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The Roles of CD137 in Mouse Atherosclerosis

마우스 동맥경화증에서 CD137의 역할

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Abstract

The Roles of CD137 in Mouse Atherosclerosis

The tumor necrosis factor receptor superfamily (TNFRSF), which includes CD40, LIGHT, and OX40, plays important roles in the development of atherosclerosis. CD137 (4-1BB), a member of the TNFRSF, is an activation-induced T cell co-stimulatory molecule. Signaling via CD137 up-regulates survival genes, enhances cell division, induces cytokine production, and prevents activation-induced cell death in T cells. CD137 has been reported to be expressed in human atherosclerotic plaque lesions. However, limited information is available on the precise role of CD137 in the development of atherosclerosis using mice models. To study the roles of CD137 in atherogenesis, we generated CD137 deficient apolipoprotