysates of macrophages were separated on 4-12% Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen). Immunoblot analysis was performed with Phospho-Akt (Ser473) (Cell Signaling #9271), Akt (Cell Signaling #9272), Phospho-mTOR (Ser2448) (Cell Signaling #2971), mTOR (Cell Signaling #2972) antibodies followed by incubation with appropriate horseradish peroxidase-associated secondary antibody before the signal was visualized by enhanced chemiluminescence (Amersham Bioscience).

8.5. Results

8.5.1. Both Trx1 and Trx80 activate Akt

Trx1 and Trx80 significantly up-regulated Akt activation in non-treated macrophages (Figure 15), as demonstrated by an *in vitro* kinase assay performed on total cellular extracts after exposure to Trx1 (1 µg/ml) or Trx80 (1 µg/ml) for 15 min. These data were confirmed through Western blot, by the increased level of the activated form of pAkt. The Trx1 did not dampen the down-regulation of Akt activity by LY294002 as Trx80 did. When cells primed with either IL-4 (15 ng/mL) or LPS (100 ng/mL), treatment with Trx1 or Trx80 did not show any further activation of Akt. This may explain that they could activate different isoforms of Akt.

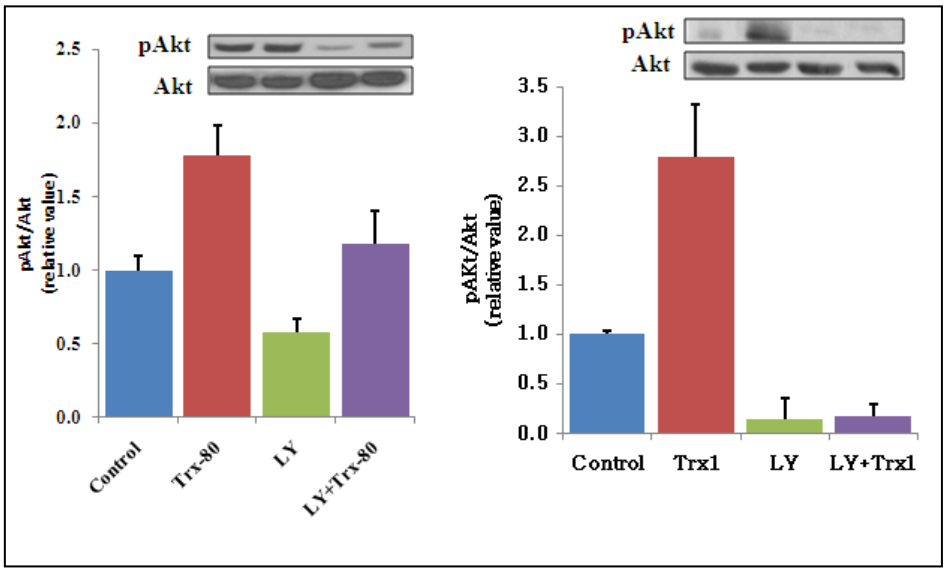

Figure 15: Both Trx1 and Trx80 activates Akt

8.5.2. Trx80, but not Trx1, activates mTOR

To determine which isoform has been activated by Trx1 and Trx80, a specific inhibitor, Akt1/2 kinase inhibitor (A6730 SIGMA), has been used. It shows IC50 = 58 nM, 210 nM, and 2.12 mM for Akt1, Akt2, and Akt3, respectively. Accordingly, either Akt1 or Akt2 has been inhibited then the cells were treated with different concentrations (0, 1, 2, and 5 µg/ml) of Trx1 or Trx80 for 15 min. As shown in Figure 15, hrTrx80 significantly activated mTOR

in a dose dependent manner only when Akt1 is inhibited whereas Trx1 did not activate it (Figure 16).

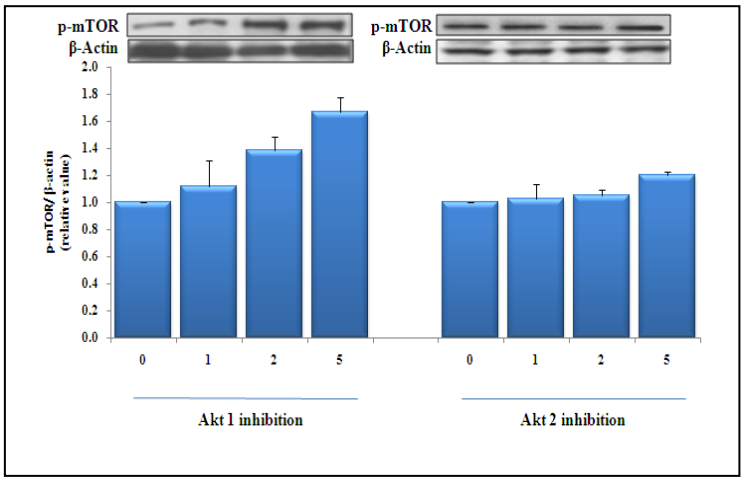

Figure 16: Trx80 significantly activates mTOR in a dose dependent manner only when Akt1 is inhibited.

8.5.3. Inhibition of mTOR downregulated the expression of inflammatory cytokines

To further explore the role of mTOR in macrophage polarization, isolated murine peritoneal macrophages were pretreated with or without rapamycin (20 nM) for 6 hrs and/or exposed to LPS (100 ng/mL) and/or hrTrx80 (1 μg/ml) stimulation. As shown in Figure 17, rapamycin (RAPA) attenuated the expression of inflammatory cytokines, IL-6 (A), IL-1β (B), and TNF-α (C) revealing the role of mTOR signaling in macrophage phenotype orientation. Interestingly, Trx80 upregulated the expression of these cytokines may be through reactivating mTOR pathway.

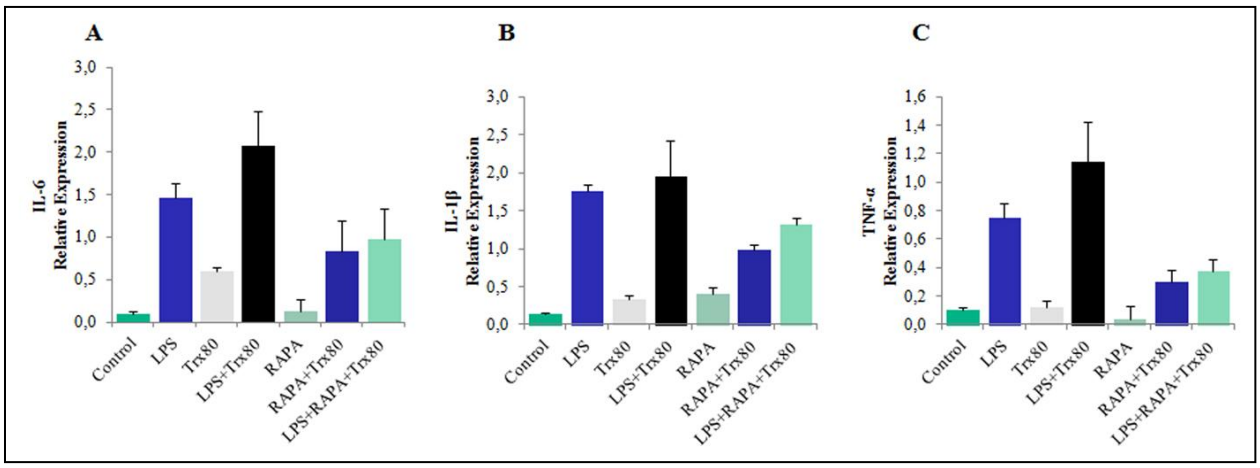

Figure 17: mTOR inhibition attenuated the expression of inflammatory cytokines.

Murine peritoneal macrophages have been pretreated with or without rapamycin (20 nM) for 6 hrs then they were stimulated with or without LPS (100 ng/mL) and/or hrTrx80 (1 μg/ml). mRNA levels of IL-6 (A), IL-1β (B), and TNF-α (C) were quantified with qPCR and normalized to GAPDH.

8.5.4. Trx80 activates mTOR in dose-dependent manner

The p70S6K (Thr389) is known to be a convincing direct substrate of mTORC1. It is often used as functional readout of mTORC1 activity, as phosphorylation of these sites by mTORC1 has been confirmed both *in vitro* and *in vivo* and is inhibited by rapamycin treatment. Therefore, to confirm our previous, murine peritoneal macrophages have been pretreated with RAPA (20 nM) for 1 hr followed by exposure to different concentrations of hrTrx80 for 30 min. Results of Western blot analysis using phospho-P70S6K and p70S6K specific antibodies showed that Trx80 activates mTOR in a dose-dependent manner (Figure 18).

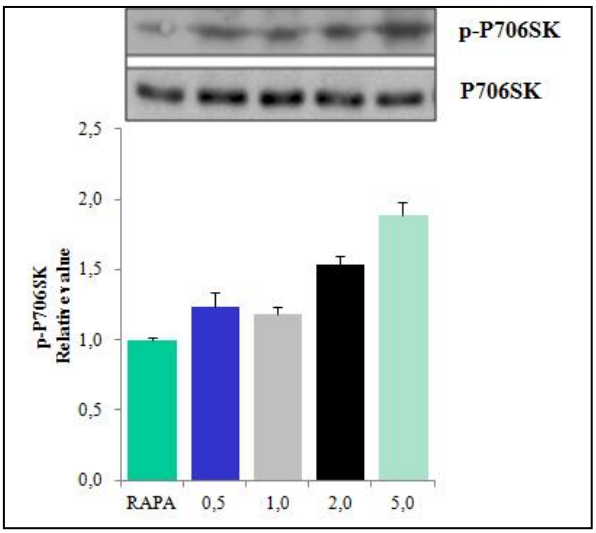

Figure 18: Trx80 activates mTOR in a dose-dependent manner.

Murine peritoneal macrophages have been pretreated with RAPA (20 nM) for 1 hr followed by stimulation with different concentrations (0, 0.5, 1, and 5 μg/ml) of hrTrx80 for 30 min. Results represent quantification of Western blots of phosphorylated p70S6K normalized total p70S6K.

8.5.4. mTOR inhibition orientes resting macrophages toward M2 phenotypes.

Freshly isolated murine peritoneal macrophages were pretreated with or without rapamycin (20 nM) for 6 hrs and/or exposed to IL-4 (15 ng/mL) and/or hrTrx80 (1 μg/ml) stimulation. As shown in Figure 19, rapamycin (RAPA) stimulated the expression of anti-inflammatory cytokines, IL-10 (A) and CD206 (B) indicating the implication of mTOR signaling in macrophage polarization. Of note, Trx80 treatment attenuated the expression of these cytokines may be through reactivating mTOR.

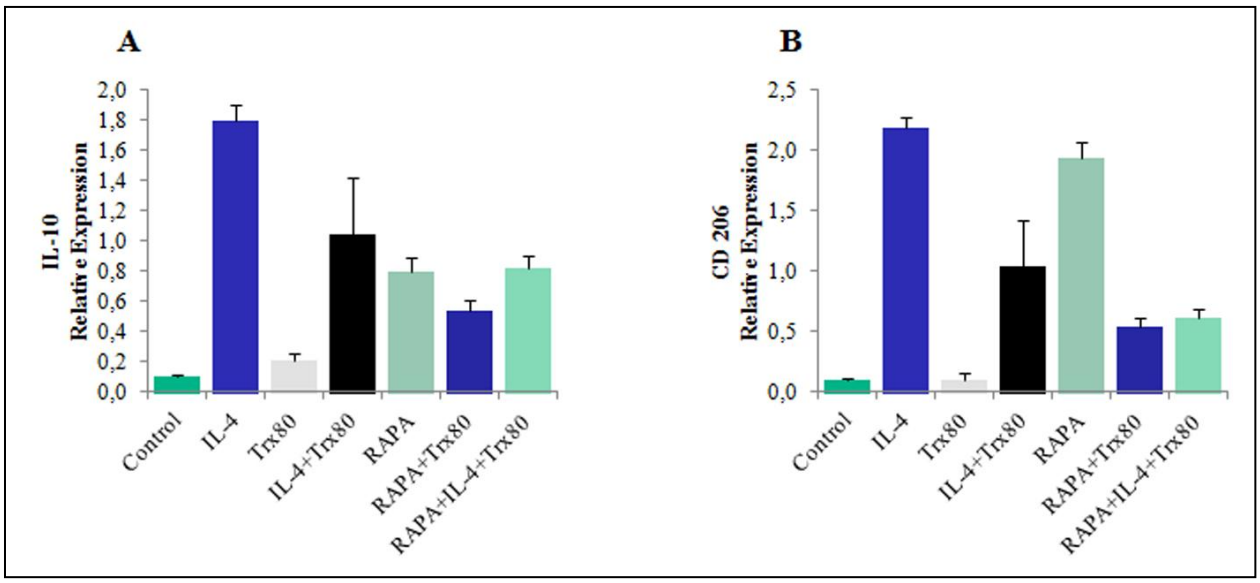

Figure 19: mTOR inhibition upregulates M2 phenotype markers

Murine peritoneal macrophages have been pretreated with or without rapamycin (20 nM) for 6 hrs and they were stimulated with or without LPS (100 ng/ml) and/or hrTrx80 (1 µg/ml) for the indicated period. The mRNA levels of IL-10 (A) and CD206 (B) were quantified with qPCR and normalized to GAPDH.

8.6. Discussion

A network of signaling molecules, transcription factors, epigenetic mechanisms, and posttranscriptional regulators underlies the different forms of macrophage activation. Canonical IRF/STAT signaling pathways are activated by IFNs and TLR signaling to skew macrophage function toward the M1 phenotype (via STAT1) or by IL-4 and IL-13 to skew toward the M2 phenotype (via STAT6) [292]. M1 macrophages upregulate IRF5, which is essential for induction of cytokines (IL-12, IL-23, TNF) involved in eliciting Th1 and Th17 responses [293]. The IL-4 type I and type II receptors activate Stat6, which in turn activates transcription of genes typical of M2 polarization, e.g., mannose receptor (*Mrc1*), resistin-like α (*Retnla, Fizz1*), and chitinase 3–like 3 (*Chi3l3, Ym1*). IL-10 activates STAT3-mediated expression of genes (*Il10, Tgfb1, Mrc1*) associated with an M2-like phenotype [292]. The nuclear receptors PPARγ and PPARδ control distinct subsets of genes associated with M2 macrophage activation and oxidative metabolism. Interestingly, STAT6 coordinates and synergizes with both PPARγ [294] and Krüppel-like factor 4 (KLF4), a member of a family of proteins that contribute to macrophage function. KLF4 cooperates with Stat6 to induce M2 genes (*Arg-1, Mrc1, Fizz1, PPARγ*) and inhibit M1 genes (*TNFa, Cox-2, CCL5, iNOS*) via sequestration of coactivators required for NF-κB activation. KLF2 regulates macrophage activation by inhibiting NF-κB/HIF-1α activities. IL-4 also induces c-Myc activity in human macrophages, which controls genes of M2 activation (*Scarb1*, *Alox15*, and *Mrc1*) as well as STAT6 and PPARγ activation [33]. Therefore, the balance between activation of STAT1 and STAT3/STAT6 finely regulates macrophage polarization and activity. A predominance of NF-κB and STAT1 activation promotes M1 macrophage polarization, resulting in cytotoxic and inflammatory functions. In contrast, a predominance of STAT3 and STAT6 activation results in M2 macrophage polarization, associated with immune suppression and tumor progression. Our results showed that both Trx1 and Trx80 activate Akt in resting

macrophages whereas in already oriented macrophages toward either M1 or M2, they could not further activate Akt. Previously, it was documented that Trx1 can activate Akt [262] [98] but here for the first time we show the effect of Trx80 on Akt/mTOR pathway. Our previous published data indicate that Trx80 binds to the macrophages cell membrane, which is later taken up by the cells and which can be detected primarily in the cytosol after extended periods of time. Interestingly, Trx80 does not colocalize with acidic compartments such as lysosomes, and is barely degraded even after 24 h [48]. Therefore, its role as a ligand and/or effector in signal transduction is evident. As mentioned earlier, we hypothesized that each molecule may activate a different isoforms of Akt, therefore, we have inhibited Akt isoforms separately. It has been found that only Trx80 specifically activate mTOR only when Akt1 is inhibited.

The past several years have seen an explosion of interest in the mTOR signaling pathway, spurred in large part by the finding that inhibition of mTORC1 signaling can significantly increase lifespan and protect from age-related diseases in mouse models. Genetically engineered mouse models have significantly added to our understanding of the role of mTOR in mammalian physiology. It is clear that mTOR signaling regulates lipid homeostasis, as treatment of rodents or humans with rapamycin leads to hyperlipidemia and hypercholesterolemia [291]. The mTOR is an evolutionarily conserved serine/threonine protein kinase implicated in a wide array of processes such as cell growth, proliferation, survival, and autophagy. It forms two distinct functional complexes, mTORC1 and mTORC2. mTORC1 activity is sensitive to inhibition by rapamycin; whereas, mTORC2 activity is resistant to the drug. mTORC1 activation leads to increased protein synthesis and cell growth whereas mTORC2 activity is mainly regulated by growth factors such as insulin and IGF-1 and regulates the activation of Akt, a kinase involved in cell proliferation and survival. Recently, Sun *el al,* [127] showed that hyperglycemia and insulin deficiency can modify macrophage function through Akt-mTOR and ERK pathways and through epigenetic effects on

macrophage precursors. Therefore, further downstream proteins need to be investigated in order to find out the exact signaling pathway employed by either Trx1 or Trx80 to stimulate macrophage polarization since specific macrophage-targeted therapies are now taking the first steps into the clinical arena.

Based on the obtained results, inhibition of mTOR activity orients resting macrophages toward M2 phenotype and impairs the polarizing effect of LPS. These results are consistent with most recent works by [295] and [296]. It has been reported that LPS-induced expression of IL-1β and IL-6 was markedly suppressed by INK128, an mTOR inhibitor, at both mRNA and protein levels [296]. Another recently published study showed that rapamycin treatment restores Akt activation simultaneous with rescue of M2 gene expression and arginase activity. Moreover, rapamycin treatment of control BMDMs modestly increased Akt signaling and M2 response [295].

Furthermore, our results show that Trx80 uses mTOR signaling pathway to exert its effect in polarizing macrophages toward M1 phenotype since it activated mTOR activity in a dose-dependent manner as demonstrated by the increased phosphorylation of P70S6K. The molecular mechanism is still not clear how Trx80 could rescue mTOR from the inhibitory action of rapamycin. It may prevent the formation of rapamycin- FK506 binding protein (FKBP12) complex since rapamycin first binds to its cellular receptor, FKBP12, and it is the rapamycin-FKBP12 complex that inhibits TOR function. This mechanism of action is conserved in eukaryotes [278].

Truncated thioredoxin, Trx80, is a natural cleavage product of Trx1with 10 kD sharing the 80 or 84 N-terminal amino acids with Trx1 [124,125]. It has been suggested that the enzyme responsible for its cleavage would be an inducible protease [126].Very recent findings, indicated that the disintegrins and metalloproteinases (ADAM10 and 17), two α-secretases processing

the amyloid β precursor protein, are responsible for Trx80 generation in brain. Up to date, there are very limited works on Trx80. Previously, it has been shown that macrophages can cleave Trx1 producing Trx80 [128] which can activates monocytes and induces up-regulation of cell surface pathogen recognition receptors, molecules essential for T-cell activation and function [126] as well as release of proinflammatory cytokines evoking inflammation [90]. Since Trx80 has very different properties from the full-length protein, lacking the disulfide reductase activity of the full-length species, and instead having pro-inflammatory cytokine-like effects on immune cells, [124,131] we further investigated the vascular proinflammatory mechanism to clarify its atherogenic implication [48]. In his study, we report that Trx80 on one hand potentiates the expression of M1 macrophages markers, through the AP-1 and Ref-1 transcription factors-independent pathway and on other and it blunts the expression of anti-inflammatory cytokines. Taken together, these studies indicate that Trx80 functions as regulator of macrophage phenotype tipping the balance toward the pro-inflammatory M1 state. As a consequence, atherosclerotic plaques become larger and probably more unstable.

GENERAL DISCUSSION

General discussion

The Trx system comprises Trx, Trx80, Trx reductase, and NADPH, besides a natural Trx inhibitor, TXNIP. This system is essential for maintaining the balance of the cellular redox status, and it is involved in the regulation of redox signaling. It is also pivotal for growth promotion, neuroprotection, inflammatory modulation, antiapoptosis, immune function, and atherosclerosis. Decades of studies have indicated that Trx is expressed in all forms of life, executing its function through its antioxidative, protein-reducing, and signal-transducing activities. The biological properties of the Trx system and its implications in several human diseases have been extensively reviewed [48]. The CVD as a dysfunction of heart and blood vessels remains the leading cause of morbidity and death worldwide, accounting for more than 80% of all fatalities occurring in the Western countries [48]. According to the European Cardiovascular Disease Statistics report in 2008, each year, CVD causes over 4.3 million cases of death in Europe and over 2.0 million fatalities in the European Union. The term CVD encompasses an array of diseases such as coronary artery disease, hypertension, congestive heart failure, and stroke, all of which are complications of atherosclerosis that begins early in life and progresses gradually, remaining usually asymptomatic for a long period of time. Macrophages orchestrate the inflammatory response in inflamed tissues, and recent work indicates that these cells can alter their phenotypes and functions accordingly in response to changes in the microenvironment [33,292,293]. Macrophages play a dynamic role in host defense and maintenance of tissue homeostasis. This necessitates a delicate balance between their proinflammatory and immunomodulatory functions to ensure appropriate responses to environmental stimuli [295]. Moreover, pathology is frequently associated with dynamic changes in macrophage activation, with classically activated M1 cells implicated in initiating and sustaining inflammation and M2 or M2-like cells associated with resolution or smoldering chronic inflammation. Several studies have reported the cardioprotective role of

Trx1 [297-301] but the molecular mechanism of Trx1 and Trx80 on the macrophage polarization were not elucidated. Therefore, the present work aimed to explore the role of both Trx1 and Trx80 on the atherosclerosis through investigating their effects on macrophage phenotypes and by which signaling pathway they exert their actions.

Our *in vivo* and *in vitro* published data confirmed that Trx1 antagonized atherosclerosis through downregulation of $p16^{INK4a}$ and suppressing nuclear translocation of AP-1 and Ref-1 which resulted in changing the balance between M1 and M2 macrophages. As a consequence, LPS-induced atherosclerotic plaque became significantly smaller and more stable. However, Trx80 potentiated M1 phenotype activation over M2 leading to a significantly increased aortic lesion area and probably more vunerable. These paradoxical effects of the Trx1 and its truncated form provide new insight into modulation of macrophage function since it is an off-target effect for a number of diverse therapeutic agents.

PPARγ agonists (thiazolidinediones) have long been used in the treatment of diabetes. The evidence linking PPARγ to M2 polarization and hence to the homeostatic role of adipose tissue macrophages (ATMs) sheds fresh new light on their mode of action. Preclinical evidence suggests that PPARγ promotes M2-like polarization and homeostatic metabolic function in ATM and that alteration of this function is a key pathogenic feature in diabetes. Other therapeutic strategies that have been reported to affect macrophage polarization include zoledronic acid (an agent used for preventing recurrence of breast cancer bone metastasis), statins, trabectedin and TLR ligands [292]. Therefore, reorienting and reshaping deranged macrophage polarization is the holy grail of macrophage therapeutic targeting.

To further explore the implicated signaling pathway in macrophage polarization, Akt-mTOR axis became a possible candidat since distinct metabolic programs are required to support energy demands of M1 and M2 macrophages. M1 macrophages rely primarily on glycolytic metabolism, mediated by HIF-1α, while M2 macrophages utilize fatty acid oxidation

mediated by PPARγ and the transcriptional coactivator, PGC-1β [295]. Furthermore, Androulidaki *et al* showed that Akt1(-/-) macrophages exhibited increased responsiveness to LPS in culture and Akt1(-/-) mice did not develop endotoxin tolerance *in vivo* [302]. Based on our obtained results, both Trx1 and Trx80 activated Akt pathway even they activate macrophages differently. This may be due to the differences in functions of Akt isoforms since it has been reported that Akt2 ablation resulted in the M2 polarization of macrophages whereas Akt1 ablation promotes their M1 polarization [247]. Interestingly, Trx80 has activated mTOR in a dose-dependent manner only when Akt1 is inhibited. This may imply the involvement of Akt-mTOR signaling network. To further confirm this result, mTOR has been inhibited by rapamycin and mRNA levels of M1 (IL-6, IL-1 β, and TNF-α) and M2 (IL-10 and CD206) markers has been quantified by qPCR. In consistent with most recently published works by [295,296], inhibition of mTOR activity oriented resting macrophages toward M2 phenotye. Furthermore, mTOR ablation suppressed the responsiveness of macrophages toward LPS.

CONCLUSIONS

Conclusions

Based on the obtained results, the following conclusions can be drawn:

- Trx1 exerts protective effects in cardiovascular diseases through promoting differentiation of macrophages into an alternative, anti- inflammatory phenotype.
- Trx80 potentiated atherosclerosis through activating macrophages toward M1 phenotype over M2 resulting in significantly increased aortic lesion area.
- Trx80 employs mTOR signaling pathway to activate macrophages toward M1 phenotype.
- Inhibition of mTOR activity orients resting macrophages toward M2 phenotype and impairs the polarizing effect of LPS.
- Trx1 may represent a promising therapeutic molecule in the treatment of CVDs keeping the opposite effects of its truncated form, Trx80, in consideration.

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APPENDICES

Appendices

Appendix I:

Mahmood DF, Jguirim-Souissi I, Khadija el H, Blondeau N, Diderot V, Amrani S, Slimane MN, Syrovets T, Simmet T, Rouis M. Peroxisome proliferator-activated receptor gamma induces apoptosis and inhibits autophagy of human monocyte-derived macrophages via induction of cathepsin L: potential role in atherosclerosis. J Biol Chem 2011;286(33):28858-66.

Appendix II:

Mahmood DF, Abderrazak A, El Hadri K, Simmet T, Rouis M. The thioredoxin system as a therapeutic target in human health and disease. Antioxid Redox Signal 2013;19(11):1266-30.

APPENDIX I

Peroxisome proliferator-activated receptor gamma induces apoptosis and inhibits autophagy of human monocyte-derived macrophages via induction of cathepsin L: potential role in atherosclerosis.

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Peroxisome Proliferator-activated Receptor γ Induces Apoptosis and Inhibits Autophagy of Human Monocyte-derived Macrophages via Induction of Cathepsin L

*POTENTIAL ROLE IN ATHEROSCLEROSIS**

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Macrophages play a pivotal role in the pathophysiology of atherosclerosis. These cells express cathepsin L (CatL), a cysteine protease that has been implicated in atherogenesis and the associated arterial remodeling. In addition, macrophages highly express peroxisome proliferator-activated receptor (PPAR) γ, a transcription factor that regulates numerous genes important for lipid and lipoprotein metabolism, for glucose homeostasis, and inflammation. Hence, PPARγ might affect macrophage function in the context of chronic inflammation such as atherogenesis. In the present study, we examined the effect of PPARγ activation on the expression of CatL in human monocyte-derived macrophages (HMDM). Activation of PPARγ by the specific agonist GW929 concentration-dependently increased the levels of CatL mRNA and protein in HMDM. By promoter analysis, we identified a functional PPAR response element-like sequence that positively regulates CatL expression. In addition, we found that PPARγ-induced CatL promotes the degradation of Bcl2 without affecting Bax protein levels. Consistently, degradation of Bcl2 could be prevented by a specific CatL inhibitor, confirming the causative role of CatL. PPARγ-induced CatL was found to decrease autophagy through reduction of beclin 1 and LC3 protein levels. The reduction of these proteins involved in autophagic cell death was antagonized either by the CatL inhibitor or by CatL knockdown. In conclusion, our data show that PPARγ can specifically induce CatL, a proatherogenic protease, in HMDM. In turn, CatL inhibits autophagy and induces apoptosis. Thus, the proatherogenic effect of CatL could be neutralized by apoptosis, a beneficial phenomenon, at least in the early stages of atherosclerosis.

Atherosclerosis is a complex pathophysiological process that involves interaction of a variety of cells within the vessel wall, including smooth muscle cells, endothelial cells, and monocytes/macrophages. The latter cells play pivotal roles in atherogenesis, mainly through accumulation of oxidatively modified LDL and production of a variety of inflammatory mediators, cytokines, and extracellular matrix-degrading enzymes (1). Rupture of atherosclerotic plaques is the most common event triggering formation of occlusive thrombi in arteries and subsequent acute ischemic events, such as acute coronary syndromes and stroke (2).

Several cysteine proteases have been implicated in atherogenesis and the associated arterial remodeling. Cysteine cathepsins are members of the papain family of proteases that degrade elastin and collagen (3). Among them, cathepsin L (CatL)[3] is one of the most potent collagenases and elastases cleaving mature insoluble elastin (3). CatB (4, 5) and CatL (6, 7) expression is enhanced in human coronary lesions, but also in human carotid lesions and abdominal aortic aneurysms (8). CatK, CatS, and CatF are also expressed in human atherosclerotic lesions (9, 10), and CatB, CatD, and CatL were found in macrophage-derived foam cells in lipid-rich plaque areas (11). The pathophysiological role of Cat is underlined by the findings that deficiency of CatS and CatL in LDL receptor knock-out mice reduced atherosclerotic lesions (6, 12). Of note, unstable plaque regions contain increased levels of active legumain, a cysteine protease that promotes intracellular processing of CatL to its mature 25-kDa form (13). The role of cathepsins in apoptosis is now widely recognized in a variety of cell types including macrophages (14, 15).

Peroxisome proliferator-activated receptor (PPAR) γ is a transcription factor that regulates a large number of genes important for lipid and lipoprotein metabolism, for glucose homeostasis, and for inflammation (16). PPARγ regulates transcription of target genes by the formation of heterodimers with

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[3] The abbreviations used are: Cat, cathepsin; PPAR, peroxisome proliferator-activated receptor; HMDM, human monocyte-derived macrophage(s); ODN, phosphorothioate-modified oligonucleotides; PTEN, Phosphatase and tensin homolog; PPRE, PPAR response element; RXR, retinoid X receptor; Trx, thioredoxin.

the retinoid X receptor (RXR) and their subsequent binding to PPAR response elements (PPRE) in the promoter regions of target genes (17). PPARγ can also repress gene expression either in a DNA binding-independent manner by interfering with other signaling pathways or in a DNA binding-dependent way through the recruitment of corepressors (18). Similar to CatL, PPARγ is highly expressed in macrophages of atherosclerotic lesions and macrophage-derived foam cells (19–21), where it may affect macrophage function and consequently atherogenesis.

PPARγ agonists have been shown to inhibit the development of atherosclerosis in apolipoprotein E-deficient ($apoE^{-/-}$) (22) and in LDL receptor-deficient ($LDLR^{-/-}$) mice (23, 24). In addition, mice transplanted with bone morrow from $PPAR\gamma^{-/-}$ chimera mice exhibited a significantly increased atherosclerosis (25, 26) suggesting an antiatherogenic role of PPARγ in macrophages.

PPARγ could inhibit atherosclerosis either by direct inhibition of proinflammatory genes (27) or indirectly through liver X receptor α (28), further, by negatively interfering with AP-1, NFκB, and STAT signaling pathways (29) or by reducing tumor necrosis factor-α, IL-1, and IL-6 secretion (30). In addition, enhanced PPARγ expression by human and murine monocytes inhibits CC chemokine receptor 2 (CCR2) and suppresses MCP-1-mediated chemotaxis (31) Atheroprotective effects of PPARγ were also implied by data showing that PPARγ agonists induce reduction of endothelial adhesion molecules by reduction of monocyte/macrophage recruitment in a mouse model of atherosclerosis (24, 32, 33). Moreover, it has been reported that ligand-mediated activation of PPARγ triggers induction of apoptosis of nonactivated or activated human macrophages (34). Concomitantly, it was observed that PPARγ inhibits the transcriptional activity of the p65/RelA subunit of NFκB, suggesting that PPAR activators induce macrophage apoptosis by negatively interfering with anti-apoptotic NFκB signaling (34). Up-regulation of phosphatase and tensin homolog by PPARγ may be another mechanism by which PPARγ could induce macrophage apoptosis (35). Only recently, we reported a new mechanistic explanation for the PPARγ-mediated induction of macrophage apoptosis. We demonstrated that PPARγ activation stimulates apoptosis in human macrophages by altering the cellular redox balance via regulation of thioredoxin-1 (Trx-1) and its endogenous inhibitor, TXNIP (the thioredoxin interaction protein, also called VDUP-1, for vitamin D_3 up-regulated protein-1) (36).

In the present study, we examined the effect of PPARγ activation on the expression of CatL in human monocyte-derived macrophages. Because several studies indicated that PPARγ activation exerts antiatherogenic effects, we hypothesized that PPARγ activation might inhibit the expression of CatL in human monocyte-derived macrophages. Surprisingly, we found that PPARγ concentration-dependently increases CatL expression.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Human Monocytes—Mononuclear cells were isolated by Ficoll gradient centrifugation from the buffy coats of healthy normolipidemic donors. Cells were cultured in RPMI 1640 supplemented with gentamycin (40 μg/ml), glutamine (0.05%) (Sigma), 10% pooled human serum (Promocell, Heidelberg, Germany) at a density of 6×10^6 cells/well in 60-mm well Primaria-plastic culture dishes (Polylabo, Strasbourg, France). Differentiation of monocytes into human monocyte-derived macrophages (HMDM) was allowed to occur spontaneously by adhesion of cells to the culture dishes and by continued maturation for the subsequent 8–12 days.

RNA Extraction and Analysis—Total cellular RNA was extracted using TRIzol (Invitrogen). For quantitative PCR, reverse transcribed CatL mRNA was quantified by real time PCR on a MX4000 apparatus (Stratagene, La Jolla, CA), using the forward primer (5′-GCATAATCCATTAGGCCACCATT-3′) and the reverse primer (5′-CAGATCTGTGGATTGGAGAGA-3′). PCR amplification was performed in a volume of 25 μl containing 100 nM of each paired primer, 4 mM $MgCl_2$, and the Brilliant quantitative PCR core reagent kit mix as recommended by the manufacturer (Stratagene). The cycling conditions were 95 °C for 10 min, followed by 40 cycles of 30 s each at 95, 55, and 72 °C. Levels of CatL were normalized to the internal control, 36B4 mRNA, using the primer set (forward: 5′-CATGCTCAACATCTCCCCCTTCTCC-3′ and reverse: 5′-GGGAAGGTGTAATCCGTCTCCACAG-3′).

Western Blotting Analyses—HMDM cells were treated with PPARα, γ, or β/δ agonists or with CatL inhibitor (Merck, ref. 219435) and then scraped into Tris buffer containing a mixture of protease inhibitors (Sigma). Protein concentrations were measured by Peterson's method with BSA as standard. The samples were denatured with SDS loading buffer and subjected to SDS-PAGE (Invitrogen SARL, Cergy-Pontoise, France). The samples were transferred to nitrocellulose membranes blocked with nonfat dried milk. The blots were incubated with monoclonal anti-human CatL antibody (Sigma; 1:500), rabbit anti-human beclin 1 antibody (Cell Signaling; 1:1000), rabbit anti-human LC3 antibody (Cell Signaling; 1:1000), rabbit anti-human Bax antibody (Cell Signaling; 1:1000), or rabbit anti-human Bcl2 antibody (Cell Signaling; 1:1000); after washing, the blots were incubated with peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) (1:500 dilution). Human β-actin (Santa Cruz; 1:1000) served as loading control. The blots were visualized with a chemiluminescence kit (Amersham Biosciences).

Immunocytochemistry—HMDM cells were cultured in chamber slides (LabTec). After treatment, HMDM were fixed with 4% paraformaldehyde/PBS, permeabilized in 0.3% Tween 20, and blocked with 5% goat serum/PBS at room temperature. HMDM were then incubated with the following rabbit polyclonal antibodies from Cell Signaling: anti-beclin-1 (1:400), anti-cleaved caspase-3 (Asp175; 1:2000) overnight. After washing, the sections were incubated in anti-IgG Alexa 488-coupled antibodies (1:1000; Molecular Probes, Leiden, Netherlands) in 5% normal goat serum. Hoechst 33342 stain was used to label HMDM nuclei.

To ensure comparable immunostaining, HMDM cultures were processed together under the same conditions. Confocal microscopy was performed with a laser scanning confocal microscope (TCS SP, Leica) equipped with a DMIRBE inverted microscope, using a Plan Apo 63×/1.4 NA oil immersion objec-

tive. Quantification was done on pictures acquired with the same settings (such as the laser power, scanning speed, and photomultiplier gain). The fluorescence intensity was digitally measured for each cell of five randomly selected fields from each culture condition (Image J; National Institutes of Health). Quantification was carried out by investigators blinded to the experimental condition.

Plasmids and Transient Transfection Assays—The human CatL promoter constructs were generated by PCR using *Pfu* polymerase (Stratagene) with human genomic DNA as a template (GenBank AF163338.1). A SmaI site-linked reverse 5′-primer (5′-CGCACCCcgGGATGCCGCTC-3′) was used with NheI site-linked forward 5′-primer (5′-ACCAAAAATgCtAGcACTAAGGAATAG-3′) to amplify the −370/+36 fragment, with NheI site-linked forward 5′-primer (5′-ATAATCCATAGgCTAGcGAGAG-3′) to amplify the −783/+36 fragment or with KpnI site-linked forward 5′-primer (5′-TTCTAAAGGTACcTAATGCTAC-3′) to amplify the −1250/+36 fragment. Inserted nucleotides used to create restriction sites are designated by lowercase characters, and restriction sites are underlined. These fragments were transferred into the promoterless firefly luciferase reporter vector pGL3-Basic (Promega, Mannheim, Germany) in the correct orientation. One firefly luciferase reporter vector in pGL3-Basic containing the human CatL promoter (−1250/+36 bp) with mutated PPRE-like (5′-GCAGGCCAGcCcCCTCCCTCC-3′) was generated using the QuikChange site-directed mutagenesis kit (Stratagene). Point mutations were introduced in the human CatLPPRE-like using *Pfu* DNA polymerase, double-stranded plasmid DNA, and complementary oligonucleotide primers containing the desired mutation. The nucleotide sequences of the constructs were verified by automated sequencing (Applied Biosystems Inc., Courtaboeuf, France). Plasmid DNA was prepared using the Qiagen Maxi Prep kit (Qiagen).

THP-1 cells, grown in 6-well culture dishes in RPMI 1640 supplemented with 10% pooled human serum, were transiently transfected with these luciferase reporter plasmids or with empty vector, using jetPEI-Man transfection reagent (Qbiogene, Illkrich, France). Transfection efficiency, analyzed by flow cytometry with a GFP expression control plasmid, was ~30%. β-Galactosidase expression vector (100 ng of a pCH110-β-Gal; GE Healthcare) was cotransfected as control for transfection efficiency. 20 h post-transfection, the medium was changed (RPMI 1640 with 1% Nutridoma HU) and supplemented with or without PPARγ agonist. After 36 h, the cells were washed with PBS, lysed in 100 μl of passive lysis buffer (Promega), and subjected to luciferase and β-galactosidase assays (37). The data were normalized to β-galactosidase and presented as fold induction compared with luciferase activity of the cells expressing promoterless control vector (basic pGL3).

Knockdown of CatL and CatB—For *in vitro* knockdown of CatL and CatB, phosphorothioate-modified oligonucleotides (ODN) (ThermoHybaid, Ulm, Germany) were used. The ODN sequences complementary to corresponding mRNA sequences devoid of secondary structures, *i.e.* the loops, were selected using available algorithms (38). The antisense ODN for CatL corresponded to the nucleotides 544–565 of human CatL mRNA (NM_001912.4) 5′-AATACAGGGAAGGGAAACACAG6–3′, and for human CatB, they corresponded to nucleotides 1087–1105 (NM_001908.3) 5′-TTCTTTAAAATACTCAGAG-3′. The control sequences contained the same set of the base pairs in a scrambled order. The sequences were analyzed for a lack of secondary structure and oligonucleotide pairing. According to blast search, the selected sequences did not show any similarity to coding mRNA. Macrophages were treated for 72 h every 24 h with 5 μM of the ODN in RPMI 1640 supplemented with 10% FCS (39). The cells were kept in the presence or absence of the PPARγ agonist for 24 h, lysed, and analyzed for protein expression by Western immunoblotting using antibodies against CatL, CatB, beclin 1, LC3, or β-actin.

Electrophoretic Mobility Shift Assays—Human PPARα, PPARγ, PPARβ/δ, and RXRα were synthesized *in vitro* using the TNT quick coupled transcription/translation system (Promega). 20-bp (5′-GTTTTGATCCAGTTTCCAGT-3′) double-stranded oligonucleotides containing the consensus PPRE sequence were used as control PPRE (40). The putative CatL PPRE-like (5′-GCAGCCAGTCTCCTCCCTCC-3′) and its mutated form (5′-GCAG**G**C**CAG**C**C**CCTCCCTCC-3′) were end-labeled with [γ-^{32}P]ATP with T4-polynucleotide kinase. Either protein (2.5 μl) was incubated for 15 min at room temperature in a total volume of 20 μl with 2.5 μg of poly(dI-dC) and 1 μg of herring sperm DNA in binding buffer before the radiolabeled probe was added. Binding reactions were incubated for a further 15 min and then resolved by 4% nondenaturing PAGE. For competition experiments, a 50-fold excess of unlabeled oligonucleotides over the labeled probe were included in the binding reaction.

ChIP Assays—Experiments were performed with a ChIP assay kit (Upstate) according to the manufacturer's procedures. Briefly, 10×10^6 cells were treated with 1% formaldehyde for 10 min at 37 °C. Subsequent procedures were performed on ice in the presence of protease inhibitors. Cells cross-linked by formaldehyde treatment were harvested, washed, and lysed. Chromatin was sonicated with five 10-s pulses at 30% amplitude (sonifier; Branson Ultrasonic Corp). After centrifugation, the supernatant was diluted 10-fold with ChIP dilution. Diluted extracts were precleared in the presence of salmon sperm DNA protein A-agarose. One-tenth of the diluted extract was kept for direct PCR (Input). The remaining extracts were incubated for 16 h at 4 °C in the presence of 1 μg of specific PPARγ antibody (Santa Cruz Biotechnology) per ml, followed by 1 h of incubation with salmon sperm DNA protein A-agarose beads. Following extensive washing, bound DNA fragments were eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$). DNA was recovered in elution buffer containing 200 mM NaCl and then incubated in the presence of proteinase K (20 μg/ml) for 1 h at 45 °C. DNA was extracted in the presence of phenol chloroform and chloroform isoamyl alcohol and ethanol-precipitated before being subjected to PCR using specific oligonucleotide primers that amplify a fragment containing the human CatL PPRE-like site (forward, 5′-ACGCCCGCGCTCTCTGGAG-3′; and reverse, 5′-TTTAGAAGAATCTGGTAAG-3′).

Statistical Analysis—Quantitative results are expressed as the means ± S.D. The results were analyzed by multi-group comparison Mann-Whitney U and Newman-Keul's tests. The differences were considered statistically significant at $p < 0.05$.

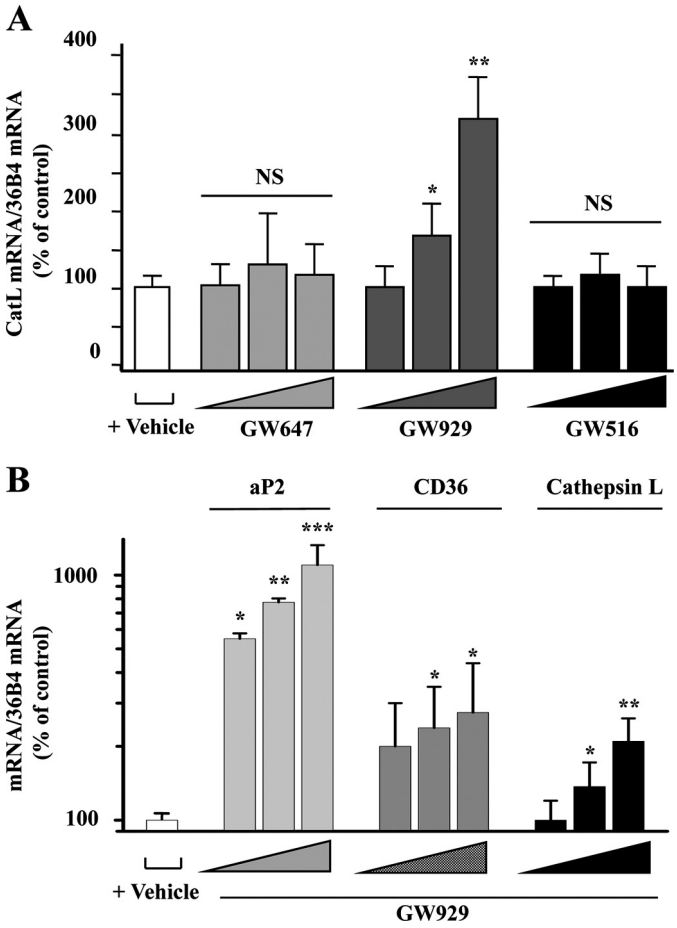

FIGURE 1. **PPARγ activation induces CatL, aP2, and CD36 mRNA expression.** HMDM cells were treated with either vehicle (control), GW647, GW929, or GW516 for 24 h. *A*, effects of PPAR activation on CatL mRNA and 36B4 mRNA (for normalization). *B*, effects of GW929 on human aP2, CD36, and CatL mRNA. Compounds were used at 0.06, 0.6, and 6 μM, and their effects on mRNA expression were analyzed by real time quantitative PCR. The results are the means ± S.D. of four separate experiments; each was performed in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *NS*, not significant, compared with control.

RESULTS

PPARγ Induces Expression of Human CatL in HMDM—To determine whether CatL is regulated by PPARα, PPARγ, or PPARβ/δ, we treated HMDM with 0.06, 0.6, and 6 μM of each agonist. Of the tested agonists, only the selective PPARγ agonist GW929 significantly increased CatL mRNA expression (100 ± 10% at 0.06 μM; 170 ± 50%, $p < 0.05$ at 0.6 μM; and 320 ± 49%, $p < 0.01$ at 6 μM *versus* control cells (100%)) (Fig. 1*A*). In addition, we investigated the effects of the PPARγ agonist GW929 on the expression of the PPARγ target genes CD36 and aP2 and correlated their induction to that of CatL. Indeed, we observed induction of both genes with the agonist GW929, and the expression was aP2 > CD36 > CatL (aP2: 550 ± 28%, $p < 0.05$ at 0.06 μM; 775 ± 28%, $p < 0.01$ at 0.6 μM; and 1,100 ± 225%, $p < 0.001$ at 6 μM *versus* control; and CD36: 200 ± 100% at 0.06 μM; 238 ± 112%, $p < 0.05$ at 0.6 μM and 275 ± 162%, $p < 0.05$ at 6 μM *versus* control) (Fig. 1*B*). In contrast, the PPARα agonist Wy14643 did not induce mRNA. However, similar to GW929, another PPARγ agonist, BRL49653, induced a significant increase in mRNA expression of CatL, aP2, and CD36 (data not shown).

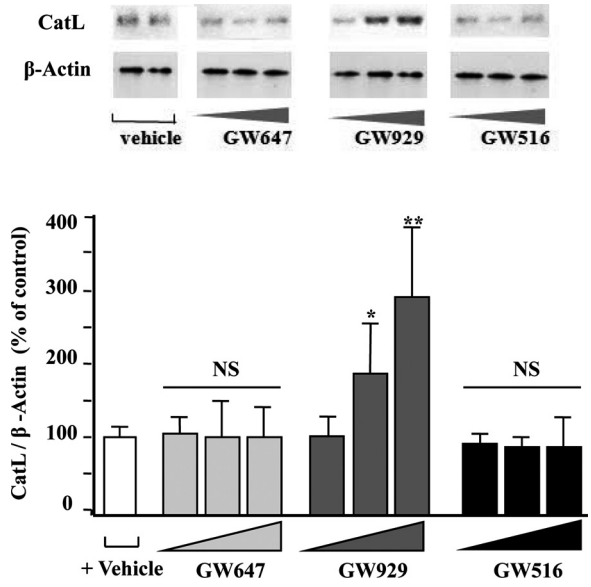

FIGURE 2. **PPARγ activation induces protein expression of CatL.** HMDM cells were treated either with vehicle (control), GW647, GW929, or GW516 (each at 0.06, 0.6, and 6 μM) for 24 h. CatL protein levels were evaluated by three independent Western immunoblots. The results are the means ± S.D.; one representative Western immunoblot was inserted for each agonist. *, $p < 0.05$; **, $p < 0.01$; *NS*, not significant, compared with control.

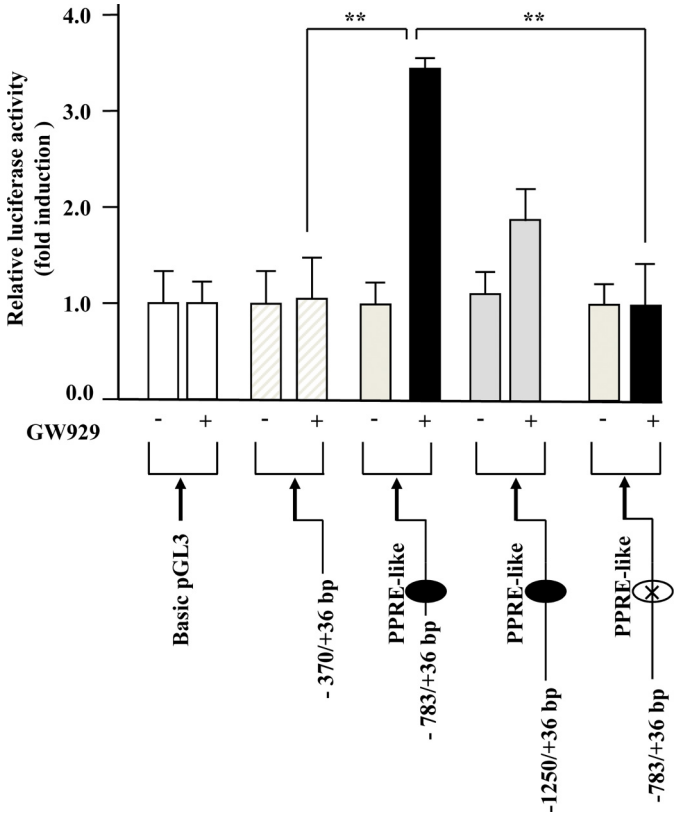

FIGURE 3. **PPARγ activation elicits CatL promoter activity.** HMDM were cotransfected with one of three constructs of the CatL promoter (pGL3−1250/+36, pGL3−783/+36, pGL3−370/+36, or PPRE-like mutated CatL-luciferase expression vectors) together with human pSG5-PPARγ and/or RXRα expression vectors and pCH110, a β-galactosidase vector for 20 h. The cells were then incubated in the presence or absence of GW929 (0.6 μM) for 36 h. The results are the means ± S.D. of three separate experiments, each performed in triplicate. **, $p < 0.01$ compared with nontreated cotransfected cells.

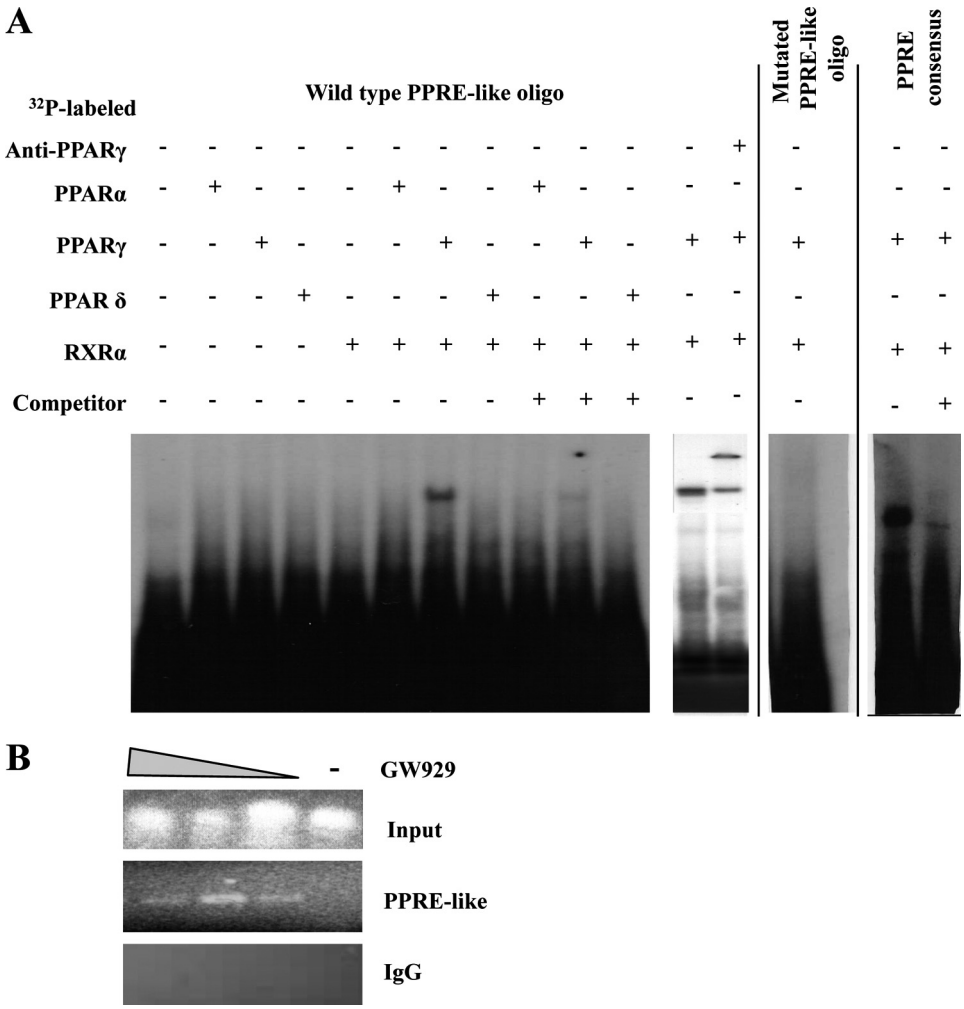

FIGURE 4. **PPARγ and RXRα bind as heterodimer to a PPRE-like site located in the 5′-flanking region of the CatL gene.** *A*, gel retardation assays with *in vitro* translated hPPARγ and hRXRγ were performed with end-labeled oligonucleotides representing the consensus DR1 PPRE, the human putative CatL PPRE-like, and a mutated version of the human putative PPRE-like. The consensus DR1 PPRE was used as unlabeled competitor. Supershift analysis was performed using a specific antibody against human PPARγ. *B*, HMDM were treated with or without GW929 (0.06, 0.6, and 6 μM) for 24 h. DNA-bound proteins were cross-linked to DNA, and after fragmentation, chromatin was precipitated with rabbit polyclonal antibody against human PPARγ or anti-rabbit IgG. As a positive control, input chromatin of the cell lysate was also amplified.

To determine whether the GW929-induced changes in mRNA expressions would also occur at the protein level, we performed immunoblot experiments. These experiments revealed a significant increase in CatL expression (180 ± 68%, $p < 0.05$ at 0.6 μM; 285 ± 1830%, $p < 0.01$ at 6 μM *versus* control cells (100%)) (Fig. 2). In contrast, treatment of HMDM with the specific PPARα GW647 or the PPAR β/δ agonist GW516 did not increase CatL expression (Fig. 2). Therefore, we focused our study on the effects of the PPARγ agonist GW929 on CatL expression.

Effects of PPARγ Activation on the CatL Promoter—Because expression of CatL was increased upon treatment of HMDM with the specific PPARγ agonist (Figs. 1 and 2), we determined whether this effect was mediated by the promoter region proximal upstream of the CatL gene. First, HMDM cells were transiently transfected with a luciferase reporter vector driven by three different human CatL promoter fragments (−1,250/+36; −783/+36 and −370/+36). Cotransfection with PPARγ and RXRα significantly stimulated the −783/+36 CatL promoter activity (3.45 ± 0.6-fold increase *versus* control with $p < 0.01$ in the presence of 0.6 μM GW929) (Fig. 3). Transcriptional activation of the CatL gene by PPARγ indicated the presence of a functional PPRE in the CatL promoter located between −783 and −370. A detailed computer analysis was performed in the region of the CatL promoter defined earlier (41). It revealed the presence of a putative PPRE-like motif, located between −652 and −662, with 91% homology with the PPRE consensus defined for PPARs (40). To prove that this putative PPRE-like sequence could be involved in the transcriptional activation of CatL by PPARγ, we next analyzed the activity of a mutated human CatL promoter fragment (−783/+36) using a luciferase reporter assay. Our results showed that cells cotransfected with the mutant CatL promoter did not respond to GW929 (Fig. 3).

CatL Promoter Contains a PPRE-like Motif That Confers Responsiveness to PPARγ—To demonstrate that the putative CatL-PPRE-like sequence could indeed bind the PPARγ/RXRα heterodimer, we performed an electrophoretic mobility shift assay. First, we verified that the *in vitro* translated RXRα and PPARγ proteins produced by the TNT quick coupled transcription/translation system bind the consensus PPRE by het-

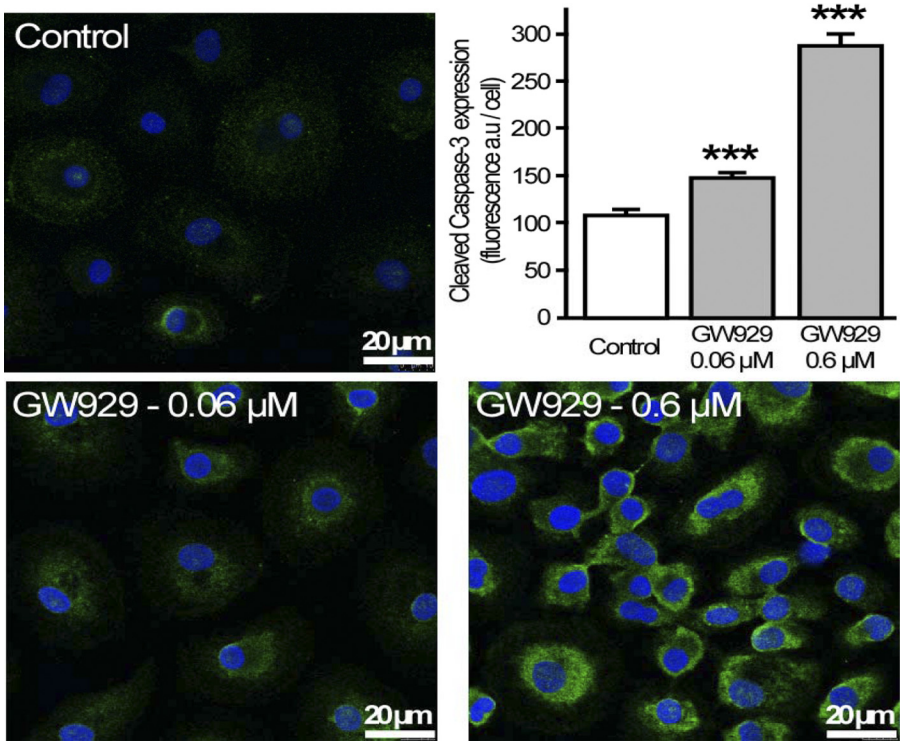

FIGURE 5. **PPARγ activation induces caspase 3 activation.** HMDM cultured in chamber slides for 7 days were treated with the PPARγ agonist GW929 for 24 h. The cells were then washed, fixed, permeabilized, and blocked. HMDM were then incubated overnight with anti-cleaved caspase-3 (Asp175) antibody, followed by anti-IgG Alexa 488-coupled antibodies. Hoechst 33342 staining was used to label HMDM nuclei. Confocal microscopy was performed with a DMIRBE inverted microscope, using a Plan Apo 63×/1.4 NA oil immersion objective. Quantification was done on pictures acquired with the same settings. The fluorescence intensity was digitally measured for each cell of five randomly selected fields from each culture condition and presented as the means ± S.D. **, $p < 0.01$; ***, $p < 0.001$, compared with control cells.

erodimerization. Second, in agreement with our previous data (36), incubation of the labeled PPRE-like sequence from the CatL promoter with *in vitro* translated PPARγ and RXRα showed formation of a retarded complex (Fig. 4*A*). This specific complex was not observed with the mutated PPRE-like oligonucleotide and disappeared when it was incubated with a 50-molar excess of unlabeled CatL-PPRE oligonucleotide as a specific competitor (Fig. 4*A*). Moreover, incubation of labeled PPRE-like oligonucleotide from CatL promoter with *in vitro* translated PPARγ and RXRα as well as specific antibody against PPARγ resulted in a supershifted band (Fig. 4*A*). Finally, the result of the ChIP experiment showed an increased fixation of the complex on the PPRE-like site upon activation of PPARγ with GW929 (Fig. 4*B*). Taken together, these results indicate that the PPARγ agonist GW929 induced CatL expression in HMDM, at least in part, through binding of PPARγ to the PPRE-like motif located between −783 and −1250 of the human CatL promoter.

CatL Increases Apoptosis in HMDM—Next we analyzed the effects of PPARγ activation on the induction of autophagy and apoptosis in HMDM. By quantitative immunocytochemistry, we found that the PPARγ agonist GW929 decreased the expression of beclin 1 and LC3, two proteins important for the formation of autophagosomes during autophagy (42). At the same time, treatment with GW929 concentration-dependently increased cleavage of caspase 3, indicating induction of apoptosis in HMDM (Fig. 5).

Next, we examined the role of GW929-induced CatL expression in macrophage apoptosis. HMDM were treated with 0, 0.6, and 6 nM of specific CatL inhibitor in the presence of the PPARγ agonist GW929 (0.6 μM), and the protein levels of Bax and Bcl-2 were determined using Western immunoblots. The results showed no significant changes in the protein levels of Bax after normalization with β-actin (Fig. 6). In contrast, a concentration-dependent increase of Bcl-2 levels was observed (Fig. 6). As a consequence, the Bax/Bcl-2 ratio was decreased (76 ± 13%, $p < 0.05$ and 51 ± 12%, $p < 0.05$ by 0.6 and 6 nM of the CatL inhibitor, respectively) (Fig. 6). A decreased ratio of Bax/Bcl-2 is consistent with a decreased susceptibility to apoptosis, indicating that CatL is involved in the PPARγ-mediated induction of macrophage apoptosis.

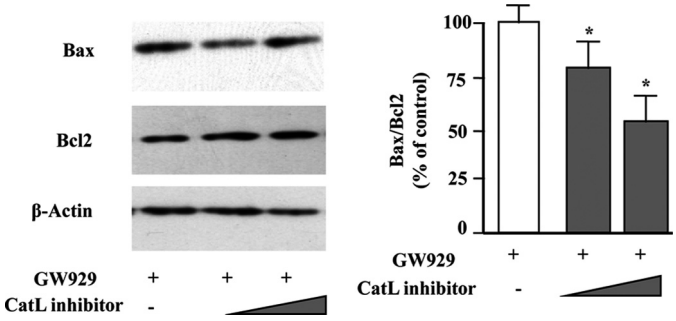

FIGURE 6. **CatL inhibitor reduces the Bax/Bcl2 ratio in PPARγ-activated macrophages.** HMDM were treated with PPARγ agonist GW929 (0.6 μM) for 24 h, followed by treatment with CatL inhibitor at 0.6 or 6 nM for additional 24 h. The cells were washed, lysed, and analyzed by Western immunoblotting. Bax, Bcl2, and β-actin protein levels were analyzed, and the results are presented as the means ± S.D. of four independent Western immunoblots. One representative Western blot for each protein was inserted. *, $p < 0.05$ compared with control.

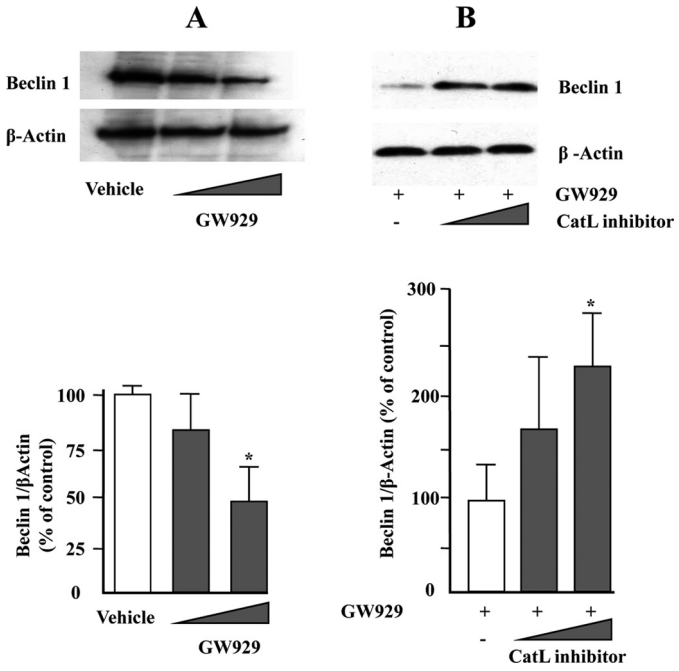

FIGURE 7. **PPARγ-induced decrease in beclin 1 levels is reversed by CatL inhibitor.** *A*, HMDM were treated with the PPARγ agonist GW929 (0.06 and 0.6 μM) for 24 h. The cells were washed and lysed, and the protein levels of beclin 1 and β-actin (for normalization) were analyzed by Western immunoblots. The results are the means ± S.D. of four independent experiments. One representative Western blot for each protein was inserted. *, $p < 0.05$ compared with control. *B*, HMDM were treated with the PPARγ agonist GW929 (0.6 μM) for 24 h. The cells were incubated for an additional 24 h in the presence or absence of the CatL inhibitor at 0.6 or 6 nM. The cells were washed and lysed, and beclin 1 and β-actin (for normalization) protein levels were analyzed. The results are presented as the means ± S.D. of four independent Western immunoblots. One representative Western blot for each protein was inserted. *, $p < 0.05$ compared with control.

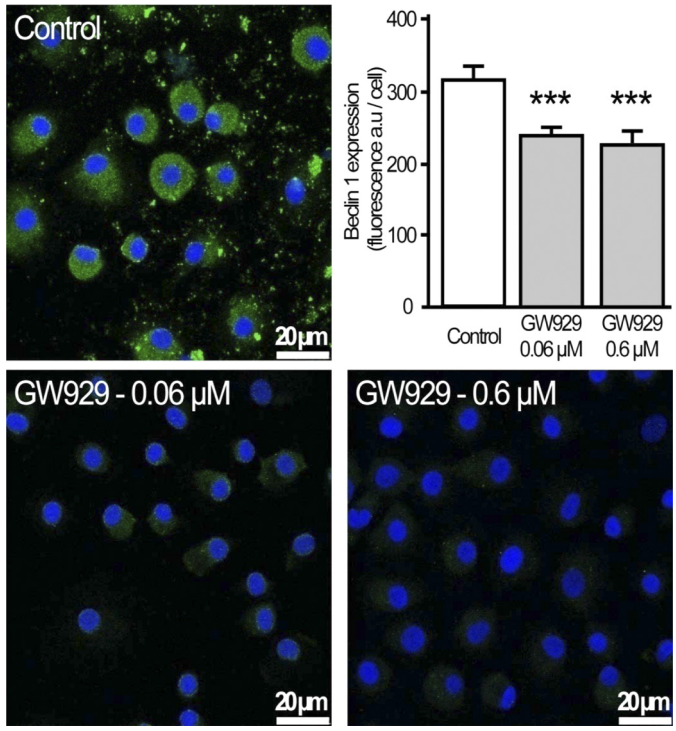

FIGURE 8. **PPARγ activation reduces beclin 1 expression in HMDM.** HMDM, cultured in chamber slides for 7 days, were rinsed and treated with the PPARγ agonist GW929 for 24 h. HMDM were incubated overnight with anti-beclin-1 antibody followed by anti-IgG Alexa 488-coupled antibodies. Hoechst 33342 staining was used to label HMDM nuclei, and all of the chamber slides were rinsed and mounted with Fluoprep mounting medium. Confocal microscopy was performed with a DMIRBE inverted microscope using a Plan Apo 63×/1.4 NA oil immersion objective. Quantification was done on pictures acquired with the same settings. The fluorescence intensity was digitally measured for each cell of five randomly selected fields from each culture condition and presented as the means ± S.D. ***, $p < 0.001$ compared with control cells.

Cathepsin L Decreases Autophagy in HMDM—Because macrophages play a central role in atherosclerotic plaque destabilization, selective induction of macrophage death is increasingly gaining attention in cardiovascular medicine because it could stabilize vulnerable, rupture-prone lesions (43). Compared with apoptosis or necrosis, autophagy might be a favorable type of cell death leading to elimination of macrophages in atherosclerotic plaques, because autophagic macrophages literally digest themselves to death (43). As a consequence of autophagy, the cytoplasmic content progressively decreases so that activation of inflammatory responses, the release of matrix degrading proteases, and the deposition of necrotic debris after postautophagic necrosis are minimal. Therefore, we examined whether autophagy is involved in mechanisms of cell death induced by PPARγ treatment. To do so, we examined the protein levels of beclin 1 (Bcl2-interacting protein) and microtubule-associated protein 1 LC3 (light chain 3), which had previously been shown to promote autophagy (42). Our results indicate that PPARγ decreases the protein expression of beclin 1 (87 ± 13% at 0.06 μM; 49 ± 21% at 0.6 μM, $p < 0.05$) (Figs. 7*A* and 8). In addition, in the presence of the CatL inhibitor, we observed a concentration-dependent increase of the protein levels of beclin 1 (177 ± 63 and 226 ± 50%, $p < 0.05$ by 0.6 and 6 nM of the CatL inhibitor, respectively) (Fig. 7*B*). Of note, we did not observe any changes in the mRNA levels of beclin 1 following either PPARγ or CatL inhibitor treatment (not shown).

Similar experiments were conducted to evaluate the level of LC3 protein. Fig. 9*A* shows a concentration-dependent inhibitory effect of PPARγ on LC3 expression (48 ± 15%, $p < 0.01$ and 39 ± 25% $p < 0.01$ by 0.06 and 0.6 μM GW929, respectively). Moreover, treatment with the CatL inhibitor concentration-dependently prevented the degradation of LC3 (162 ± 14%, $p < 0.05$ and 220 ± 25%, $p < 0.01$ by 0.6 and 6 nM of the CatL inhibitor, respectively) (Fig. 9*B*). Finally, knockdown of CatL in PPARγ agonist-treated HMDM (Fig. 10*A*) protects beclin 1 and LC3 from degradation (Fig. 10*B*). In contrast, knockdown of CatB in identically treated cells did not affect the expression of both proteins (Fig. 10*B*), confirming the specific effect of CatL on beclin 1 and LC3 degradation.

DISCUSSION

Our study shows that PPARγ activation increases the levels of CatL mRNA and protein in HMDM. Using promoter analysis, we identified a functional PPRE-like sequence in the CatL promoter region that confers increased CatL regulation by PPARγ. In addition, we show that PPARγ-induced CatL decreases the levels of Bcl2 and increases those of BAX, indicating involvement of CatL in the PPARγ-mediated induction of macrophage apoptosis. We also found that PPARγ-induced CatL decreases autophagy through the reduction of beclin 1 and LC3. The reduction of proteins involved in autophagic cell

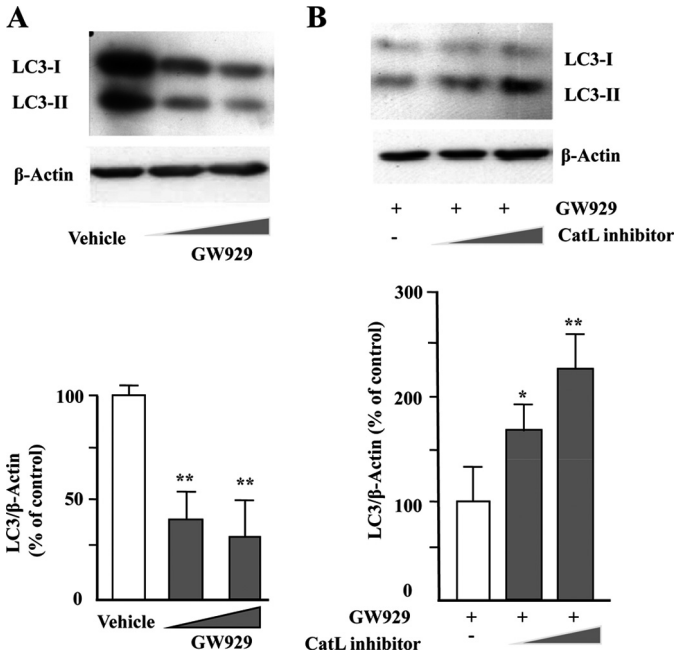

FIGURE 9. **PPARγ activation induces a CatL-dependent decrease of autophagy markers.** *A*, HMDM were treated with the PPARγ agonist GW929 (0.06 and 0.6 μM) for 24 h. The cells were analyzed for LC3 and β-actin (for normalization) expression using Western immunoblotting. The results are presented as the means ± S.D. of four independent Western blots. One representative Western blot for each protein was inserted. **, $p < 0.01$ compared with control. *B*, HMDM were treated with the PPARγ agonist GW929 (0.6 μM) for 24 h followed by incubation in the presence or absence of CatL inhibitor at 0.6 or 6 nM for additional 24 h. The cells were washed and lysed, and LC3 and β-actin (for normalization) protein levels were evaluated. The results are the means ± S.D. of four independent Western immunoblots. One representative Western blot for each protein was inserted. *, $p < 0.05$; **, $p < 0.01$ compared with control.

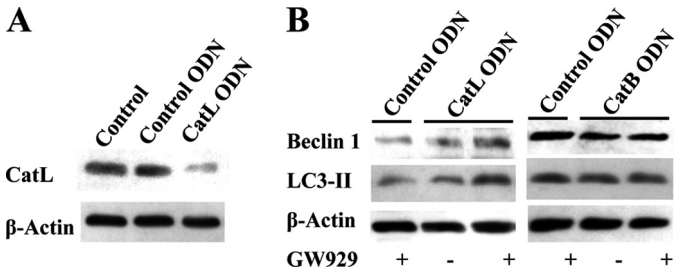

FIGURE 10. ***In vitro* knockdown of CatL reverses the PPARγ-mediated decrease of autophagy markers.** *A*, down-regulation of CatL by phosphothioate ODN. HMDM were treated with 5 μM ODN complementary to the corresponding mRNA sequence devoid of secondary structures for 72 h. Down-regulation of CatL was analyzed by Western blotting. A representative blot is shown. The control ODN contained the same set of the bases in a scrambled order. *B*, HDMD were treated for 72 h with 5 μM of either ODN followed by treatment with the PPARγ agonist GW929 (0.6 μM) for additional 24 h. Expression of CatL, beclin 1, LC3, or β-actin was analyzed by Western immunoblotting. Representative blots are shown.

death was antagonized by a CatL inhibitor following CatL knockdown in PPARγ agonist-treated HMDM. To the best of our knowledge, this is the first study showing that PPARγ inhibits autophagy and stimulates apoptosis through induction of CatL in human macrophages. Previous studies have shown that PPARγ can induce apoptosis through other pathways. Thus, Trx-1, a 12-kDa protein, which plays a major role in the regulation of the cellular redox balance, can interact with ASK-1 and inhibits apoptosis (44). In addition, we have previously shown that the PPARγ agonist GW929 concentration-dependently increased HMDM expression of VDUP-1, a specific endogenous inhibitor for Trx-1, suggesting that PPARγ activation stimulates apoptosis in human macrophages by altering the cellular redox balance via up-regulation of VDUP-1 (36). Trx-1 may also antagonize apoptosis by binding to PTEN and the subsequent inhibition of its phosphatase activity (45). The reduced expression/activity of Trx-1 in cells treated with PPARγ agonists would increase PTEN activity and contribute to decreased cell survival by inhibition of Akt signaling. Furthermore, it has been demonstrated that activation of PPARγ by its selective ligand rosiglitazone directly up-regulates PTEN expression in several cell types including macrophages and reduces the rate of macrophage proliferation (35). Taken together, PPARγ may increase macrophage apoptosis in several ways, such as direct up-regulation of PTEN gene expression, through reduction of Trx-1 activity or, as we have demonstrated in the present study, by increasing the CatL expression, or by a combination of these mechanisms.

It is important to note that death of lesional macrophages by apoptosis was observed throughout all stages of atherosclerosis. However, the consequences of this event were considered to be very different in early *versus* late atherosclerotic lesions (46). One explanation based on *in vivo* investigations is that phagocytic clearance of apoptotic macrophages in early atherosclerotic lesions is highly efficient (47–50), whereas phagocytic clearance in advanced lesions is less effective because of the high number of apoptotic macrophages in advanced atherosclerotic lesions (47, 51–53).

Recently, Liu *et al.* (54) reported that macrophage apoptosis suppresses the development of atherosclerosis in LDLR$^{-/-}$ mice. On this background, our results would imply that the PPARγ-mediated increase in CatL expression in macrophages may represent a new approach for the inhibition of atherosclerosis progression. However, CatL was reported to induce rather than to reduce the progression of atherosclerosis (6, 12).

On the other hand, PPARγ activation was found to exert both atherogenic and anti-atherogenic effects. In general, the beneficial effects of PPARγ activation are considered to be greater than its atherogenic effects (55). We therefore hypothesized that the induction of CatL by PPARγ activation could eliminate macrophages by apoptosis or/and autophagy and thus counteract atherogenesis. Autophagy is a catabolic pathway for bulk turnover of long-lived proteins and organelles via lysosomal degradation. A growing body of evidence suggests that autophagy is playing a role in many diseases by promoting or preventing their progression (56). In atherosclerotic plaques, basal autophagy is a survival mechanism safeguarding plaque cells against oxidative injury, metabolic stress, and inflammation, by removing harmful oxidatively modified proteins and damaged components. Hence, autophagy is anti-apoptotic and contributes to cellular recovery. Basal autophagy can be intensified by appropriate drugs. Hence, pharmacological approaches have been developed to stabilize rupture-prone plaques through selective induction of macrophage autophagic death, without affecting the plaque stabilizing smooth muscle cells (for review see Ref. 56).

Because macrophages play a central role in atherosclerotic plaque destabilization, selective induction of macrophage death aiming at stabilization of vulnerable, rupture-prone lesions is gaining increased attention (43). Compared with apoptosis or necrosis, autophagy seems to be a favorable type of cell death to eliminate atherogenic macrophages within atherosclerotic plaques. As a consequence, the release of matrix degrading proteases, the deposition of necrotic debris, and the activation of inflammatory responses would be minimal.

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APPENDIX II

The thioredoxin system as a therapeutic target in human health and disease

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COMPREHENSIVE INVITED REVIEW

The Thioredoxin System as a Therapeutic Target in Human Health and Disease

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Thomas Simmet,[2] and Mustapha Rouis[1]

Abstract

The thioredoxin (Trx) system comprises Trx, truncated Trx (Trx-80), Trx reductase, and NADPH, besides a natural Trx inhibitor, the thioredoxin-interacting protein (TXNIP). This system is essential for maintaining the balance of the cellular redox status, and it is involved in the regulation of redox signaling. It is also pivotal for growth promotion, neuroprotection, inflammatory modulation, antiapoptosis, immune function, and atherosclerosis. As an ubiquitous and multifunctional protein, Trx is expressed in all forms of life, executing its function through its antioxidative, protein-reducing, and signal-transducing activities. In this review, the biological properties of the Trx system are highlighted, and its implications in several human diseases are discussed, including cardiovascular diseases, heart failure, stroke, inflammation, metabolic syndrome, neurodegenerative diseases, arthritis, and cancer. The last chapter addresses the emerging therapeutic approaches targeting the Trx system in human diseases. *Antioxid. Redox Signal.* 00, 000–000.

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I. Oxidative Stress—the Underlying Cause of Many Diseases

Free radicals, characterized by an unpaired electron, are highly reactive molecules generated predominantly during cellular respiration, metabolism, and phagocytosis (Fig. 1). In addition, low levels of free radicals produced in response to growth factors and cytokines are key components of cellular metabolism (251, 254). The mitochondrial respiratory chain is the major intracellular source of reactive oxygen species (ROS), oxygen-containing chemically reactive molecules, and free radicals under normal physiologic and pathologic conditions (225). ROS and reactive nitrogen species (RNS) are both physiologically necessary and potentially destructive. Moderate levels of ROS play specific roles in the modulation of several cellular events, including signal transduction, proliferation, and gene expression (279). High ROS/RNS levels can cause damage to key cellular components such as lipids, proteins, and nucleic acids, possibly leading to subsequent cell death by necrosis or apoptosis (261) (Fig. 2). Oxidation of any of these substrates, if uncontrolled, can theoretically contribute to disease development. Indeed, an increasing body of evidence suggests that the production of ROS/RNS and, subsequently, oxidative/nitrosative stress and damage are linked to either the primary or secondary pathophysiological mechanisms of multiple disorders, including cancer, diabetes, atherosclerosis, coronary heart disease (CHD), and cataract. Interestingly, these diseases are degenerative processes involved in the rate of aging and in age-related diseases (216). An imbalance favoring the cellular production of free radicals in amounts exceeding the cellular defense capacities is referred to as oxidative stress (22, 92, 252). Therefore, cells have evolved several strategies (enzymatic and nonenzymatic) to overcome free radical-induced oxidative stress, including preventive and repairing mechanisms, physical barriers, and antioxidant defenses. Nonenzymatic antioxidants are ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants. Enzymatic antioxidant defenses include superoxide dismutase, GSH peroxidase, catalase, glutaredoxin, thioredoxin reductase (TrxR), and thioredoxin (Trx) (432).

II. The Trx System

In 1964, Trx1 was first isolated from *Escherichia coli* by Laurent *et al.*, reporting that *E. coli* also contained an enzyme, TrxR, which catalyzes its reduction (223). Through its redox-active cysteine residues, the dithiol Trx plays a vital role in

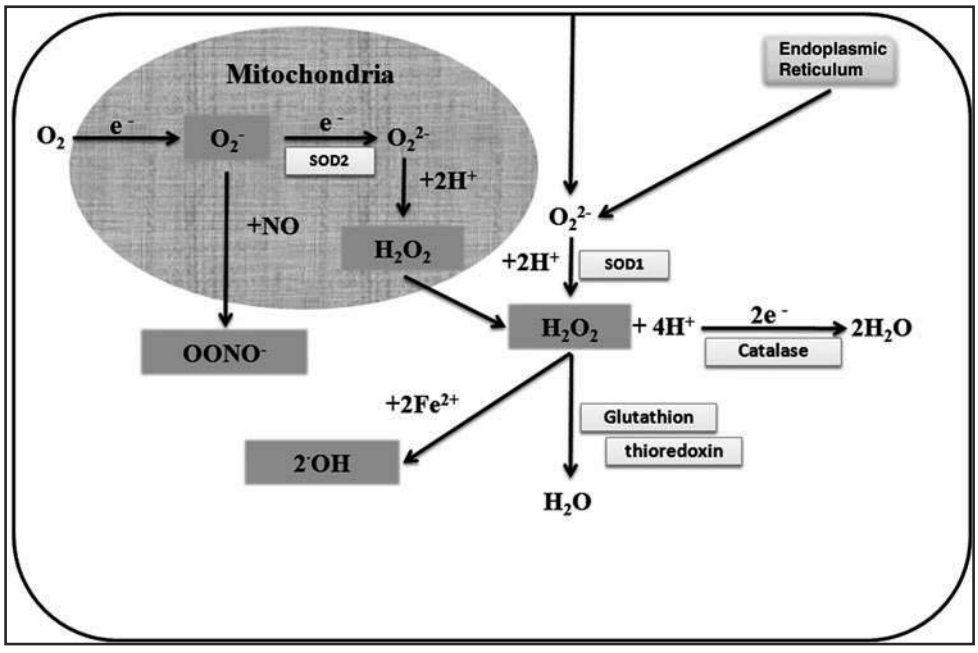

FIG. 1. Generation of reactive oxygen species (ROS). The mitochondria, the endoplasmic reticulum, and the NADPH oxidase system are the major generating sites of ROS, oxygen-containing chemically reactive molecules, through cellular respiration, metabolism, and phagocytosis. Superoxide generated by mitochondria is converted by superoxide dismutase 2 (SOD2) to hydrogen peroxide (H_2O_2). The later one can be converted either to H_2O by catalase or to hydroxyl radicals (·OH) in the presence Fe^{2+}. Further, H_2O_2 can also be reduced by antioxidants such as glutathione and thioredoxin (Trx) [adapted from (420)].

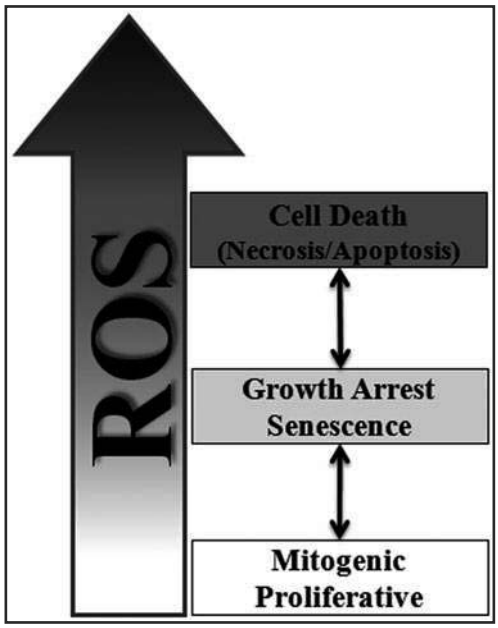

FIG. 2. Cellular effects of ROS. Different levels of ROS, which change the cellular redox status, may result in different cellular responses; low levels of ROS, particularly of H_2O_2, are mitogenic and promote cell proliferation, whereas intermediate levels result in either temporary or permanent growth arrest, such as replicative senescence. Very severe oxidative stress causes ultimately cell death *via* either apoptotic or necrotic mechanisms [adapted from (261)].

maintaining the cellular environment in a reduced state. The dithiol moieties of Trx are reduced by receiving electrons from NADPH in the presence of TrxR. Reduced Trx in turn reduces proteins with disulfide bonds by transferring electrons from its reactive cysteines through thiol–disulfide exchange reactions. Thus, NADPH, TrxR, Trx, and thioredoxin-interacting protein (TXNIP), the endogenous Trx inhibitor, are collectively termed the Trx system (Fig. 3) (17, 117, 269, 312). There are three distinct forms of human Trx, encoded by separate genes: cytosolic Trx (Trx1), mitochondrial Trx (Trx2), and a Trx variant that is highly expressed in spermatozoa (SpTrx/Trx3) (138, 272, 399).

Depending on its subcellular localization, Trx exerts different roles (Table 1). It can be found in the extracellular environment, the cytoplasm, and the nucleus (15). In the extracellular environment, Trx exhibits a chemokine-like activity (312), whereas in the cytoplasm, it regulates the cellular redox environment and also the activity of certain proteins. In the nucleus, Trx1 has been shown to interact with many transcription factors such as redox factor-1 (Ref-1), hypoxia-inducible factor-1 α (HIF-1α), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p53, activator protein-1 (AP-1), Nrf-2, glucocorticoid receptor, estrogen receptor (ER), and others (116, 130, 131, 162, 163, 370), thereby regulating gene expression. Intracellular Trx1 localizes mainly in the cytoplasm. However, several factors induce nuclear translocation of Trx1, despite the lack of a nuclear localization signal (NLS). This nuclear translocation, through karyopherin-α (372), suggests that Trx1 may be associated with signaling molecules that bridge the cytoplasmic and nuclear compartment (272). By contrast, the translocation of Trx1 to the membrane requires binding of TXNIP (453).

Trx-2 is a mitochondrial redox protein containing the Trx1 active-site Trp-Cys-Gly-Pro-Cys. It is encoded by a nuclear gene, *TRX2*, with a mitochondrial targeting signal peptide. Initially, Trx2 has been cloned from a rat heart library by Spyrou *et al.*, who reported that this gene encodes a 18-kDa protein (399). Later, Trx2 also has been cloned from human osteosarcoma cells (81) and human embryonal stem cells (67). The *TRX2* gene is expressed in virtually all organs and tissues with the highest expression level in the brain (200, 360). Trx-2 plays important roles during embryonic development, since $TRX2^{-/+}$ heterozygous mice appeared normal, even though they displayed decreased levels of Trx2, whereas early embryonic lethality was seen in the $TRX2^{-/-}$ homozygous embryos (311). Furthermore, overexpression of the Trx2 can facilitate the development of resistance to ROS-induced apoptosis (67), as well as resistance of human embryo kidney cells to etoposide (81). The Trx2 system appears to have a more important role in preventing mitochondrial dysfunction than the mitochondrial GSH system in endothelial cells under conditions that mimic a septic insult (246), because inhibition of Trx2 resulted in a higher mitochondrial metabolic activity, lower ATP/ADP ratios, lower oxygen consumption, increased lactate formation, and evidence of caspase 3 and 7 activation (246). Like Trx1, Trx2 is important for cell viability and prevents apoptosis-induced cell death, since its knockdown in endothelial cell increases tumor necrosis factor (TNF)/apoptosis signal-regulated kinase 1 (ASK-1)-induced cytochrome c release and cell death (477). Moreover, Trx-2

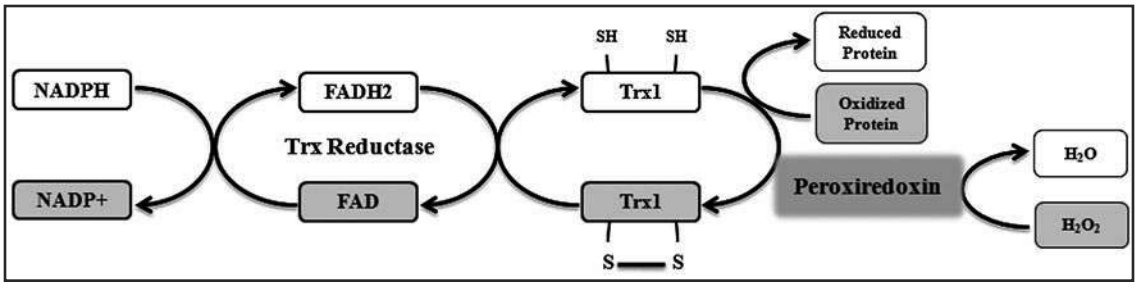

FIG. 3. The redox-cycling reactions of the Trx system. The dithiol moieties of Trx are reduced by receiving electrons from NADPH in the presence of thioredoxin reductase (TrxR). Reduced Trx1 in turn reduces oxidized proteins with disulfide bonds through thiol–disulfide exchange reactions. Thus, NADPH, TrxR, and Trx are collectively termed the Trx system. Further, Trx can scavenge free radicals indirectly through peroxiredoxin.

Table 1. Cellular Occurrence and the Subcellular Distribution of Thioredoxin

Subcellular distribution	*Roles*	*Targets*	*Cells*	*References*
Cytoplasm	Antioxidant	Oxidized proteins, DNA, lipids	All cells	(9, 17, 33, 175, 243, 297, 409, 427)
Cytoplasm	Antiapoptotic	ASK-1	Mv1Lu, L929, and 293 cells	(362)
Cytoplasm	Signaling transduction	Akt and PTEN regulation	Neuroblastic neoplasms and neuroblastoma cell lines	(366)
Cytoplasm	Anti-inflammatory	Downregulation of MCP-1	Trx-1-overexpressing/knockdown EA.hy 926 and bovine aortic ECs	(61)
Cytoplasm	Immunomodulation	Regulation of Tregs	Tregs	(288)
Translocation to nucleus	Gene regulation	Histone deacetylase 4	Cardiac myocyte	(4)
Translocation to nucleus	Gene regulation	Hypoxia inducible factor-1α	HeLa cells	(99)
Translocation to nucleus		Glucocorticoid receptor	COS7, CV-1, and HeLa cells	(256)
Nucleus	Gene regulation	NF-κB, AP-1, and Ref-1	L929 and HeLa cells	(61, 370)
Cell surface	Immunomodulation	Inhibition of complement deposition	HUVEC	(213)
Extracellular	Chemokine	Immunomodulation chemoattractant	*In vitro*: human monocytes, PMNs, and T-cell *In vivo*: Air pouch in mice	(33, 312)

AP-1, activator protein-1; ASK-1, apoptosis signal-regulated kinase 1; HUVEC, human umbilical vein endothelial cell; MCP-1, monocyte chemoattractant protein-1; PTEN, phosphatase and tensin homology; Ref-1, redox factor-1; Tregs, regulatory T-cells; Trx, thioredoxin.

improves the endothelial cell function and reduces atherosclerotic lesions in the apolipoprotein E-deficient mouse model *via* reducing oxidative stress and increasing NO bioavailability (474).

Among other isoforms of Trx, spermatid-specific thioredoxin-3 (SpTrx3) has been characterized, which is exclusively expressed in the testis and localized to the Golgi apparatus of mammalian spermatocytes and spermatids. Further, SpTrx3 is more abundant in defective spermatozoa from infertile men. In addition, it constitutes a novel sperm postobstruction autoantigen (188) [reviewed by (283)].

In this review, we will focus mainly on the Trx1 isoform, and the other Trx system members are discussed when required. In the following sections, we use the abbreviation Trx referring mainly to all Trx isoforms and when the role is restricted to one isoform and not others, as it will be indicated.

A. *The* Trx-1 *gene*

The gene encoding human Trx1 is located on chromosome 9 at bands 9q31. Its coding region spans over 13 kb and is organized in five exons separated by four introns. The *Trx1* promoter contains two transcription start sites (Fig. 4). Initial studies involving the *Trx1* promoter region identified the first transcription start site (denoted as TSS1) at -110 bp (with respect to the ATG start codon) by mapping the 5′-end of the cDNA clone to a genomic sequence (418). Three specificity protein 1 (Sp1)-binding sites were also located between -244 and -183 bp (418) and have been found to enhance transcription of the *Trx1* gene (39). In contrast, a second transcription start site has been previously mapped (using a primer extension analysis) to -74 bp with a TATA box (TATAAA) located at -102 bp (195). The TATA box is located downstream of TSS1 and is therefore only relevant for transcription initiation at TSS2. Luciferase assays, utilizing various wild-type and mutated *Trx1* promoter fragments, revealed the roles for the oxidative stress-responsive element (ORE), antioxidant-response element (ARE), three Sp1-binding sites, and the TATA box in the activation of the *Trx1* gene (31, 292, 319, 445).

B. *Structure of Trx-1*

All Trx proteins in different organisms share a common structure, consisting of four α-helices and five β-sheets constituting a protein of 12 kDa (123, 165). They have a highly conserved active-site sequence, Cys^{32}-Gly-Pro-Cys^{35}(CXXC) (32), which links the second β-strand to the second α-helix and forms the first turn of the second helix, making it a compact globular protein with a high temperature stability (6, 146, 339, 461). In addition to the two cysteine residues, human Trx1, but not bacterial Trxs, contain three other critical structural cysteines residues at positions -62, -69, and -73, providing Trx1 with unique biological properties (Fig. 5) (143, 219). These cysteine residues of Trx1 can undergo post-translational modifications such as thiol oxidation, glutathionylation, and *S*-nitrosylation regulating its activity (see below). Both Cys62 and Cys69 are the sites of *S*-nitrosylation (144, 408, 447), whereas Cys73 is a multimodification site, undergoing nitrosylation (284, 443, 454), glutathionylation (50), dimerization (447), or 4-hydroxy-2-nonenal modification (130).

Because the first N-terminal methionine is mostly removed after translation by an N-terminal methionine excision process, Trx1 found in human tissues consists mainly of 104 amino acids starting from the second N-terminal valine (445).

C. *Truncated Trx*

To date, only limited research has been performed on the biological roles of the truncated form of Trx (Trx-80). Previously, it was termed eosinophil cytotoxicity-enhancing factor due to its eosinophil cytotoxicity, and it has been first

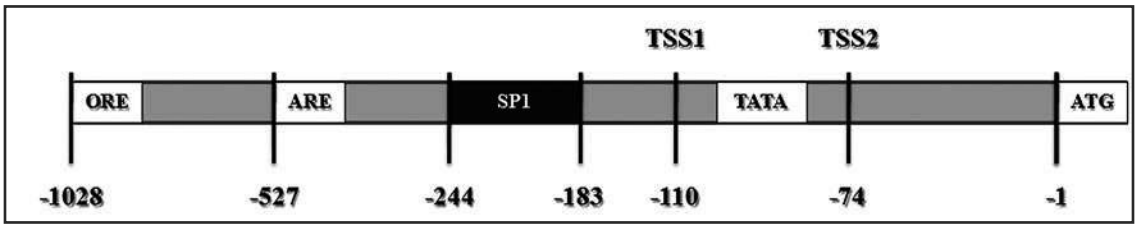

FIG. 4. Map of the human *Trx* promoter region. The first transcription start site (TSS1) is located at −110 bp, while three specificity protein 1 (Sp1)-binding sites are also located between −244 and −183 bp. The TSS2 is just at −74 bp with a TATA box (TATAAA) located at −102 bp. ORE, oxidative stress responsive element; ARE, antioxidant response element.

detected in the plasma of patients suffering from severe schistosomiasis (88, 231, 388). Trx-80 (10 kDa) is a natural cleavage product of Trx1, sharing the 80 or 84 N-terminal amino acids with Trx1 (Fig. 5) (326, 329). It has been suggested that the enzyme responsible for its cleavage would be an inducible protease (229). Very recent findings indicated that the disintegrins and metalloproteinases (ADAM10 and 17), two α-secretases processing the amyloid β precursor protein, are responsible for Trx-80 generation in the brain (128). Further work will have to establish other possible candidates in different tissues and under different pathophysiological conditions.

Macrophages are capable of cleaving full-length Trx1 to yield Trx-80, which in contrast to the cytosolic localization of Trx1 is present mainly at the surface of monocytes (327). Trx-80 is expressed also by U937 monocytes, cytotrophoblasts, and $CD4^+$ T-cells (90, 388). Recently, human brain samples and human primary cultures were shown to produce Trx-80 and polymerize it into very stable aggregates migrating at ~30 kDa in SDS-PAGE (128).

While the levels of Trx-80 ranged from 2 to 175 ng/ml, those of Trx1 ranged from 16 to 55 ng/ml without any correlation to Trx-80 concentrations, which increased significantly under inflammatory conditions (79, 328). Trx-80 activates monocytes and induces the upregulation of the cell surface pathogen recognition receptors, molecules essential for T-cell activation and function (229) as well as the release of the proinflammatory cytokines evoking inflammation (33). Induction of human monocytes is associated with phenotypic cell changes (326), which differentiate into a novel line type named by Cortes-Bratti *et al*. Trx-80 activated monocytes with an intrinsic function, contributing to the first line of defense against a foreign intruder and triggering innate immunity (79). Thus, Trx-80-activated monocytes could be more effective against bacteria and parasites *via* CD14 receptor upregulation and other pathogen recognition receptors such as CD1 and the mannose receptor (79, 327, 328). Rheumatoid arthritis (RA) synoviocytes express the truncated form of Trx-80, and treatment with the proinflammatory cytokines IL-1β and/or TNF-α increases Trx-80 cell expression, playing an important role in the establishment and/or the development of RA autoimmunity. Lemarechal *et al*. reported that synoviocytes represent important target cells of Trx-80, which respond in a concentration-dependent manner (229). Further, in contrast to Trx1, Trx-80 activates the classical and alternative pathways of complement activation through binding to the complement initiators C1q and properdin, leading to the production of the anaphylatoxin C5a (213).

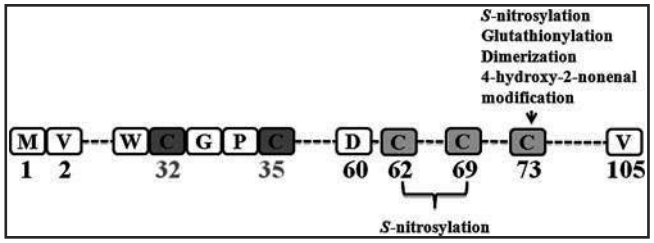

FIG. 5. Cysteine residues of the highly conserved active-site sequence Cys32-Gly-Pro-Cys35 (CXXC) of Trx. In addition to the two cysteine residues, human Trx, but not bacterial Trxs, contains three other, critical structural cysteine residues, at positions −62, −69, and −73, providing unique biological properties to Trx. These cystine residues of Trx can undergo post-translational modifications. Both Cys62 and 69 are the sites of *S*-nitrosylation, whereas Cys73 is a multi-modification site, undergoing nitrosylation, glutathionylation, dimerization, or 4-hydroxy-2-nonenal modification (5).

D. The Trx reductases

TrxR, a homodimeric, relatively thermostable, selenium-containing flavoprotein oxidoreductase (129, 315), catalyzes the NADPH-dependent reduction of Trxs disulfide and of numerous other oxidized cell constituents (16). TrxR is important for cell proliferation, antioxidant function, and redox signaling (403, 422). These enzymes have three isoforms in mammals: TrxR1 in the cytosol, TrxR2 in the mitochondria, and TrxR3 or Trx GSH reductase present primarily in the testes (166); in humans, they are encoded by three different genes, *TNXRD1*, *TNXRD2*, and *TNXRD3*, respectively (16).

The C-terminal extension with the characteristic motif Gly-Cys-Se-Cys-Gly carrying the essential selenocysteine residue is unique for mammalian TrxRs, and it forms a selenenylsulfide in the oxidized enzyme. Further, this C-terminal extension enables TrxR to extend the electron transport chain from the catalytic disulfide to the enzyme surface and to prevent the enzyme from acting as a GSH reductase by blocking the redox-active disulfide (365). Due to their flexible C-terminal tail, TrxRs have a broad range of substrates, including glutaredoxin 2, protein disulfide isomerase, and granulysin, and also some nonprotein substrates such as selenite, dehydroascorbate, lipoic acid, ubiquinone, cytochrome c, or the cancer drugs motexafin, gadolinium, and alloxan (discussed below) (16, 138).

Overexpression of TrxR1 has been observed in many tumors and cancer cells (156, 166, 184, 239, 240, 247), rendering it an interesting target candidate for chemotherapy (discussed in section XIII). TrxR1 in cancer cells is essential for self-sufficiency of growth, progression into the S phase (466), tumor progression, and metastasis (465). However, knockout

of TrxR1 in mice exposed to a liver carcinogen showed a significantly increased incidence for chemically induced liver cancer (49). Thus, the dual role of TrxR in cancers, prevention/promotion, may depend on the stage of cancer development and on tissues as well.

E. Trx-interacting protein

Human Trx-binding protein-2 (TBP-2)/vitamin D_3-upregulated protein 1 (VDUP1)/TXNIP is a negative regulator of Trx1 function, because it interacts with the active center of Trx1, thereby inhibiting its reducing activity (63, 193, 325, 373). It is one of the six members of the mammalian α-arrestin family. The α-arrestins are structurally related to the β-arrestins, which are well-characterized mediators of G-protein-coupled receptor signaling and endocytosis. TXNIP expression is ubiquitous and is induced by a variety of stresses, including UV light, γ-rays, heat shock, and H_2O_2, as well as glucose (78, 281). TXNIP overexpression renders cells more susceptible to oxidative stress and promotes apoptosis (368, 384, 444). Further, peroxisome proliferator-activated receptor-gamma (PPARγ) activation stimulates apoptosis in human macrophages by altering the cellular redox balance *via* regulation of TXNIP (35). In addition to its inhibitory role, TXNIP plays important roles in lipid and glucose metabolism (72, 73, 98, 383, 400), inflammation (10, 335, 478), cardiac function (471), and carcinogenesis (310). Interestingly, the ability of TXNIP to inhibit glucose uptake was found to occur independent of Trx1 binding (397).

F. Regulation and post-translational modifications of Trx

In spite of being cell cycle dependent, the expression of Trx1 can be upregulated by a variety of stress stimuli, including hypoxia, *Staphylococcus aureus* protein, lipopolysaccharide (LPS), O_2, H_2O_2, phorbol ester, viral infection, photochemical oxidative stress, X-radiation, and UV irradiation (219, 462).

As mentioned earlier, Trx1 can undergo post-translational modifications. Glutathionylation of Trx1 leads to a reduction in the enzymatic activity of Trx1 under conditions of oxidative stress, which it seems to regain again, indicating the ability of Trx1 to degluthionylate itself by some means of autoactivation (143). *S*-Nitrosylation is the covalent addition of an NO moiety onto a cysteine thiol. It is a dynamic post-translational modification for the regulation of protein functions. *S*-nitrosylation of protein thiols may occur on exposure of specific redox-active motifs to NO, or as a result of transnitrosation/transfer reactions from low-mass carrier *S*-nitrosothiols or transfer from other protein *S*-nitrosothiols (124). Available lines of evidence imply S-nitrosylation in cardiovascular, pulmonary, musculoskeletal, and neurological dysfunctions, as well as in cancer [see (114) and (376) for more details]. In contrast to other proteins such as caspases (258), peroxiredoxin 2 (PRX2) (104), or methionine adenosyl transferase (331), which are inhibited, the activity of Trx1 is increased upon *S*-nitrosylation (144); consistently, purified Trx1 is sensitive to *S*-nitrosylation. Stimulation of HEK-293 cells with *S*-nitrosoglutathione resulted in Trx1 *S*-nitrosylation and consequently ASK-1 activation (401), whereas others found that *S*-nitrosylation occurring at Cys69 enables Trx1 to scavenge ROS, to preserve its redox regulatory activity, and to act as an antiapoptotic protein in endothelial cells (144). Interestingly, the Trx system can act as a major *S*-nitrothiol–caspase-3 denitrosylating protein (30), because denitrosylation by Trx1 alone in the absence of TrxR1 was ineffective. Further, Trx2 mediates Fas-induced denitrosylation of mitochondrion-associated *S*-nitrothiol–caspase-3 and promotes apoptotic signaling (30, 377). Cys73 of Trx1 was shown to be responsible for the transnitrosylation of caspase 3 at Cys163, because mutation of Cys73 to Ser yielded a mutant exhibiting a normal disulfide reduction activity, which was resistant to nitrosylation, and which lacked the transnitrosylation activity toward caspase 3 (284). On the other hand, the redox states of the Cys32 and Cys35 seem to be crucial regulators determining the nitrosylation of Trx1 at Cys73 and its ability to transnitrosylate target proteins (155, 454). Therefore, under certain conditions, Trx1 plays a major role in protein *S*-denitrosylation, and it can also trans-*S*-nitrosylate other proteins (155, 285, 376, 377).

G. Regulation of cell signaling by Trx1

Depending on its subcellular localization, Trx1 exerts different roles. It can be found in the extracellular environment, on the cell surface and intracellularly. Trx1 shows redox regulatory functions in the signal transduction and transcriptional mechanisms.

1. Extracellular Trx1. In spite of lacking a signal peptide, Trx1 can be secreted by cells through an unknown mechanism (101, 359). Extracellular Trx1, either secreted or exogenously added, triggers a variety of physiologic or pathologic functions (Fig. 6). Until now, no binding of Trx1 to a specific cell surface receptor has been characterized. It seems that Trx1 acts as an autocrine growth factor and in synergy with other cytokines as a potent costimulatory molecule from the outside of cells (439).

Although it has not yet been extensively studied, Trx1 has been reported as a lipid raft (LR)-associated protein. LRs are specialized domains of the plasma membrane that cluster specific proteins and provide a dynamic scaffold for organizing cellular processes such as signal transduction (Fig. 7) (398). There is convincing evidence that the LR-redox-signaling platforms may mediate the actions of Trx1 on the leukocyte–endothelial cell interaction related to redox regulation during inflammation (139). On the other hand, LR clusters get endocytosed and form redoxosomes that mainly produce ROS as signaling molecules to evoke intracellular downstream responses. Trx1 can be internalized into the cells through LR-mediated endocytosis (218). Increasing evidence suggests that LR redox signaling may contribute to infection, host defense, and to the development of different diseases such as atherosclerosis, obesity or metabolic syndrome, and tumor progression (190). Further studies are needed to investigate the molecular mechanisms involved in the formation of this membrane-signaling complex highlighting the possible role of Trx1 through these platforms.

2. Cytoplasmic Trx1. Disulfide-reducing action is extremely important in regenerating oxidized proteins, which may render many signaling proteins inactive. Trx-dependent redox regulation of cellular signaling and stress response occurs through several mechanisms such as redox balance regulation or protein/protein interaction (Fig. 8). Here we

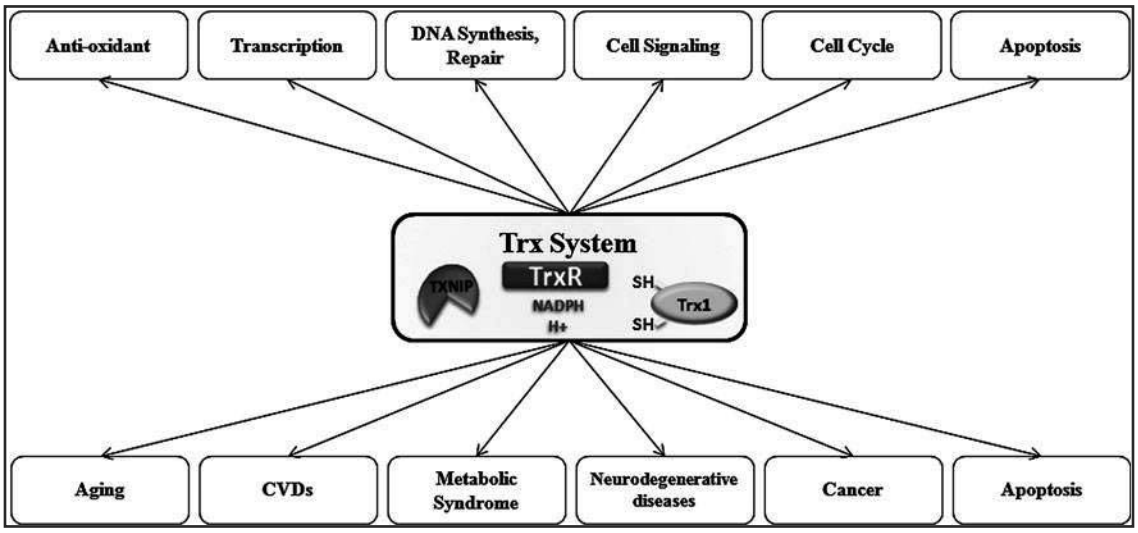

FIG. 6. The regulatory role of the Trx system. The Trx system regulates several cellular processes, including cell cycle, growth, cell signaling, and apoptosis. Accordingly, these effects can manifest themselves in different diseases.

highlight the involvement of Trx1 in mitogen-activated protein kinases (MAPKs) and Ca^{2+} signaling.

a. Mitogen-activated protein kinases. ASK-1 is an upstream MAP kinase kinase kinase that regulates the c-Jun N-terminal kinases (JNK) and p38 MAPK leading to stress-induced apoptosis and inflammation. ROS-activated ASK-1 mediates p38 signaling that may induce nonapoptotic outcomes, such as differentiation (69) and TLR4-mediated innate immunity (270). Importantly, reduced Trx1 can bind to the amino-terminal portion of ASK-1, thereby inhibiting the kinase activity and ultimately leading to ASK-1 ubiquitination and degradation (Fig. 8) (243, 362). The final actor in this triad is TXNIP, which binds to the catalytic cysteines of Trx1, and thus inhibits the Trx1 activity and its ability to bind to ASK-1. Trx1 has also been shown to activate certain components of the MEKK1-JNK signaling pathway, resulting in IκB degradation and NF-κB activation (82).

On the other hand, Trx1 knockdown suppressed epidermal growth factor-induced extracellular signal-regulated kinase (ERK)1/2 activation, suggesting that Trx1 plays a positive regulatory role in ERK1/2 signaling (287).

In response to extracellular growth or survival factors, the Akt signaling pathway becomes activated to coordinate various cellular events and eventually controls physiological processes such as cell proliferation, cell growth, cell migration, and survival (84). On the other hand, the phosphatase and tensin homology (PTEN) phosphatase exerts the function of terminating Akt signaling (232). Therefore, PTEN inactivation by oxidative stress is a physiological mechanism by which Akt becomes activated (42). It has been demonstrated that Trx1 activates the Akt pathway, either directly or indirectly, by interacting with or inhibiting PTEN (197, 226, 278).

b. Calcium signaling. Oxidation of the thiol systems has long been known to cause cell death due to dysregulation of Ca^{2+} homeostasis. Transient receptor potential (TRP) channels are cation channels that mainly mediate the influx of Na^{+} and Ca^{2+} across the plasma membrane into the cytoplasm. TRPC5 homotetrameric channels and TRPC5/TRPC1

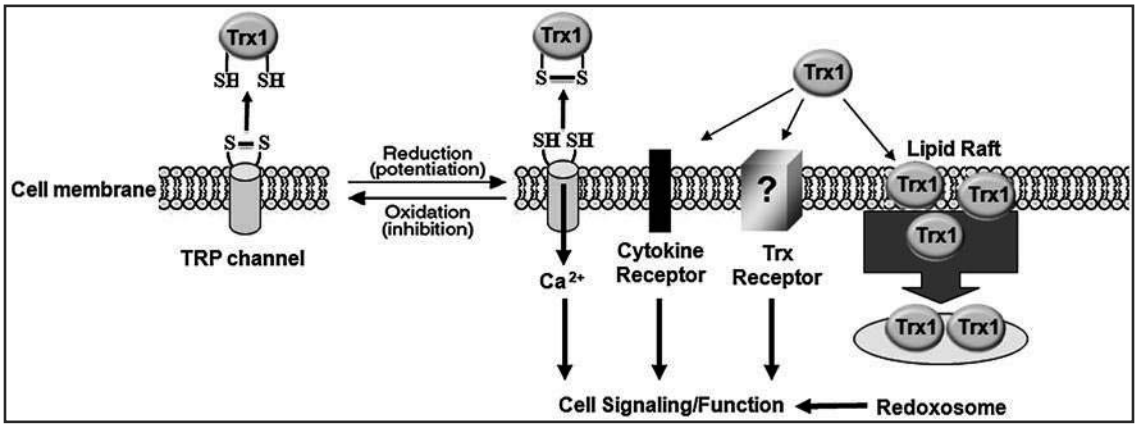

FIG. 7. Possible signaling pathway of extracellular Trx. Trx has been reported as a lipid raft (LR)-associated protein. Trx may enter the cell through LRs, subsequently forming the redoxsome, endosomes responsible for early receptor-mediated signaling in nonphagocytic cells. Reduced Trx can also break a disulfide bond in the extracellular loop of the transient receptor potential-C5 (TRPC5) channel, thereby increasing the Ca^{2+} ion influx of the channel.

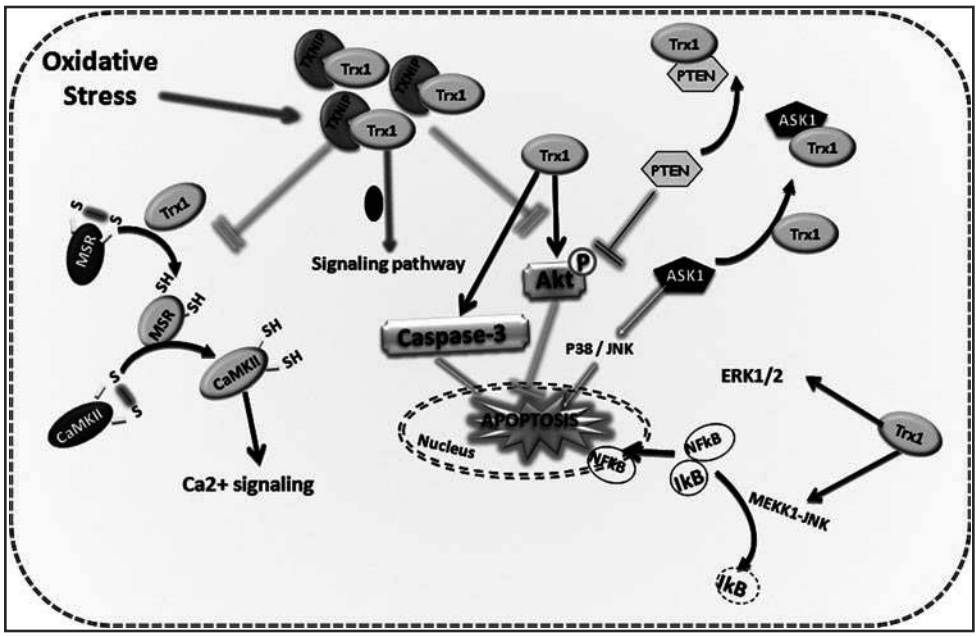

FIG. 8. Cytoplasmic Trx and cell signaling. Reduced Trx can inhibit apoptosis through binding to ASK-1. Further, Trx activates the Akt pathway either directly or indirectly, by interacting with and thereby inhibiting PTEN activation. Trx can activate also certain components of the MEKK1-JNK signaling pathway, resulting in IκB degradation and NF-κB activation. The TXNIP inhibits Trx activities. ASK-1, apoptosis signal-regulated kinase 1; MEKK1, MAP/ERK kinase kinase-1; PTEN, phosphatase and tensin homology; TXNIP, thioredoxin-interacting protein; CaMKII, Ca^{2+}–calmodulin-dependent protein kinase II; MSR, methionine sulfoxide reductase.

heterotetrameric channels, expressed in HEK293 cells, are activated by extracellular Trx1. Reduced Trx1 breaks a disulfide bond in the extracellular loop adjacent to the ion selectivity filter of TRPC5 and increase the Ca^{2+} influx of the channel (457). On the other hand, in mammalian tissues, activities of the phosphatase calcineurin and Ca^{2+}–calmodulin-dependent kinase II (CaMKII) have been demonstrated to be reversibly altered through oxidation of specific methionines within their calmodulin-binding and autoinhibitory sites, respectively. Trx-dependent reduction by methionine sulfoxide reductase limits these methionine oxidations and promotes the Ca^{2+} signaling pathways, which are important to stress responses (34).

3. Nuclear Trx1. Oxidants in the cytoplasm induce oxidative stress, which triggers transcriptional activation of the defense systems protecting the cells from uncontrolled oxidative reactions. It seems that a fine-tuned balance between the antioxidative and oxidative systems likewise exists in the nucleus (Fig. 9). Trx1 was found to be translocated into the nucleus in response to oxidative stress, despite the lack of an NLS (163). Schroeder *et al* clearly demonstrated that Trx1 is imported into the nucleus in a karyopherin-α-dependent manner, and this nuclear import of Trx1 is required for the inhibition of apoptosis (372). In addition, TrxC32S/C35S can still be imported into the nucleus. However, Cys32 and Cys35 are required for the ability of Trx1 to bind to the transcription factors and thereby modulating their activity. Because the nuclear import of Trx1 is greatly impaired when Cys69 is mutated to serine, one may speculate that a modification of Trx1 has to occur in the cytoplasm to allow Trx1 to be imported into the nucleus. Trx1 then promotes the antiapoptotic effects by binding to several transcription factors and increasing transcription factor binding to the genes through the ARE (372).

Trx1 reduces Ref-1, a bifunctional protein with a redox activity and a separate DNA repair (APE-1 and endonuclease) activity. Ref-1 is exclusively localized in the nuclei (455), and its redox activity maintains conserved Cys residues of redox-dependent transcription factors, including HIF-1α, NF-κB, p53, AP-1, Nrf-2, glucocorticoid receptor, and ER (116, 130, 131, 162, 163, 370). Further, inducible activation of HIF1-α protein in response to hypoxia is produced, at least in part, by thiol redox regulation of the C-terminal transactivation domain activity of the Trx1 and Ref-1 system, leading to the interaction with CREB-binding protein (CBP)/p300 (99). Ref-1 was recognized to reduce the c-Fos and c-Jun members of AP-1 (1). This redox modulation occurred at a conserved Cys residue (Lys–Arg) present in both c-Fos and c-Jun. Reductions of c-Fos and c-Jun by Ref-1 stimulate AP-1 binding to DNA, and this stimulatory effect is significantly elevated by reduced Trx1 (1). Additionally, an important role of Trx1 in the AP-1 activity is mediated by an interaction with Jun activation domain-binding protein (Jab1) (175).

NF-κB is a p50/p65 heterodimer, and the Cys62 residue must be reduced for the p50 subunit to facilitate DNA binding, indicating that the redox state of the nuclear compartment is critical for NF-κB activation (110). The Trx1 system modulates the NF-κB transcriptional activity through Cys62 of the p50 subunit (36, 163).

The p53 transcription factor controls the expression of a wide range of genes in response to DNA damage, chemotherapeutic drugs, growth factors, and oxidants (180). Ten Cys residues are located within the DNA-binding domain of p53, and all are susceptible to oxidation, resulting in an alteration of the DNA-binding properties of p53. Trx1 enhances the p53 DNA-binding activity directly or through Ref-1 mediation (427).

Nrf-2 is a prototypic redox-signaling pathway (207). Nrf-2 is bound to the actin-associated binding protein (Keap-1) in the cytoplasm. Oxidation or alkylation of Cys residues in Keap-1 provides a signal to release Nrf-2, which translocates to the nucleus and activates transcription *via* the ARE within the control regions for the antioxidant and detoxification genes (305). Hansen *et al.* showed that oxidant-induced Nrf-2/Keap-1 dissociation in the cytoplasm is regulated by the GSH

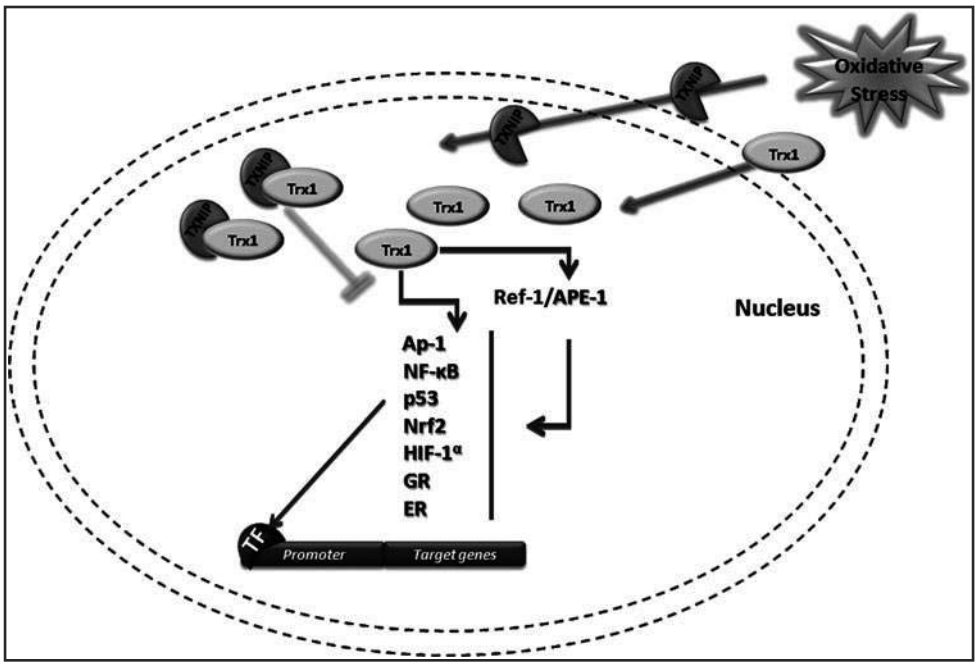

FIG. 9. Nuclear Trx regulates the nuclear redox state and transcriptional activity. Despite the lack of a nuclear localization signal, Trx can translocate into the nucleus in response to oxidative stress, where it regulates different transcription factors. Ref-1, redox factor-1; HIF-1α, hypoxia-inducible factor-1 α; AP-1, activator protein-1; Nrf-2, nuclear factor erythroid-2 related factor 2; ER, estrogen receptor; GR, glucocorticoid receptor.

system, whereas the DNA-binding activity of Nrf-2 in the nucleus is controlled by nuclear Trx1, indicating a dual regulation (152).

Trx1 also mediates the interplay between cellular redox signaling and glucocorticoid receptor signaling *via* direct association with its conserved DNA-binding domain (256). The function of the ER, another nuclear receptor, is strongly influenced by its redox state and is modulated by endogenous Trx1 (159). Indeed, Trx1 inhibits the binding of PPAR-α to the PPAR-response element by modulating its AF-1 transactivation domain (241).

TXNIP inhibits the intracellular Trx1 activity by forming a mixed disulfide bond between the Trx1 active-site Cys32 and TXNIP Cys247 (325). In addition, TXNIP can also mediate the nuclear localization of Trx1 (4), because it can interact with importin alpha 1, a component of the nuclear import machinery (307).

Interestingly, TXNIP-null hepatocytes exhibit reduced glucose production that can be rescued by reintroducing TXNIP-WT. Overexpression of the TXNIP mutant C247S failed to restore the liver glucose production, although the Trx1 activity was not affected (74). In addition, the TXNIP/Trx1 complex has been shown to mediate the activation of plasma membrane signaling, thereby promoting cell survival and migration (453). The TXNIP/Trx1 effects could be related to TXNIP as a scaffold protein, as an α-arrestin protein. These data strongly suggest that the TXNIP/Trx1 complex can have major signaling properties in a specific subcellular localization, independent of the inhibition of the Trx1 activity. It is clear that phosphorylation of Trx1 at T100 plays an important role in its cytoprotective activity in cancer cells (66).

III. General Functions of Trx

The Trx system is a crucial and essential system for the protection against oxidative stress, for the maintenance of the cellular redox balance, and for the regulation of differentiation and cell fate [recently reviewed (248)]. This wide range of cellular functions of Trx system leads into its involvement in a variety of diseases (Fig. 6) (309). Knockout of either Trx1 or Trx2 in mice is embryonically lethal (268, 311). The regulation of the cellular redox balance is critically determined by the activity of several antioxidant systems. Trx1 seems to exert most of its antioxidant properties through peroxiredoxin (PRX), which uses the –SH groups as reducing equivalents. Trx1 reduces the oxidized form of PRX, and reduced PRX in turn scavenges ROS, such as H_2O_2 (57, 145).

Trx1 acts as a hydrogen donor for the enzymes involved in reducing reactions, for example, ribonucleotide reductase (473), PRX, and methionine sulfoxide reductases (361, 411). Trx1 reduces protein disulfide bonds and other cysteine oxidations in the respective cellular compartments (155).

As mentioned above, Trx1 and Trx2 expression is essential for early differentiation and morphogenesis of the mouse embryos, since embryos of *TXN1*- (268) and *TXN2*-null (311) mice are not viable, whereas heterozygous mice are fertile. The role of Trx system has been studied in transgenic (Tg) animal models of different human diseases. Tg mice overexpressing Trx1 showed increased resistance to oxidative stress (2, 298), and a longer life span (286, 298, 332) suppressed UV-induced inflammation (318), a higher resistance against the teratogenic effects of hyperglycaemia (201), attenuated osteopenia (149) and nephropathy (147) associated with streptozotocin-induced diabetes, increased availability of glucose for fetal growth (428), attenuated apoptosis of cardiomyocyte and myocardial fibrosis after myocardial infarction (2), and a decreased local macrophage migration inhibitory factor resulting in a suppressed Th2-driven airway inflammation (419). Further, Tg mice with cardiac-specific overexpression of a dominant-negative mutant (C32S/C35S) of Trx1 showed increased markers of oxidative stress and exhibited cardiac hypertrophy with a maintained cardiac function at the baseline (460). The role of Trx2 in the endothelial cell function has been studied using endothelial cell-specific

transgenesis, and it was found that Trx2 improved the endothelial cell function and reduced atherosclerotic lesions in the apolipoprotein E-deficient mouse model (474). Interestingly, cardiomyocyte size enlargement and hypertension due to chronic angiotensin II infusion were significantly diminished in Tg mice that overexpress Trx2 compared to the wild type (451).

A. Role of Trx inapoptosis

Apoptosis is a stringently controlled process of cell death that is fundamental for the normal development of organs. Evidence now suggests that the initiation and execution of apoptosis are triggered by changes in the redox environment (248, 306). Trx1 has been shown to be involved in apoptotic processes in at least two different ways (Fig. 8). First, the redox status of one of the key determinants of the apoptotic process, caspase-3, is maintained by Trx1 (284, 285, 425). Secondly, ASK-1, an enzyme involved with regulating apoptotic events, is a substrate for Trx1, which binds to ASK-1 and inhibits its apoptotic activity (248). This molecule, ASK-1, has since been identified as mitogen-activated protein (MAP) kinase kinase kinase, and oxidation of Trx1 causes the release of this binding, followed by the induction of apoptosis *via* the activation of p38 MAP kinase and c-Jun-NH_2-terminal kinase (362). Experiments with various lymphoid cells also confirmed the involvement of Trx1 in the apoptotic process. Sato *et al.* implicated the human Trx1, in the regulation of lymphocyte function, most likely *via* the oxidation of this protein (367). This regulatory process may prove to be of significance in the redox-sensitive pathway of apoptosis induced by oxidative stress and the oxidation of sulfhydryl groups. Also, when lymphoid cells were cultured in the absence of L-cystine and GSH, the addition of recombinant Trx1 to these cultures was found to block partially the apoptotic process in a concentration-dependent manner (162). Moreover, it has been shown that Trx2 plays an important role in the mitochondrial pathway of apoptosis, since, compared to the wild-type mice, $TXN2^{+/-}$ mice displayed increased caspase-3 and caspase-9 activities as well as an increased mitochondrial cytochrome c release (333).

B. Trx regulates glucocorticoid and ERs

Glucocorticoids, as a major peripheral effector of the hypothalamic-pituitary-adrenal axis, play an essential role in re-establishing the homeostatic status in every peripheral tissue in humans. Upon binding of glucocorticoids to their receptor, they promote the dissociation of heat shock proteins, and the ligand–receptor complex translocates to the nucleus, where it binds to palindromic DNA sequences. The redox status of cysteine residues of glucocrticoids maintains their structure and binding abilities (58, 389). Previously, it has been revealed that Trx1 is required for generating the ligand-binding conformation of the glucocorticoid receptor. Antibody-mediated sequestration of either Trx or TrxR inhibited the ligand-binding activity of the glucocorticoid receptor (136). The same group conducted another study to show the direct effect of Trx1 on the DNA binding of the gluccocorticoid receptor. They have inhibited the binding ability of receptors by methyl methane thiosulfonate (MMTS), which can be reversed by adding dithiothreitol (DTT). Surprisingly, the promoting effect of partially purified Trx1 on DNA binding of MMTS-treated receptors in the presence of DTT was not affected by anti-Trx1 serum (414).

Additional studies on the role of Trx1 on the responsiveness to glucocorticoids revealed that the downregulation of Trx1 by antisense ODN or treatment with H_2O_2 negatively modulated the glucocorticoid receptor function and decreased the glucocorticoid-inducible gene expression. However, the cellular responsiveness to glucocorticoids was rescued by overexpression of Trx1 (255, 256).

In vitro and *in vivo* results showed that the specific DNA-binding activity of recombinant ERs was inhibited by sulfhydryl-modifying reagents and restored by the addition of recombinant Trx1 protein in an electrophoretic mobility-shift assay, revealing that the transcriptional activity of ERs is strongly influenced by their redox state, which is reversibly modulated by Trx1 (159). Moreover, treatment of ovariectomized adult mice with a time course of estradiol resulted in a rapid decrease of the expression of TXNIP, but increased the levels of cytosolic Trx1 and TrxR1 as well as Trx2. This clearly suggests that rapid estradiol-mediated activation of the Trx1 pathway is an important step in the response of the mammalian uterus to estrogen (87). Therefore, Trx and TrxR are the members of an interconnected network of proteins, which collectively help to maintain the structural integrity and activity of ER-alpha, its associated coregulatory proteins, and other complex members (349).

IV. The Trx System and Aging

Multicellular organisms generally undergo qualitative changes with time (aging) that are associated with progressive degeneration of biological functions, increased susceptibility to diseases, and increased probability of death within a given time period (93). Age-specific mortality rates from heart disease and stroke increase exponentially with age throughout the later years of life, accounting for more than 40% of all deaths among people aged 65–74 years and almost for 60% of all fatalities at the age of 85 years and older (430).

Cellular aging is commonly associated with the instability of the mitochondrial and nuclear genomes as well as oxidative protein damage in response to environmental and genetic stress (437). Several theories have been proposed to explain the molecular mechanisms behind aging and aging-related diseases. The free-radical theory of aging was first contented by Harman in 1956, proposing that free oxygen radicals cause cumulative oxidative damage, which eventually results in aging and age-related diseases in humans and animals. Subsequently, he refined his theory and suggested that the mitochondria play a key role in the aging process, because these organelles are the major source and likewise a major target of free radicals (378). Further, the capacities of the cellular antioxidant systems will decline with age, leading to a gradual loss of the free radical/antioxidant balance and subsequently to the accumulation of oxidative damage during the aging process (446). Aging is a major risk factor for cardiovascular diseases (CVDs). It has been demonstrated that endothelium-dependent vasodilatation and the basal release of NO are reduced during aging. Further studies showed that Trx1 can improve the endothelial function and is able to rescue endothelial cells from age-induced disorders (12). It has also been shown that TrxR in aging muscle cells plays a role in the sensitization of cells to oxidative challenge and for the

increased susceptibility to the mitochondrial pathway of apoptosis (357). It is important to note that overexpression of human Trx1 in Tg was shown to suppress oxidative stress damage and to elongate the lifespan (286). Moreover, telomerase activity, another determinant of longevity, was found to be higher in Trx1-Tg mice in comparison to wild mice (286). Nevertheless, Tg mice overexpressing Trx1 are indeed resistant to oxidative stress, and survival studies demonstrated that male Tg $(TXN1)^{+/0}$ mice have a significantly reduced mortality rate in their earlier part of life span compared with the wild-type littermates, whereas no significant changes were observed in females (332). Moreover, neither male nor female Tg $TXN1^{+/0}$ mice showed changes in their maximum life span. These findings suggested that the increased levels of Trx1 in the Tg $TXN1^{+/0}$ mice were correlated to increased resistance to oxidative stress, which could be beneficial in the earlier part of the life span, but would not extend the maximum life span in the C57BL/6 mice (332). It has been reported that $TXN2^{+/-}$ mice show a reduced mitochondrial function as indicated by reduced ATP production, reduced activity of electron transport chain complexes, and subsequently increased ROS generation. Accordingly, these mice have a higher oxidative damage of cellular components (185, 333) and no longer lifespan than their age-matched wild-type counterparts (334).

Methionine sulfoxide reductase A (MsrA) is another antioxidant enzyme known to repair efficiently oxidized methionine residues within proteins. Thus, in light of the oxidative stress theory of aging, it would be expected that MsrA contributes to the regulation of the aging process in mammals. While $MsrA^{-/-}$ mice are more susceptible to oxidative stress induced by paraquat, no difference between $MsrA^{-/-}$ and control mice in either their median or their maximum lifespan was observed (363).

Further, Trx1 has been associated with a wide variety of diseases with oxidative imbalance, including cancer, human immunodeficiency virus infection, neurodegenerative diseases, and CVDs (117).

V. The Trx System in CVD

CVD as a dysfunction of heart and blood vessels remains the leading cause of morbidity and death worldwide, accounting for more than 80% of all fatalities occurring in the Western countries (119, 244, 352, 356). According to the European Cardiovascular Disease Statistics report in 2008, each year, CVD causes over 4.3 million cases of death in Europe and over 2.0 million fatalities in the European Union (351). The risk factors for CVD can be either nonmodifiable, such as age, gender, ethnicity, and genetics, or modifiable, such as elevated serum lipids, high blood pressure, physical inactivity, obesity, and smoking (60, 186).

The term CVD encompasses an array of diseases such as coronary artery disease (CAD), hypertension, congestive heart failure, and stroke, all of which are complications of atherosclerosis that begins early in life and progresses gradually, remaining usually asymptomatic for a long period of time (252).

The majority of CVD results from complications of atherosclerosis, which is characterized by the formation of plaques consisting of foam cells, immune cells, vascular endothelial cells, smooth muscle cells, platelets, the extracellular matrix, and a lipid-rich core with extensive necrosis and fibrosis of surrounding tissues (121, 235, 250, 252). Available evidence supports the notion of a central role of oxidative stress in the atherosclerotic process, and the correlation between increased oxidative stress and vascular disease in as much as most risk factors leads to dramatic increases in ROS (53, 59, 113, 161, 186, 313, 342, 434).

The events of atherogenesis are well documented and have been reviewed extensively. Of crucial importance is the involvement of both innate and acquired immunity in various stages of atherosclerosis, in which leukocyte recruitment and expression of proinflammatory cytokines characterize early atherogenesis (153). In this scenario, macrophages play a central role in lipid metabolism, immune responses (233, 276, 385), and the maintenance of inflammation (263). Therefore, atherosclerosis can be considered as a chronic inflammatory disease of the arterial wall with the inflammatory process that plays a key role in all stages of the pathogenesis, including formation, progression, and the rupture of atherosclerotic plaques (236, 342).

Growing evidence indicates that overproduction of ROS under pathophysiological conditions is an integral component in the development of CVD such as atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy, and congestive heart failure (252, 432), because they activate various signaling pathways that underlie the vascular inflammation in atherogenesis (Fig. 10). Therefore, Trx1 may play a beneficial role antagonizing CVD. In the isolated perfused hearts adapted to ischemia and reperfusion by short cycles of ischemia/reperfusion (I/R), the protection is apparently afforded by the enhanced induction of Trx1, because ROS increase when Trx1 is inhibited by a suitable inhibitor (423). Further studies conducted in animal models *in vivo* suggest that Trx1 has many beneficial functions in the heart (269, 460). Only recently, it has been demonstrated that impaired angiogenesis and myocardial dysfunction can be counteracted by an adenoviral vector-based gene therapy encoding Trx1 (364).

Several studies indicate that Trx1 plays a role in the pathogenesis of atherosclerosis. NO and peroxynitrite contribute to the damage to smooth muscle and endothelial cells seen in atherosclerotic plaques (324) with increased Trx and TrxR mRNAs (404). Trx1 prevents the NO-dependent inhibition of purified NO synthase. Further, the endogenous levels of Trx1 are critical for restoring the NO-induced loss of the eNOS activity, because overexpression of Trx1 prevents the NO-induced loss of the eNOS catalytic activity (Fig. 10) (410, 476). It has been well documented that the mechanisms responsible for the improved angiogenesis as well as cardioprotection mediated by resveratrol operate both *in vitro* and *in vivo* through the induction of vascular endothelial growth factor (VEGF) expression triggered by Trx1 and HO-1. A significant increase in the Trx1 expression was observed at a baseline level in the myocardium of streptozotocin-induced diabetic rats after I/R. A protective role for Trx1 has also been implicated in cardiac hypertrophy. Several studies support the concept that Trx1, as an antioxidant, inhibits and protects from cardiac hypertrophy in animal models. Tg mice with a cardiac-specific overexpression of a dominant-negative Trx1 mutant showed a loss of the endogenous oxidoreductase activity of Trx1 (5, 206, 280, 476).

In patients with CAD, high homocysteine levels may cause low Trx1 activity, which is closely correlated to the extent and

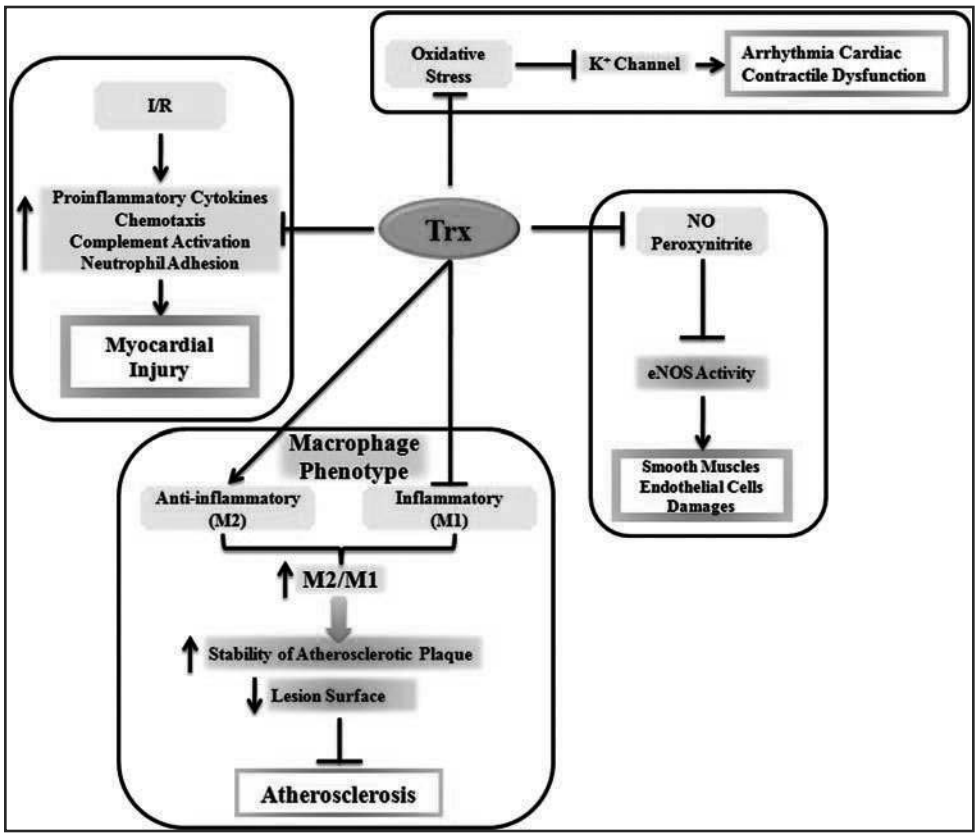

FIG. 10. Protective roles of Trx in cardiovascular diseases. Trx can attenuate ischemia–reperfusion injuries by inhibiting the expression of proinflammatory cytokines, chemotaxis, complement activation, and neutrophil adhesion. Further, Trx can prevent the smooth muscle and endothelial cell damage by protecting eNOS against NO and peroxynitrite. As a powerful antioxidant, Trx can also prevent lethal arrhythmia and cardiac contractile dysfunction by protecting the K^+ channel and by inhibiting electrical instability. Recently, it has been shown that Trx can antagonize atherosclerosis, because it can potentiate the anti-inflammatory macrophage phenotype. (To see this illustration in color, the reader is referred to the Web version of this article at www.liebertpub.com/ars.)

severity of the CAD (454). During I/R, Trx1 also inhibits the expression of proinflammatory cytokines, chemotaxis, complement activation, and neutrophil adhesion. This is an important issue, because I/R stimulates cell adhesion molecules and the migration of neutrophils into myocardial tissue. Subsequent production of inflammatory cytokines and ROS enhances myocardial injury. Another potentially important mechanism is the effect of Trx1 upon ion channel remodeling. Downregulation of the K^+ channel by oxidative stress could cause lethal arrhythmia and cardiac contractile dysfunction. Upregulation of Trx1 may prevent downregulation of the Kv4 channel and the subsequent electrical instability during I/R (269). Additionally, exogenous Trx1 exerts distinct cytoprotective effects on the cerebral I/R injury in mice by means of its redox-regulating activity (158). Further, it has been demonstrated that *S*-nitrosation of Trx1 potentiates its protective activity against myocardial I/R (408).

It has been suggested that there is a possible association between Trx1 secretion and the severity of heart failure. Trx1 is increased in both inflammatory cells and myocytes during myocarditis (461). Further, increased Trx1 levels have been observed in several oxidative stress-associated CVDs; for instance, serum Trx1 levels were significantly increased in patients with abdominal aortic aneurysm (AAA) relative to healthy subjects. Indeed, the levels correlate well with the size and expansion of AAA, suggesting its potential role as a biomarker of AAA evolution (262). Recently, we have demonstrated that Trx1 can promote differentiation of macrophages into an alternative, anti-inflammatory phenotype (Fig. 10) that may explain its protective effects in CVDs (142). Indeed, we have shown that Trx1 induced downregulation of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ and significantly promoted the polarization of anti-inflammatory M2 macrophages in cells exposed to IL-4 or IL-4/IL-13 *in vitro*, as evidenced by the expression of the CD206, also called mannose receptor, and IL-10 markers (97). In addition, Trx1 induced downregulation of nuclear translocation of AP-1 and Ref-1 and significantly reduced the LPS-induced differentiation of inflammatory M1 macrophages, as indicated by the decreased expression of the M1 cytokines TNF-α and MCP-1 (97). Consistently, Trx1 administered to hyperlipoproteinemic ApoE2.Ki mice challenged with either LPS or IL-4 significantly induced the M2 phenotype while inhibiting differentiation of macrophages into the M1 phenotype in the liver and thymus. ApoE2.Ki mice challenged with LPS for 5 weeks developed severe atherosclerotic lesions enriched with macrophages expressing predominantly M1 over M2 markers. In contrast, however, daily injections of Trx1 shifted the phenotype pattern of lesional macrophages in these animals to predominantly M2 over M1, and the aortic lesion area was significantly reduced (97).

Further, a recent analysis of $TXNIP^{-/-}$ mice revealed suppression of genes that participate in mitochondrial metabolism (470). Consequently, $TXNIP^{-/-}$ mitochondria were functionally and structurally altered, showing reduced oxygen consumption and ultrastructural derangements (470). TXNIP deletion would therefore enhance the I/R damage. Surprisingly, the $TXNIP^{-/-}$ hearts had a greater recovery of cardiac function after an I/R insult (470). Similarly, cardiomyocyte-specific TXNIP deletion reduced the infarct size after reversible coronary ligation. Thus, in addition to reduced mitochondrial function, deletion of TXNIP enhanced anaerobic glycolysis (470). While the mitochondrial ATP synthesis was minimally decreased by TXNIP ablation, the cellular ATP

content and lactate formation were higher in the *TXNIP*$^{-/-}$ hearts after I/R injury (470). Inhibition of glycolytic metabolism abolished the protection of the heart by TXNIP deficiency under hypoxic conditions. Thus, although TXNIP deletion suppresses the mitochondrial function, protection from myocardial ischemia is increased as a result from a coordinated shift to enhanced anaerobic metabolism, which supplies an energy source outside mitochondria (470).

VI. Physical Exercise and the Trx System

Moderate physical exercise is a deterrent for several diseases such as CVD, type 2 diabetes (TD2), obesity, hypertension, hyperlipidemia, and endothelial dysfunction (127, 343, 380), and its beneficial effects have been studied in different animal models (343, 380). Nevertheless, exercise is known to induce oxidative stress in animals and in humans (224, 381), and this would appear incompatible with its beneficial effects. To explain this paradox, some proposed that in case of atherosclerosis, for example, either the beneficial effects of moderate physical exercise would overwhelm the deleterious effects of oxidative stress, or the exercise-induced oxidative stress might be beneficial by inducing arterial antioxidant enzymes (322, 381, 450). Thus, many studies report the induction of antioxidant enzymes by exercise in different species, including humans (21, 109, 204). For example, Trx1 expression was found at its maximal levels in the cytosol and in the nucleus in peripheral blood monocytes after 12 and 24 h of a single bout of a 30-min swimming exercise (402). A similar profile was observed when cells were exposed *in vitro* to H_2O_2. It has been reported that *Trx1* gene expression is enhanced through the oxidative stress-response element located within its promoter (407). Therefore, exercise-induced oxidative stress may affect Trx1 induction.

In the case of hypercholesterolemia, which is known to represent a significant risk factor for CAD, was recently suggested to have direct negative effects on myocardial function due to increase ROS generation and increased myocyte death potentially through the disruption of the mitochondrial function (106, 168, 320, 369). Pigs with familial hypercholesterolemia (FH) showed increased oxidative stress, depicted by increased protein nitrosylation, and decreased levels of reduced GSH in cardiac mitochondria (275). Expression of mitochondrial antioxidant enzymes, Mn-SOD, Trx2, and PRX3, was greatly reduced in pigs with FH (275). Chronic exercise training was able to reduce mitochondrial oxidative stress, and return Mn-SOD, Trx2, and PRX3 to normal (275). Equally, in the case of diabetes, regular exercise plays a preventive and therapeutic role. Thus, it has been reported that the levels of Trx1 protein and activity and TXNIP protein in the brain of untrained rats were similar in diabetic and nondiabetic animals. However, exercise training increases Trx1 protein in nondiabetic animals without affecting the TXNIP levels, whereas diabetes inhibited the effect of training on Trx1 protein and also increased TXNIP mRNA (222).

It is now well accepted that a moderate physical exercise has beneficial effects on health by reducing the risk of several diseases. In contrast, however, an extreme endurance exercise was suggested to be detrimental, because very high levels of ROS were generated by excessive oxygen consumption (11). Exhaustive exercise also exhibits strong effects on the immune system. Such effects have been attributed to the changes in the cellular composition of peripheral blood as well as to changes in the expression of certain genes (76, 105, 396). Among the changes in gene expression, Trx1 was found to be significantly upregulated (479). In addition, Trx1 was also found to be upregulated in plasma by exhaustive exercise (265). As mentioned earlier, Trx1 has been reported to be a sensitive oxidative stress marker, with its levels increasing in relation to hydrogen peroxide (H_2O_2), ultraviolet irradiation, and inflammation (33, 165, 299). It is associated with the signal transduction of cellular redox regulation and with the protection against oxidative stress (299). High levels of Trx1 in peripheral blood cells or in plasma suggest an elevated oxidative stress and an enhancement of the protective antioxidant system through the activation of the *Trx1* gene expression (265, 479). However, the appropriate level of physical exercise to promote good health is not defined yet. Nevertheless, age, gender, ethnicity, and general health conditions might be considered.

VII. The Txr System in the Metabolic Syndrome

Abdominal obesity, hyperlipidemia, insulin resistance, and hypertension are the predominant underlying risk factors for the metabolic syndrome. The pathogenesis of metabolic disorders is characterized by an endothelial cell activation, adipocyte hypertrophy, free-fatty-acid accumulation (375), and infiltration of peripheral blood monocytes, which gradually differentiate into macrophages (345, 393). Chronic inflammation of the arterial wall and the endothelial dysfunction are considered as potent driving forces in the pathogenesis of the atherosclerosis, the main cause of myocardial infarction, stoke, and gangrene (120, 345). There has been a dramatic increase in the incidence of the metabolic syndrome, and it is highly correlated with the atherosclerosis prevalence, which contributes to 50% of the death among the Western industrialized countries (277). Considering this alarming increase, the identification of the molecular and cellular pathways implicated in the metabolic syndrome has attracted considerable attention. Among the members of the Trx system, TXNIP has obtained a particular attention because of its direct involvement that has been extensively reviewed [see (198, 397)].

TD2 is a chronic metabolic disorder mediated by sterile inflammation (85) and characterized by insulin resistance and pancreatic β-cell dysfunction (196). TD2 is one of the major risk factors for CHD, stroke, peripheral arterial diseases, and other vascular complications (358). The proatherosclerotic effects of TD2 are the result of increased oxidative stress (374) due to generation of ROS and other free radicals. Chronic exposure to high glucose levels generates a toxic metabolic environment termed glucotoxicity, which is commonly associated with lipotoxicity (199, 354). Several signaling molecules are involved in the induction of β-cell dysfunction, and inhibition of such molecules might provide a basis for new antidiabetic therapies. ROS and TXNIP are the most important mediators involved in the β-cell glucotoxicity pathway. A chronic increase of glucose and free-fatty-acid levels enhances ROS generation in part by the mitochondria and endoplasmic reticulum. Excess of ROS perturb several mechanisms regulating the β-cell function and induce cellular stress and death (227). Therefore, high levels of Trx1 protein could be effective in suppressing the progression of diabetes. In fact, Tg overexpression of Trx1 ameliorates glucose intolerance, enhances

pancreatic duodenal homeobox factor-1 (PDX-1) and MafA expression, and preserves β-cell functions, especially the insulin-secreting capacity. Its protective effects are also apparent in the early phase of the β-cell failure, where it suppresses insulin hypersecretion induced by hyperglycemia and the ROS production associated with the glucotoxicity (459). Recently, Yin *et al.* demonstrated a highly significant correlation between chronic hyperglycemia and myocardial I/R injury by enhancing nitrative inactivation of Trx1. They also showed that blocking nitration or application of exogenous recombinant human Trx1 can be a promising novel strategy for the attenuation of TD2 cardiac complications (463).

Indeed, ROS perturb the expression of multiple genes involved in the regulation of β-cell functions such as proinsulin production through decreased expression and activity of transcription factors, especially PDX-1 and MafA, a basic leucine-zipper transcription factor (154, 202, 227). In addition to ROS, TXNIP expression is highly induced by glucose in pancreatic islets and vascular cells (424). Under stress conditions, the Trx1/TXNIP complex dissociates in an ROS-dependent manner (478). Thus, free Trx1 reduces oxidized proteins and scavenges free radicals (227). However, TXNIP binds to the cytosolic multiprotein complex NLRP3 inflammasome and changes its function from Trx1 repressor to NLRP3 activator (Fig. 11) (91). Upon stimulation, NALP3 oligomerizes the adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), which in turn activates caspase-1 and induces the cleavage, activation, and liberation of the proinflammatory cytokine IL-1β (91, 167). Induction of the active form of IL-1β mediates β-cell dysfunction and apoptosis. Chronic hyperglycemia enhances the TXNIP expression, which inhibits Trx1 glucose uptake through its arrestin domain independent of its binding to Trx1 protein (Fig. 12) (291). Thus, it has been considered as a key mediator of the TD2 pathogenesis through its deleterious effects on pancreatic cells, especially glucotoxicity and β-cell apoptosis (282). In addition to its stress–response activity, recent evidence indicates that under physiological conditions, TXNIP protein is involved in glucose and free-fatty-acid regulation, underlining its important role in stress, inflammation, and physiologic conditions (172). Leibowitz *et al.* demonstrated that in hyperglycemia, partial knockdown of

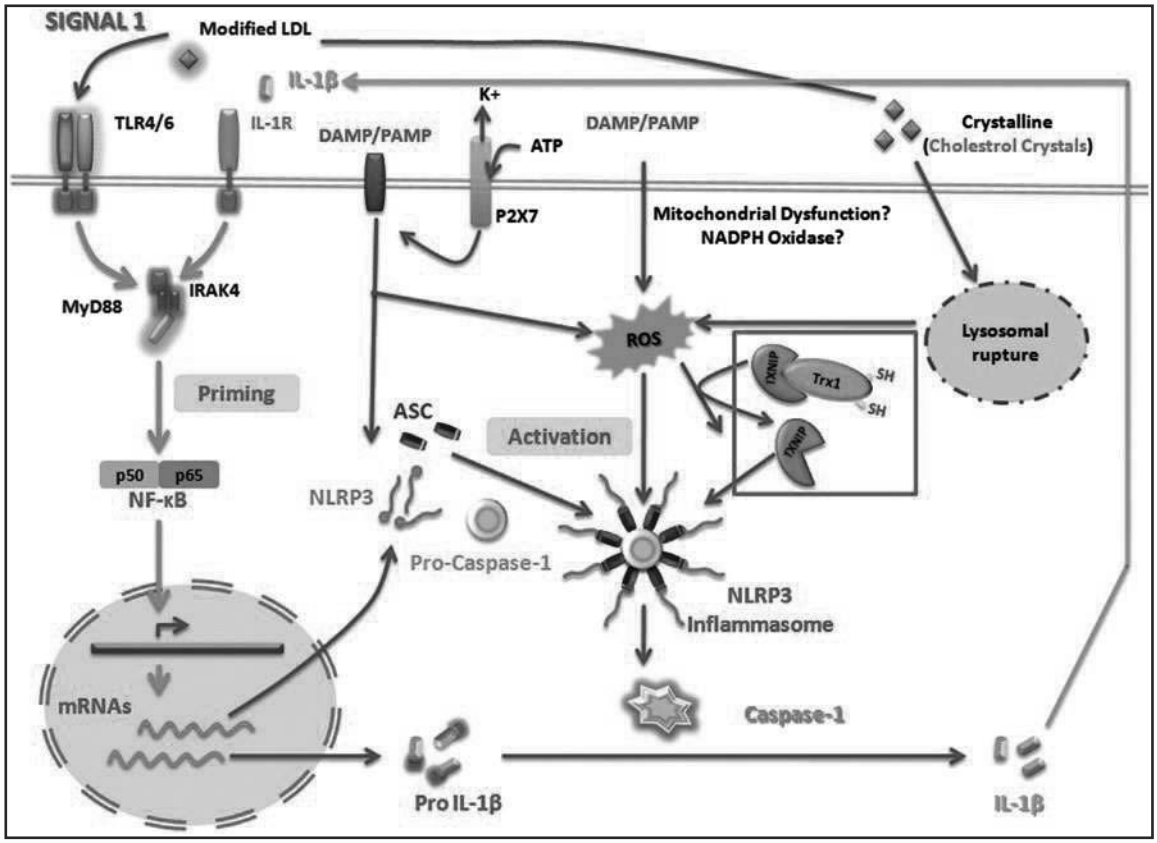

FIG. 11. TXNIP activates the NLRP3 inflammasome. Cells of the innate immune system, namely, macrophages and dendritic cells, express the sensors for danger signals. These include the family of transmembrane Toll-like receptors (TLRs), RIG-1-like helicases (RLRs), and the nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs). They are involved in innate immune recognition of pathogen-associated molecular patterns (PAMPs) as well as the intracellular and extracellular damage-associated molecular patterns (DAMPs). Several members of the NLR family such as NLRP1, NLRP3, and NLRC4 have been shown to assemble into large multiprotein complexes named inflammasomes to control the caspase-1 activity. The stimuli, PAMPs, DAMPs, cholesterol crystals, modified low-density lipoprotein (LDL), ATP, K^+ efflux, ROS production and increased TXNIP, can activate the inflammasome. As a result, NLRP3 recruits ASC and procaspase-1 to form a multimeric protein complex, which leads to the cleavage of the procaspase 1 into the active form. Caspase-1 can then cleave the proforms of two potent, proinflammatory cytokines, IL-1β and IL-18, in the cytoplasm [adapted from (85, 189, 192, 264, 293, 371)]. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars.)

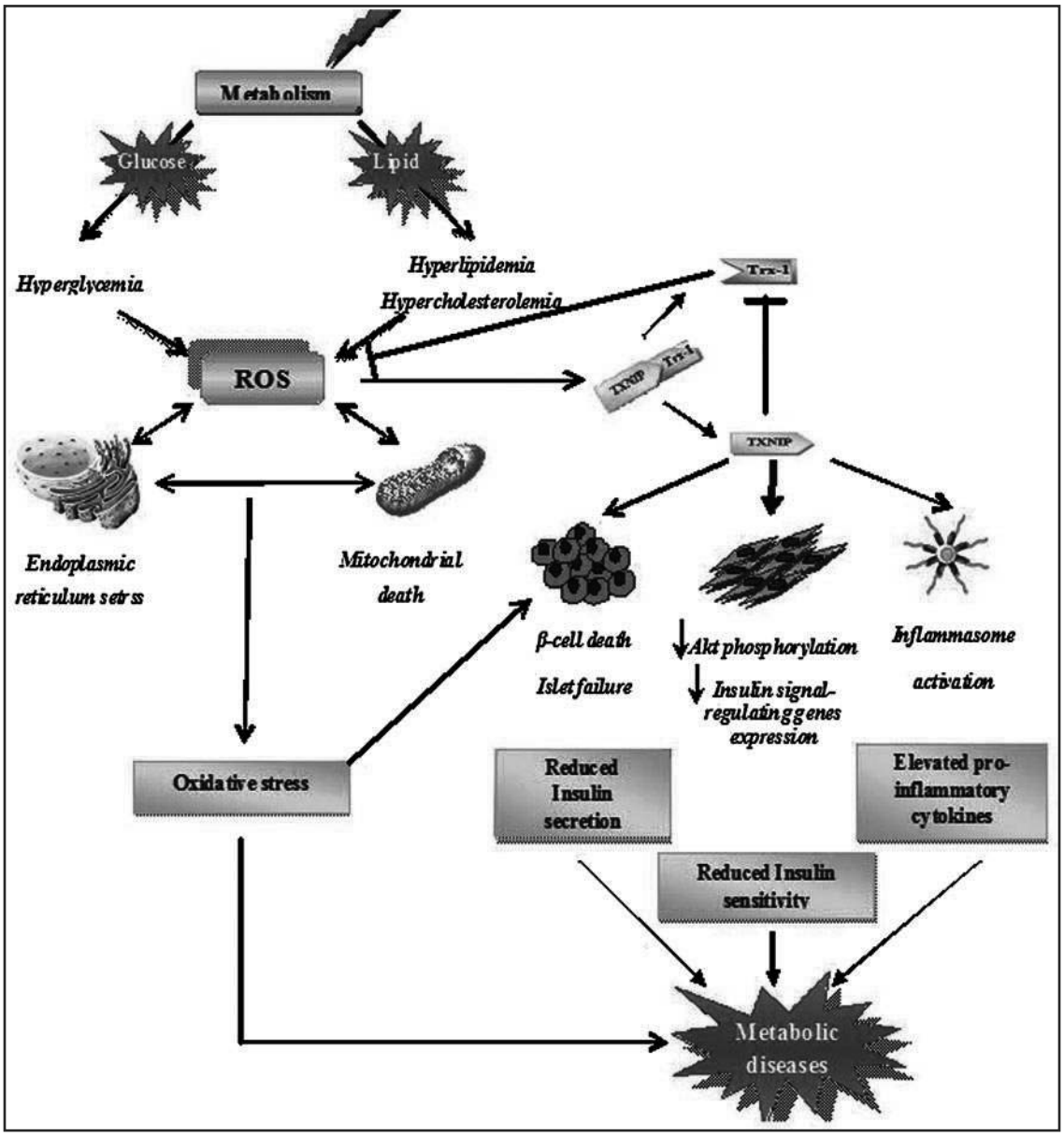

FIG. 12. ROS and TXNIP, the two critical inducers of metabolic diseases. Chronic hyperglycemia and prolonged exposure to high free-acid levels lead to an excess production of ROS generated by the mitochondria and the endoplasmic reticulum. An excess of ROS facilitates ER dysfunction, enhances mitochondrial DNA damage, and impairs the mitochondrial membrane integrity, leading to cytochrome c release and mitochondrial cell apoptosis. This stress condition impairs the β-cell function and survival. It decreases mRNA expression of genes implicated in the β-cell function (proinsulin) and enhances its degradation pathway. It induces the cell death pathway through the activation of proapoptotic kinases (mitogen-activated protein kinases) such as c-Jun N-terminal kinase. Chronic hyperglycemia induces TXNIP expression, and ROS dissociate the TXNIP-Trx complex. The dissociation of the complex increases the TXNIP availability, which impairs several regulatory systems such as muscles and the pancreas. (i) It activates the NLRP3 inflammasome, leading to the liberation of the active forms of the proinflammatory cytokines IL-18 and IL-1β. (ii) It decreases the cellular glucose uptake and (iii) decreases the insulin secretion by β-cells through activation of the expression of an uncoupling protein (UCP-2). The resultant proinflammatory state worsens the cellular environment, leading to the development and progression of metabolic diseases, especially atherosclerosis. (To see this illustration in color, the reader is referred to the Web version of this article at www.liebertpub.com/ars.)

TXNIP in an insulinoma-1E β-cell line is sufficient to prevent β-cell glucotoxicity (227). Considerable attention gained recently on the role of TXNIP in the induction and progression of microvascular complications in diabetes such as diabetic retinopathy and nephropathy. TXNIP mRNA and protein are highly expressed in renal mesangial cells (148, 341), neurons (335), and retinal cells (336). The blockade of TXNIP decreases oxidative stress (336) and protects the diabetic subjects from complications.

Further, several findings highlight the role of TXNIP in adipose tissue, which is considered to be a central metabolic regulator in human metabolism, due to its regulation of

energy balance, insulin sensitivity, and adiposity (72). A nonsense mutation in the *TXNIP* gene has been detected in the mutant mouse strain, HcB-19/deem (HcB-19), characterized by a familial combined hyperlipidemia, suggesting a crucial role of TXNIP in the lipid metabolism (40, 172).

Disruption of TXNIP in diabetic obese mice (ob/ob) ameliorates glucose metabolism through enhancement of its uptake in adipose tissue and skeletal muscle associated with the activation of the insulin receptor substrate-1 (IRS-1)/Akt signaling pathway and glucose-stimulated insulin secretion without amelioration of obesity. In addition to insulin signaling genes, the expression and activity of PPARs and their target genes (72), *Scd1*, *Fabp3*, *Acaca*, and *Pdk4*, were increased, especially in skeletal muscle, the liver, and adipocytes of $TXNIP^{-/-}$ mice, suggesting their involvement in the amelioration of insulin responsiveness (469). Li *et al.* investigated the defense mechanisms against metabolic stress induced by free fatty acids to identify new therapeutic targets for CVD (234). They found that AMPK is the relevant signaling pathway reducing the intracellular excess of oxidative stress through upregulation of the Trx1 protein. In fact, AMPK directly phosphorylates the residues of the C-terminal domain of the Fork-head transcription factor 3 (FOXO3) (134), thereby inducing its nuclear translocation. After activation, FOXO3 directly binds to the *Trx1* promoter and forms a transcriptional complex, increasing the expression of Trx1 and protecting cells from metabolic stress (234).

VIII. The Trx System in Immunity

Trx1 was initially identified as a soluble factor synthesized by HTLV- or EBV-transformed lymphoblastoid cells playing a key role in the growth stimulation of transformed T- and B-cells (211, 464). The *Trx1* gene expression is upregulated by the prototypic Th1-cytokine IFN-γ. This regulation is mediated through the Jak, PI3K/Akt, and ERK pathways and induces activation of distinct transcription factors. Under stress conditions, Trx1 protects the immune cells from apoptosis and counter-regulates the production of IL-4 and IFN-γ, thereby maintaining the immune cell homeostasis (211). Regulatory T-cells express Trx1 as a major antioxidative molecule, which is critical for maintaining a high density of their thiol surface as the first line of the antioxidative defense pathways (288).

TXNIP is highly expressed by immune cells, and it plays a regulatory role in the immune system (317). Natural killer (NK) cells are a type of cytotoxic lymphocytes playing a major role in the innate immune system defending against virus-infected cells and tumor cells (55, 141). During NK cell maturation, TXNIP regulates the transcription of the common receptor-beta subunit (CD122) of IL-2/IL-15 (71), the expression of which is an important factor for lineage specification and differentiation of NK cells (316). Reduction of NK cells in $TXNIP^{-/-}$ mice prevents the expression of cytotoxic T-lymphocytes and depletion of the lymph node and splenic CD8+ cell activity (71). On the other hand, the number of immune cell subsets in intraepithelial lymphocytes and lamina propria lymphocytes is highly increased in $TXNIP^{-/-}$ mice (71). This increase has been explained by the regulatory functions of TXNIP in cell cycle progression and proliferation (71, 150). Dendritic cells are potent antigen-presenting cells that possess the ability to stimulate naïve T cells that play a crucial role as messengers between the innate and adaptive immunity (14). TXNIP is a regulator of DC-mediated T-cell activation, specifically of IL-12p70, IL-12p40, and IL-6 expression (395). Vitamin D_3 is involved in the immune system as an autocrine and paracrine signaling system (435). Its biological effects are mediated by the vitamin D receptor, which is highly expressed in macrophages, DC, and in both $CD4^+$ and $CD8^+$ T-lymphocytes (395). Several reports highlight the crucial effects of vitamin D_3 and its receptor agonists on the phenotype and the function of immune cells, especially DC (3, 135), for which TXNIP protein appears to be dispensable for the signal transduction pathways (395).

Using Tg mice that overexpress Trx1, it was found that the release of histamine from mast cells was suppressed, suggesting a protective effect of Trx1 against allergic inflammation (394).

IX. The Trx System and Cancer

The role of Trx1 in cancer is controversial. Indeed, there are two opposite views, one of which implicates the elevated levels of Trx1 as a stimulator of cancer cell growth, whereas the other defines the elevated expression of the Trx1 as a response to the significantly increased oxidative stress. Several studies revealed an overexpression of TXNIP in many forms of cancers, such as nonsmall-cell lung cancer (208, 392), renal cell carcinoma (237), melanoma (240), thyroid cancer (239), breast carcinoma (56, 174), cervical carcinoma (160), and colorectal cancer (344) (Fig. 13). Further, the Trx system is essential for cancer cell survival and growth by enhancing the sensitivity to other growth factors and/or inhibition of spontaneous apoptosis and decreased sensitivity to drug-induced apoptosis. In addition, Trx1 induces HIF-1α, which increases the production of VEGF and leads to tumor angiogenesis and drug resistance (198). Moreover, high expression levels of both Trx and TrxRs have been reported in many cancer cells, including neoplastic liver cells, suggesting that the increase in cytosolic TrxR1 is important for survival and the promotion of cancer progression (390).

On the other hand, the endogenous inhibitor of Trx1, TXNIP, which also possesses tumor-suppressive functions, is dramatically downregulated in various human cancers, including bladder cancer. Thus, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN)-induced bladder cancer was found in 100% of $TXNIP^{-/-}$ mice at week 8 of BBN administration, but only in 22% of wild-type mice at the same time (470). Moreover, in $TXNIP^{-/-}$ mice, the immunohistochemical analysis showed enhanced expression of the C-X-C chemokine receptor type 4 (CXCR4), the receptor of cell-derived factor-1 (SDF-1), and of phosphor-extracellular signal-regulated kinase (pERK) in urothelial cells during BBN-induced bladder carcinogenesis (470). Subcutaneous injection of the CXCR4 antagonist, TF14016, attenuated pERK in urothelial cells and suppressed bladder cancerogenesis (470). This result indicates that TXNIP negatively regulates bladder carcinogenesis.

However, to date, there is no evidence showing Trx-induced tumorigenesis. Instead, Trx1-overexpressing Tg mice are functionally normal and do not show any increase in malignancies (286, 404, 468). Additionally, it is well known that the Trx system is involved in the antioxidant defense and probably in the prevention of cancer *via* removal of carcinogenic oxidants or by repair of oxidized proteins (Fig. 13) (18).

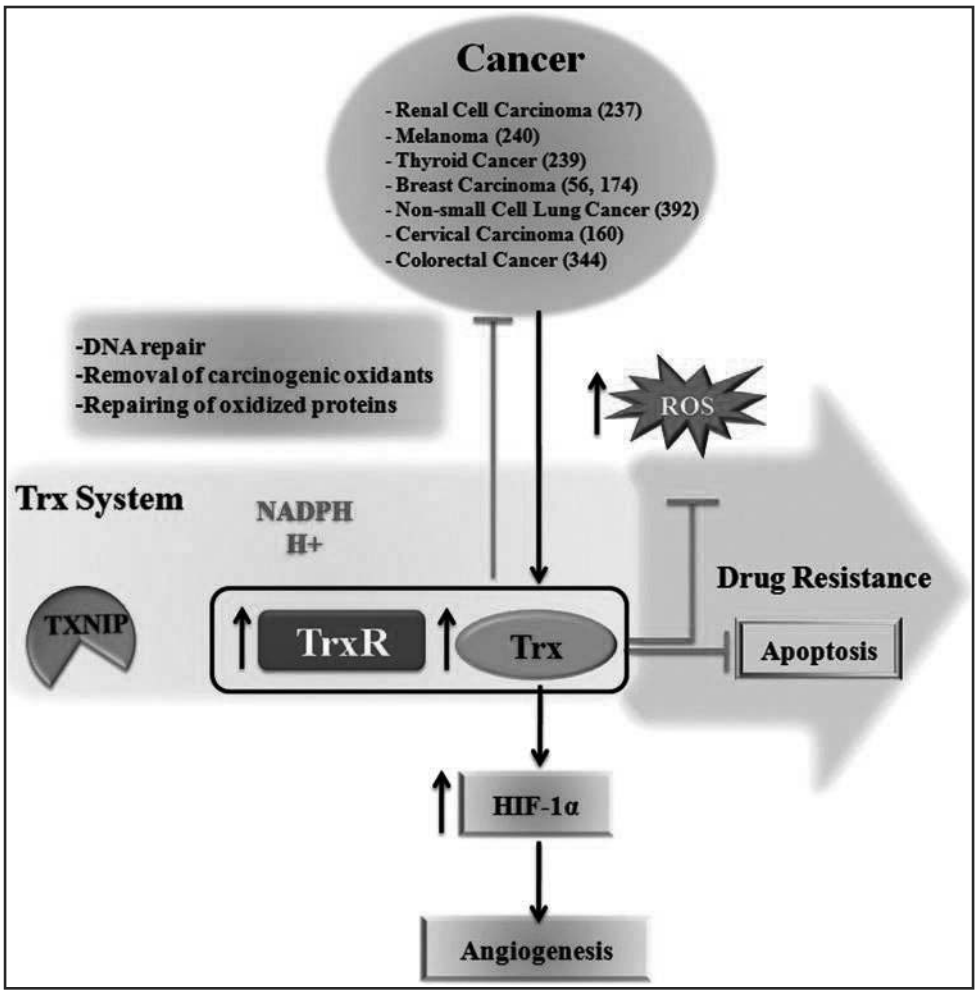

FIG. 13. The role of the Trx system in cancer. Trx can act as anticancer agents by protecting cells from carcinogenic oxidants, ROS, or by repairing oxidized proteins, whereas it can also protect and enhance angiogenesis in different types of cancers by upregulating HIF-1α. Since almost all anticancer drugs depend on increased ROS levels, targeting the Trx system could be of a therapeutic interest during chemotherapy. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars.)

X. The Trx System in Neurodegenerative Diseases

Neurodegenerative diseases are characterized by a progressive neuronal dysfunction that initially affects selected groups of neurons in specialized neuronal circuits. Alzheimer's disease (AD), frontotemporal dementia, Parkinson's disease (PD), and other neurodegenerative disorders are a major health problem in both developed and developing countries (122). Since the brain utilizes a high amount of oxygen and contains oxidizable polyunsaturated fatty acids and redox-active metals, it is highly vulnerable to oxidative damage. Oxidative stress increases with age and can therefore be considered as an important causative factor in several neurodegenerative diseases, typical for older individuals (432). Consistently, the available evidence suggests that the altered redox homeostasis and the increased oxidative stress, which have been primarily implicated in the pathogenesis of multiple sclerosis, are triggers for the activation of a brain stress response (330).

A. Alzheimer's disease

Alzheimer's disease represents the most common neurodegenerative disease, accounting for more than 50% of all types of dementia (337). Within the United States alone, the prevalence estimates indicate that AD affects 2.4 million individuals aged 70 and older. With increasing age, AD progressively affects more individuals (43, 95). Oxidative stress has been consistently linked to aging-related neurodegenerative diseases, which are characterized by progressive dysfunction and death of neurons. Additionally, oxidative stress is also associated with the dysfunction of the mitochondria and endoplasmic reticulum, inducing protein misfolding and neuronal apoptosis (126). The role of Trx1 in AD seems to be important, since the brain tissue of patients suffering from AD shows low Trx1 levels (272, 405) and a marked accumulation of the β-amyloid peptide (245). Akterin *et al.* studied the levels of Grx1 and Trx1 in the AD brain as well as their effects on β-amyloid peptide neurotoxicity. They have reported a reduced Trx1 expression in both the frontal cortex and the hippocampus CA1 regions of patients suffering from AD. Further, in human SH-SY5Y neuroblastoma cells, treatment with the β-amyloid peptide caused a strong and early oxidation of both Grx1 and Trx1 that was transient with time, whereas overexpression of Trx1 completely protected cells from the β-amyloid peptide toxicity as indicated by the cell viability (9). Surprisingly, compared to normal brain tissue, Trx-80 levels were drastically reduced in AD, even in the areas with abundant inflammatory changes, suggesting that Trx-80 deficiency could be a specific feature of the disease (128). The same authors also showed that Trx-80 is able to inhibit β-amyloid aggregation *in vitro*, as well as the toxicity of β-amyloid in SH-SY5Y cells, indicating that improving the

levels of Trx1, its activity, or cleavage to Trx-80 might be beneficial to counteract the toxic effects of β-amyloid (128). In fact, this role of Trx-80 depends basically on its physical property rather than on its function as a cytokine, opening new insights into further roles of this molecule that await exploration.

To study the role β-amyloid protein in the pathogenesis of age-related macular degeneration and glaucoma, Lamoke *et al.* have used a Tg mouse overexpressing β-amyloid. In this model, it was shown that the TrxR1 activity was drastically reduced at 14 months of age, resulting in the loss of the Trx1 function (221). Further, during aging and loss of the antioxidant function, both the hippocampus and frontal cortex are subject to oxidative stress and consequently to selective neuronal degradation (436). Additionally, the role of Trx1 in the nervous system is not only defined through its antioxidant action but also through the enhancement of the effect of nerve growth factor and other signal transduction pathways (Fig. 14) (266). These findings imply that an acquired or genetic dysfunction of Trx or TrxR could predispose neurons for degeneration (379).

PRXs, together with Trx and TrxR, represent an antioxidant enzymic system of growing significance in the context of neuronal physiology and pathology. Overexpression, knockdown, and knockout approaches have demonstrated an important role for PRXs in protecting neurons from oxidative insults. It is also becoming clear that neuronal PRXs are subjected to post-translational modifications that impair their function as a part of disease pathology. Conversely, components of this pathway are also subject to dynamic upregulation, for example, *via* endogenous synaptic activity-dependent signaling and induction of the Nrf-2-dependent phase II response. As such, the Trx-PRX system represents a potential therapeutic target for the central nervous system disorders associated with oxidative stress (29). Further, the data suggest a signaling pathway by which PRX2 suppresses ischemia-induced neuronal apoptosis. Enhanced neuronal expression and activity of PRX2 protect against ischemic neuronal injury by directly modulating the redox-sensitive Trx-ASK-1 signaling complex (62).

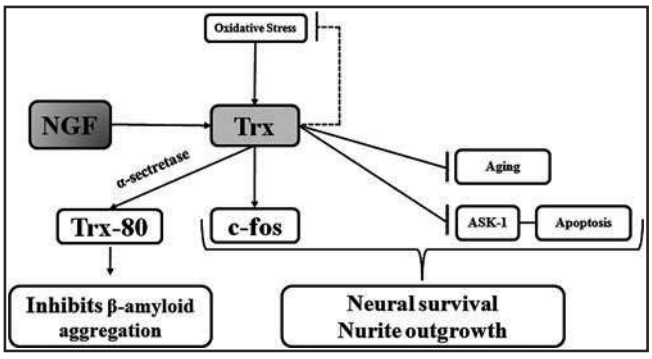

FIG. 14. The role of Trx in Alzheimer's disease. Trx plays a role in the nervous system not only through its anti-apoptotic, antiaging, and antioxidant actions, but also by enhancing the effect of nerve growth factor (NGF) and signal transduction pathways. NGF has profound effects on neurons, including promotion of survival and differentiation through multiple signaling pathways such as c-fos. Further, Trx can be cleaved by α-secretase producing Trx80, which inhibits β-amyloid aggregation, consequently reducing its toxicity.

B. Parkinson's disease

PD is the second highly prevalent disease, after AD, affecting 0.3% of the general population and 1%–3% of the population older than 65 (77, 86). To date, several genetic forms have been identified causing both dominantly and recessively inherited PD, but environmental factors may also contribute to the disease risk (177). As of yet, there are numerous animal models, ranging from toxins, such as 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which induce dopaminergic neuronal loss, to Tg models with null mutations of *Parkin*, *DJ-1*, and *PINK1* or point mutations of genes located in different PARK loci that carry the same genetic mutations as patients suffering from familial PD. For the dominantly inherited gain-of-function mutations such as in α-synuclein and leucine-rich repeat kinase 2 (*LRRK2*), the Tg mouse models have been created, in which extra copies of the gene have been introduced into the mouse genome (412). Although each model represents different aspects of PD, none of these models manifest the full spectrum of the PD pathology (173). PD is characterized by a chronic, progressive, and profound loss of neuromelanin-containing dopaminergic neurons in the pars compacta of the substantia nigra with the presence of eosinophillic, intracytoplamic, proteinaceous inclusions termed as Lewy bodies and dystrophic Lewy neurites in surviving neurons (112). The cells of the substantia nigra use the neurotransmitter dopamine to communicate with the neurons of the stratium. Thus, a reduction in the nigral dopamine levels results in a decrease in stratial dopamine that is believed to cause PD symptoms. A majority of studies explored the effect of oxidative stress that contributes to the cascade of events leading to dopaminergic neuron degeneration in PD (432). Further, monoamine oxidase (MAO), which catalyzes the oxidative deamination of dopamine and other monoamine transmitters, is considered to be associated with a selective loss of dopamine neurons in the substantia nigra (75, 302, 303). Metabolism of monoamines by MAO is a major source of H_2O_2, which is normally inactivated by GSH peroxidase, but it can be converted in a Fenton reaction in the presence of Fe^{2+} ions into the highly reactive hydroxyl radical. This radical has widespread deleterious effects, which can cause neuronal damage and death. When GSH levels are low and MAO and iron are increased, the possibility of a diversion of H_2O_2 *via* the Fenton reaction is correspondingly increased, with consequent increases in oxidative neuronal damage (472). Additionally, MAO-B can produce a neurotoxin (1-methyl-4-phenylpyridinium ion [MPP^+]) from the protoxin (MPTP), which causes a severe and irreversible PD-like syndrome in animals and humans. MPP^+ decreases the Trx1 expression, and its overoxidation (65) induces apoptosis *via* a caspase-12-dependent pathway, and inhibits the mitochondrial respiration at complex I, whereas Trx1 overexpression *in vitro* and *in vivo* affords a cytoprotective and neuroprotective function against MPTP (24, 25).

It has been demonstrated that PRX2, the most abundant neuronal PRX, plays a critical role in protecting dopaminergic neurons from PD-relevant toxin-induced cell death by regulating the Trx1–ASK-1 interactions and inhibiting the

subsequent activation of the ASK-1-dependent cell death pathways (171).

DJ-1 is a multifunctional protein that acts as an antioxidant, a transcriptional regulator, and a stabilizer of nuclear factor erythroid-2 related factor 2 (Nrf-2), and its loss is leading to neurodegeneration (41). Further, an oxidative modification of DJ-1 has been detected in brain tissue of patients suffering from PD and AD as well (68). DJ-1 can act as a redox sensor that protects the cells against oxidative stress by promoting the Akt pathway and by suppressing ASK-1. It has been shown that DJ-1 coimmunoprecipitates with ASK-1 upon H_2O_2 exposure after dissociation of Trx1 (133). The direct binding of DJ-1 to ASK-1 is through its Cys-106 with the N-terminus of ASK-1, forming mixed disulfide bonds (438).

Recent findings demonstrate that DJ-1 protects cells from oxidative stress by inducing Trx1 expression *via* the transcription factor Nrf-2 (Fig. 15). DJ-1 activates Nrf-2 by increasing its protein levels, promoting its nuclear translocation and enhancing its binding to the ARE consensus sequence in the *Trx1* promoter. Thus, in the presence of DJ-1, the Nrf-2-mediated increased Trx1 levels help cells to cope up with oxidative stress (177).

XI. Trx System in RA

RA is a systemic and chronic inflammation (125) characterized by a progressive damage in the synovial membrane of joints (321) and massive infiltration of a variety of immune cells, including macrophages, T-cells, B-cells, and dendritic cells. This inflammatory process is associated with an excessive production of cytokines, chemokines, and growth factors (108), which contribute to synovial hyperplasia and cartilage and bone erosion (321). ROS, the key drivers of RA pathogenesis, are excessively produced in the site of synovitis leading to the dysregulation of the cellular redox state (194).

Compared with other joint diseases, such as gout, osteoarthritis, and reactive arthritis (187, 273, 467), Trx1 expression is significantly increased in the synovial fluid of inflamed joints (229) and the serum of RA subjects (187, 273). Thus, the anti-inflammatory protein Trx1 can be considered as an important biomarker of the disease pathogenesis, indicating a significantly elevated level of oxidative stress among patients suffering from RA (187). To regulate the redox state, Trx1 inhibits the induction of IL18/IL-2 or induces the extravasation of inflammatory cells into the sites of inflammation (228, 294). Matrix metalloproteinase (MMP) enzymes are upregulated in RA and degrade all components of the extracellular matrix (44). Trx1 activates the TRP family through donation of electrons to the cysteine residues of the TRPC5 subclass. The ion channel activation suppresses the MMP expression, suggesting a protective role for Trx1, which provides opportunities to develop therapeutics against the RA progression, especially cartilage and bone degradation (28). Reduced Trx1 and TrxR1 levels have been implicated in the hyperplasia of synovial cells and their contribution to the persistence of the radical stress conditions and inflammation (194). To reduce ROS-induced oxidative stress (230), TrxR promotes SOD activity, regenerates ascorbate, and detoxifies H_2O_2 and lipid peroxides (38, 83, 274). In addition, TrxR inhibits synovial cell apoptosis caused by ROS, which are expressed by neutrophils, macrophages, and synovial cells (273). Despite the progress and evidence supporting the role of TrxR as an important effector in the inflamed synovial cells, target interactions and the exact function in the progression of RA need a more detailed exploration (194). According to Maurice *et al.*, exogenous H_2O_2 and excess TNFα levels in the joints of patients suffering from RA increase the expression of Trx1. At the inflamed site, the proinflammatory cytokine TNFα induces Trx1 expression in fibroblast-like synoviocytes (273). Further, a recent study illustrates that the Trx/TrxR system is upregulated in an inflammation-independent manner. At the first stage of the disease, TrxR is expressed under the endogenous oxidative stress environment in response to the proliferation of synovial cells. However, in later-stage RA, the Trx/TrxR system is involved in the survival of the synovial cells (194).

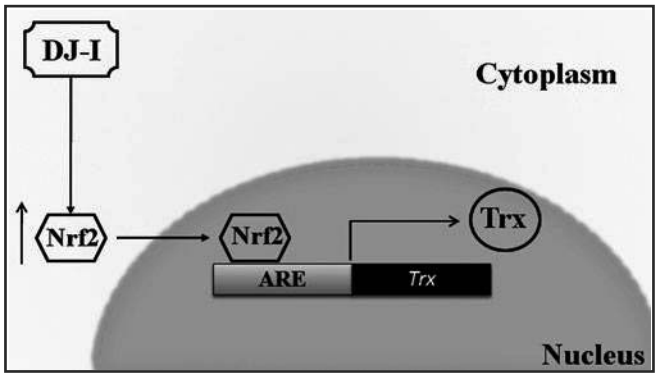

FIG. 15. Dj-1 protects cells from oxidative stress by inducing Trx1 expression *via* the transcription factor Nrf-2. DJ-1 proteins are multifunctional proteins that play roles in oncogenesis, male fertility, and control of protein–RNA interactions. Further, they protect the cells from oxidative stress by inducing Trx1 expression *via* factor Nrf-2.

XII. Trx in Inflammatory Airway Diseases

Airway epithelial tissue is an important physical barrier (111) that protects the lungs from stimulation by the large variety of inhaled stress stimuli such as irritants (111), allergens (70), and oxidants (210). The oxidant/antioxidant imbalance and inflammation are the key mechanisms in the development and manifestation of pulmonary diseases such as asthma (426). Bronchial asthma is a complex airway inflammation (458) resulting from excess of mucus secretion, edema, and infiltration of immune cells, including T-lymphocytes, eosinophils, and other inflammatory cells (458). After recruitment into the epithelial tissue, eosinophils release ROS such as superoxide and H_2O_2 and cytotoxic products, especially eosinophil granule proteins, leading to hyperresponsiveness. To reduce the epithelial damage processes, the inflamed lungs induce the cellular antioxidant defense mechanisms (415) involving nonenzymatic antioxidants (GSH, vitamins C, and E), enzymatic antioxidants (superoxide dismutases and catalase), and other proteins such as heme oxygenase-1 and PRX (210). The serum Trx1 levels are highly correlated with asthma exacerbation and allergic inflammation (458). Pneumocytes and bronchial epithelial cells synthesize Trx1 (415), which directly enters the cells to suppress the hyper-responsiveness through its anti-inflammatory, antioxidant, and cytoprotective lung defense properties (301).

In fact, it indirectly suppresses the development of airway remodeling through inhibition of chemokines and Th2 cytokine expression by Trx1-induced Th1 cytokines (176). On the other hand, it directly inhibits the airway hyper-responsiveness and reduces allergic inflammation by inhibition of lymphocyte functions and eosinophil chemotaxis through the suppression of the CC chemokine (217).

In addition, Trx1 inhibits the eosinophil-chemoattractant cytokine eotaxin-stimulated activation of the ERK1/2 and p38 MAPK signaling pathway through the regulation of the eotaxin/CCR3 signaling pathway, which is considered as an important mechanism in cell chemotaxis (217). In hyperresponsiveness, which is a key feature of bronchial asthma, Trx1 can also suppress and scavenge intracellular ROS synthesized by recruited cells into the airway epithelium (219). It upregulates the expression of IL-1α, IL-1β, IL-1 receptor antagonist (176, 178), T-helper 1 cytokines, and IL-18 (301), enhancing the Th1- and Th2-driven immune responses (301). In addition, it inhibits the production of lung proteins such as macrophage inflammatory protein-1α, IL-13, and eotaxin (176, 178) through the modulation of MIF production (183). Regarding its properties and capacities, the Trx1 protein may serve as a target molecule for further therapeutic investigations. Through its antioxidant and anti-inflammatory properties, Trx1 plays a crucial role in the inhibition of inflammatory processes and the regulation of the cellular redox status in respiratory diseases. It has been shown that in chronic obstructive pulmonary disease, Trx1 moved into the nucleus and enhanced the NF-κB-DNA binding activity in an oxidative stress-dependent manner (301). Protective properties of Trx1 play a key role in modulation of the development of acute lung injury (48). In interstitial lung diseases, Trx1 suppressed IL-18/IL-2-induced interstitial cell infiltration to prevent the lung tissue damage (181). Rancourt *et al.* studied the Trx1 effect on the rheological properties of mucus and sputum obtained from patients with cystic fibrosis (348). They showed that treatment with Trx1 changes the rheological properties, enhances the liquid fraction, and diminishes the viscoelasticity of the cystic fibrosis sputum. Thus, the development of the mucus-reducing systems has the potential to reduce viscosity and to treat cystic fibrosis (348). In the lung transplantation, Trx1 might act as a biomarker (456) to evaluate the severity of graft rejection (323). In viral pneumonia (456), Trx1 has been demonstrated to play a fundamental defense role against a viral pneumonia infection through regulation of ROS expression caused by the influenza virus and through the modulation of the redox-dependent signal transduction (300). Despite these important findings, further studies are necessary to clarify the mechanisms and functional roles of Trx1 involved in systemic inflammation and the pathology of pulmonary diseases.

XIII. The Trx System as a Therapeutic Target

As discussed above, redox control is an important determinant of cellular function and viability. The Trx system, highly conserved and ubiquitous in all cells, is a major antioxidant system. In addition to its direct antioxidant activity, it stimulates cell growth, inhibits apoptosis, activates numerous transcription factors, and regulates the immune function. Therefore, it is involved in a whole range of pathophysiological conditions representing a promising therapeutic target (80). High levels of Trx1 expression have been associated with aggressive cancers, poor patient prognosis, and resistance to some chemotherapy treatments. In contrast, low levels of Trx1 can cause diseases that develop because of an imbalance between the antioxidant systems and oxidative stress (416). Generally, the therapeutic approaches can be either through the addition of recombinant human thioredoxin-1 (rhTrx1), using gene delivery, inhibitors/inducers, or mutant Trx1 molecules that target specific pathways or that exhibit increased stability using synthetic molecules.

A. Thioredoxin

Since rhTrx1 was shown to possess antioxidant, anti-inflammatory, and antiapoptotic functions that demonstrate its cytoprotective effect, Trx1 may be an intriguing therapeutic agent for the diseases related to oxidative stress, acute inflammation, and apoptosis/necrosis to which most of the CVDs are related (7). Cardiac myosin-induced myocarditis is an experimental autoimmune myocarditis (EAM) model used to investigate the autoimmunological mechanisms in inflammatory heart diseases. It resembles fulminant myocarditis in humans, and rhTrx1 was found to attenuate EAM by suppressing chemokine expression and leukocyte chemotaxis in mice (242). Additionally, this anti-inflammatory action of Trx1 may be useful for the treatment of diseases such as the acute respiratory distress syndrome (238). Exogenous Trx also protects the cells from H_2O_2 or hypoxia (182). Intravenously administered Trx1 attenuates the I/R injury in animal models. Consistently, circulating Trx1 suppresses neutrophil adhesion on endothelial cells and extravasation into the inflammatory sites (295).

Moreover, exogenously administered Trx1 inhibits neutrophil chemotaxis, prolongs survival, and limits the pathology in animal models of I/R (118, 386, 408, 475) and sepsis (164).

One study also showed that Tg overexpression, or injection of recombinant Trx1, protects mice from choroidal neovascularization in a laser-burn model of age-related macular degeneration. The therapeutic effect of Trx1 was found to involve the interaction with factor H and inhibition of complement activation and leukocyte infiltration (179).

Intravenous infusion of rhTrx1 (10 mg/kg), commencing at the start of reperfusion, reduced the infarct volume and improved the neurological outcome in a murine model of transient focal ischemia (158). Interestingly, *S*-nitrosylated rhTrx1 shows better cardioprotective effects against the ischemic hearts in a mouse model than its non-nitrosylated counterpart (408, 409). Further, continuous intravenous administration of rhTrx1 suppresses the LPS-induced bronchoalveolar neutrophil infiltration by an antichemotactic effect. Administration of rhTrx1 did not promote the tumor growth nor did it affect the chemosensitivity in the xenotransplantation model, suggesting the potential safety of rhTrx1 therapy for cancer patients (426).

Transglutaminase 2 (TG2) is an inducible transamidating acyltransferase that catalyzes Ca^{2+}-dependent protein modifications, and it acts as a multifunctional molecular player in various cellular processes, ranging from intracellular signaling to apoptosis and pathological conditions such as autoimmune and Huntington disease (107). Jin *et al.* have reported that rhTrx1 can recognize and reduce the disulfide bond of TG2 with a high specificity to activate its oxidized form (191).

As mentioned earlier, the therapeutic effects of Trx1 have been observed in various animal models. In pancreatic β-cells, specific expression of Trx1 prevents autoimmune and streptozotocin-induced diabetes (169). Trx1 overexpression in Tg mice attenuates both focal ischemic damage (404, 405) and adriamycin-induced cardiotoxicity (387), and protects against influenza virus-induced pneumonia (300).

The increased level of Trx1 seen in many human cancers may hamper the therapeutic efficacy by scavenging the ROS involved in the mechanism of chemotherapeutic drugs or radiation (238).

Several inhibitors of the Trx system have been identified [see (289)] that could be either nonspecific, for example, the nitrosoureas, or specific such as compounds of the PX12 series and quinol compounds. Auranofin, chloro(triethylphosphine) gold, and aurothiomalate can inhibit TrxR2, causing a mitochondrial membrane permeability transition (353). PMX464, a benzothiazole-substituted quinol compound, targets Cys32 and Cys35 of the Trx1 active site (448), exerting antiproliferative effects on the tumor cell lines as well as endothelial cells (290).

Generally, unsymmetrical 2-imidazolyl disulfides can alter the cellular redox state and can also inhibit the growth of cancer cells *in vitro*. Moreover, these molecules show an antitumor activity *in vivo* against human tumor xenografts in SCID mice (215, 314). These effects can be explained by their abilities to interact with both Trx1 and TrxR1, as substrates or inhibitors depending upon the structure of the thiolating substituents. The reaction of disulfides with the conserved catalytic-site cysteine residue of Trx1 causes a slower thiolation of Cys73 of Trx1 outside the conserved catalytic site. This Cys73-thiolated Trx1 is not a substrate for the reduction by TrxR1 (214). The semisynthetic Trx inhibitor, 1-methylpropyl 2-imidazolyl disulfide (PX-12), is currently being explored in clinical trials. It irreversibly thioalkylates the Cys73 residue of Trx, and upon *in vitro* data, it might have a promising *in vivo* antitumor activity. PX-12 inhibition of Trx1 results in subsequent inhibition of the hypoxia-induced increase in HIF-1α protein and VEGF secretion (449). It was tested in phase I pharmacokinetic and pharmacodynamic studies in patients with advanced solid tumors and was found to lower plasma Trx1 concentrations in a dose-dependent manner. PX-12 was tolerated up to a dose of 226 mg/m^2 by a 3-h infusion (347). However, a randomized phase II study of PX-12 in patients with advanced cancer of the pancreas after progression after a gemcitabine-containing combination resulted in the lack of a significant antitumor activity. Although these patients had unexpectedly low Trx1 baseline levels, PX-12 does not appear to be active in unselected patients with previously treated advanced pancreatic cancer. Accordingly, the study was terminated (26, 346).

Another interesting group of molecules that target the Trx system are histone deacetylase (HDAC) inhibitors, such as suberoylanilide hydroxamic acid (SAHA). SAHA can trigger both mitochondrion-mediated apoptosis and caspase-independent autophagic cell death, since it can upregulate TXNIP and downregulate Trx1 (45). HDAC inhibitors increase the Trx1 levels in normal cells, but not transformed cells, which is likely to be one of the reasons why HDAC inhibitors preferentially kill cancer cells (260). The mechanism underlying this selectivity could be due to the SAHA-induced increase in the Trx1 activity in normal cells that is not associated with any increase in ROS accumulation. Transfection of transformed cells with Trx1 small interfering RNA caused a marked decrease in the level of Trx1 protein with an increase in ROS, a decrease in cell proliferation, and an increase in sensitivity to SAHA-induced cell death. Thus, Trx1, independent of the apoptotic pathway, is an important determinant of resistance of cells to the HDACi-induced cell death (429).

Among natural products, polyphenols, curcumin, and some flavonoids, including myricetin and quercetin-like polyphenols, quinones, and terpenoids, have been shown to affect the Trx/TrxR system at different levels (80). As mentioned above, one of the strategies to make use of the Trx system as a therapeutic target is the administration of natural products mainly from edible plants such as fragrant unsaturated aldehydes that induce Trx1 and activate ARE (267). Curcumin, a phytopolyphenol of the Asian spice turmeric (*Curcuma longa*), has been claimed to possess a wide range of beneficial effects in different human diseases [see (132, 157)], primarily through its regulation of several survival and cytoprotective signaling pathways (64, 96, 203, 205, 257, 350, 355). Moreover, this compound can also turn to be a prooxidant in the cells to produce ROS and to induce oxidative stress (151, 220). Interestingly, curcumin can also modify TrxR1 to inhibit its physiological function of Trx1 reduction and further switches this enzyme to an NADPH oxidase generating ROS, and downregulating the level and enzymatic activity of Trx1 (46), which is in contrast to Mandal *et al.*, who reported that after an intense light exposure, curcumin significantly induced the expression of Trx1 in the retinas of curcumin-fed rats (257). These controversial roles of curcumin on Trx1 may be attributed to the differences in the animals models used and the studied tissues.

It has been demonstrated the ability of 3-mer and 4-mer oligopeptides bearing the catalytic motif CXXC of the Trx1 active site, or the modified CXC motif, to efficiently reverse the cellular oxidative stress induced by the disruption of the TrxR-Trx system using auranofin (23). As discussed above, rhTrx1 has been suggested as a new therapeutic approach for the treatment of severe acute respiratory diseases (7, 102, 296). Therefore, Kim *et al.* have investigated the protective properties of a Trx-mimetic oligopeptide, *N*-acetyl cysteine-proline-cysteine amide (CB3), in allergic airway diseases. They showed that injection CB3 was effective in attenuating asthmatic pathological features in the lungs of mice that inhaled ovalbumin. Treatment of OVA-challenged mice with CB3 resulted in the upregulation of the GSH levels, as well as inhibition of the NF-κB and p38 MAPK activity (212).

All mentioned approaches exploring the therapeutic application of Trx1 remain struggling with its bioavailability and its delivery to the target tissues. Therefore, the Trx gene therapy could be one of the candidate techniques. Since Trx1 expression in the liver exerts its effects by conferring resistance to stress and cell death in various types, it has been suggested that the adenovirus-mediated Trx gene therapy could represent a promising approach for liver diseases (421). Another study showed that the infarcted myocardium of rats with streptozotocin-induced diabetes can be significantly improved in terms of myocardial angiogenesis as well as myocardial function by the Trx1 gene therapy (364). Recently, a recombinant adenovirus vector carrying hTrx has been constructed with a virus that can express efficiently Trx in HEK293 cells (170).

B. Trx reductase

A large body of evidence indicates that TrxRs are key players not only in the antioxidant defense and the cellular redox system, but also in growth control and in the selenium metabolism (289) [see (16, 166)]. Therefore, overactivation/dysfunction of TrxRs is closely related to various diseases, especially in tumor development (47). For the same reason, over the last years, an increasing number of research groups became engaged in the discovery and development of compounds acting on TrxR (431) such as gold compounds, platinum compounds, arsenic trioxide, motexafin gadolinium, nitrous compounds, and various flavonoids (417). By now, gold compounds are well-known inhibitors of TrxR (51). Aurothioglucose and auranofin (137) are two gold compounds predominantly used in the treatment of RA, but gold compounds have also been used for a long time in the treatment of other diseases. Aurothioglucose at a dose of 0.025 mg/g body weight was found to give extensive and prolonged TrxR inhibition in all tissues examined using a mouse model—notably, without any evident phenotype (16). In chromaffin cells, auranofin abolishes the depolarization-induced catecholamine release and induces phosphorylation of the MAPK ERK1/2, p38 mitogen-activated protein kinase (p38 MAPK), and JNK (253).

Most cancer cells have a high level of expression of Trx and TrxR, which has been assumed to be a protection against apoptosis and to promote cell growth (27, 297, 340, 431). As many tumors expressing elevated levels of TrxR are resistant to chemotherapy, TrxR targeting may contribute to prevent or reverse the resistance mechanisms. There is evidence that the expression of TrxR correlates with the apoptotic resistance in various cancer cell types (431). In line with that, several cancer drugs are known inhibitors of TrxR (54, 100, 103, 240, 304, 466) such as cyclophosphamide (441, 452), nitrosoureas, cisplatin (19), diaziquone, doxorubicin (271), motexafin gadolinium (156), arsenic trioxide (247), and potential cancer chemoprevention agents such as curcumin (103), or the flavonoids quercetin and myricetin (249). Ifosfamide inhibits the TrxR activity *in vivo* and leads to a dramatic deceleration in tumor progression. The inhibition of TrxR activity is relatively specific, since other antioxidant parameters were not significantly altered. Interestingly, the ifosfamide-treated cancer cells with their decreased TrxR activity lost tumorigenicity after being inoculated into mice (442). Further, as a downstream effect of TrxR1 inactivation, ethaselen caused a dose-dependent Trx oxidation and enhanced the levels of the cellular ROS in A549 cells, suggesting ethaselen as the first selenium-containing inhibitor of mammalian TrxR1 (440).

As mentioned earlier, increased levels of Trx have been implicated in resistance to certain chemotherapeutic agents, rendering the combination of such drugs with the Trx/TrxR inhibitors an attractive option. Smart *et al.* (391) combined ionizing radiation and the TrxR inhibitor IV-2 (an imidazolyl disulfide) and demonstrated that the clonogenic survival of HeLa cervical cancer cells was significantly more decreased by the combination therapy than with radiation alone. They also demonstrated in overexpression studies that a HeLa cell line with a Cys–Ser mutation of TrxR was significantly more sensitive to radiation than cells with wild-type TrxR when pretreated with the Trx inhibitor IV-2. These results identify Trx/TrxR as potential targets, whose inhibition enhances the cytotoxic effects of radiation (289). Targeting such an inhibitor to TrxR of *Plasmodium falciparum* can provide an attractive target for selectivity and a rational drug design, because *P. falciparum* TrxR, in contrast to mammalian TrxR, does not contain selenocysteine (413)

The tumor suppressor p53 is also a substrate for TrxR, and in the cells with C-terminal mutants of TrxR lacking the catalytic selenocysteine, p53 is conformationally deranged, leading to induction of apoptosis (52). The TrxR1 levels are increased in some human tumors (240), and in astrocytoma, they are associated with the tumor grade (140). The knockdown of TrxR1 with siRNA inhibits the growth and metastasis of lung tumors in mice (465). The evidence for TrxR1 as a causative factor for cancer is not as strong as for Trx1, and its role as a cancer drug target is largely as a surrogate target for Trx1 (338). The selectivity of an inhibitor is obviously one major hurdle in the pursuit of a successful TrxR inhibitor as a therapeutic drug. The majority of the inhibitors described above are targeting the selenol/thiol within the C-terminal redox-active site. Since the Cys thiol is essential in many enzymes, the specific inhibition of TrxR without detrimental effects on other enzymes is urgently needed. TrxR is a dimeric enzyme, and developing molecules that disrupt the dimer formation might be a novel strategy for finding highly specific TrxR inhibitors (47). With some drugs, the enzyme is actually converted to an NADPH oxidase, promoting cell death (17, 238).

C. Trx-interacting protein

As a natural inhibitor of Trx1, TXNIP represents an interesting therapeutic target. Its roles have been reviewed in the previous sections in different pathologies such as metabolic syndrome, inflammatory disease, and CVDs. TXNIP is an early-response gene highly induced by diabetes and hyperglycemia, and recently, it has been demonstrated that high glucose levels sustain TXNIP upregulation in the Muller glia and evoke a program of cellular defense/survival mechanisms that ultimately lead to oxidative stress, ER stress/inflammation, autophagy, and apoptosis, identifying TXNIP as a potential target to ameliorate blinding as complications of diabetic retinopathy (89). Further, TXNIP has also been reported to act as a transcriptional repressor (150, 282), suggesting that *TXNIP* could be a tumor suppressor gene, which is commonly silenced by genetic or epigenetic mechanisms in cancer cells. TXNIP is upregulated by vitamin D_3 (66), SAHA (45), 5-fluorouracil (406), deprivation of serum, or IL-2 in the cell culture (8, 308), and further by carcinogens (94) and some stress stimuli such as H_2O_2, TGF-β (150), UV, and heat shock (209). Point mutations or knockout of the *TXNIP* gene in a mouse model is associated with a higher incidence of hepatocellular carcinoma (382). Therefore, reactivation of TXNIP expression by various approaches could be a new way for targeting cancer cells.

On the other hand, it has been shown that the TXNIP deficiency impaired the activation of the NLRP3 inflammasome and the subsequent secretion of IL-1β (478). Therefore, inhibitors targeting TXNIP/NLRP3 to diminish inflammasome activation might represent an interesting therapeutical approach against inflammatory complications associated with tissue damage, metabolic stress, and infection (13, 20, 37, 115, 259, 433).

XIV. Conclusions

The ubiquitous Trx system comprises Trx and its truncated form (Trx-80), TrxR, NADPH, and TXNIP. It regulates not only cellular redox homeostasis and acts as a principal antioxidant defense system, but it also affects energy metabolism, modulates the immunological response, and controls cell growth and survival. On the background of this wide range of effects, it is not surprising that it plays an important role in health and disease. Knockout of either Trx or TrxR is embryonically lethal, revealing their crucial roles during embryonic development. However, the functions of those components are not limited to this stage of development, but they also participate in pathologic conditions such as CVDs, metabolic syndrome, cancer, aging, neurodegenerative diseases, inflammatory airway diseases, and RA. Further, TXNIP can have a unique function independent of its role as a negative regulator of Trx. Therefore, this system represents a promising target for therapeutic interventions employing inhibitors or inducers of the Trx system, rhTrx, gene delivery, or mutant Trx molecules that may target distinct pathways and that may allow targeted pharmacological interventions.

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Abbreviations Used

AAA = abdominal aortic aneurysm
AD = Alzheimer's disease
AP-1 = activator protein-1
ARE = antioxidant-response elements
ASK-1 = apoptosis signal-regulated kinase 1
BBN = *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine
CAD = coronary artery disease
CaMKII = Ca^{2+}–calmodulin-dependent kinase II
CHD = coronary heart diseases
CVD = cardiovascular disease
CXCR4 = C-X-C chemokine receptor type 4
DAMPs = damage-associated molecular patterns
DTT = dithiothreitol
EAM = experimental autoimmune myocarditis
ER = estrogen receptor
ERK = extracellular signal-regulated kinase
FH = familial hypercholesterolemia
FOXO3 = Fork-head transcription factor 3
GSH = glutathione
HDAC = histone deacetylase
HIF-1α = hypoxia-inducible factor-1 α
I/R = ischemia/reperfusion
JNK = C-Jun N-terminal kinases
LDL = low-density lipoprotein
LR = lipid raft
MAO = monoamine oxidase
MAPKs = mitogen-activated protein kinases
MMP = matrix metalloproteinase
MMTS = methyl methane thiosulfonate
MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MsrA = methionine sulfoxide reductase A
NGF = nerve growth factor
NK = natural killer
NLRs = leucine-rich repeat-containing receptors
NLS = nuclear localization signal
Nrf-2 = nuclear factor erythroid-2-related factor 2
ORE = oxidative stress-responsive element
PAMPs = pathogen-associated molecular patterns
PD = Parkinson's disease
PDX-1 = pancreatic duodenal homeobox factor-1
pERK = phosphor-extracellular signal-regulated kinase
PPARγ = peroxisome proliferator-activated receptor-gamma
PRX = peroxiredoxin
PTEN = phosphatase and tensin homology
PX-12 = 1-methylpropyl 2-imidazolyl disulfide
Ref-1 = redox factor-1
RNS = reactive nitrogen species
ROS = reactive oxygen species
SAHA = suberoylanilide hydroxamic acid
Sp1 = specificity protein 1
SpTrx3 = spermatid-specific thioredoxin-3
TBP-2 = Trx-binding protein-2
TD2 = type 2 diabetes
Tg = transgenic
TG2 = transglutaminase 2
TLRs = Toll-like receptors
Tregs = regulatory T-cells
TRP = transient receptor potential
Trx = thioredoxin
TrxR = thioredoxin reductase
TSS1 = transcription start site
TXNIP = Trx-interacting protein
VDUP1 = vitamin D_3-upregulated protein 1
VEGF = vascular endothelial growth factor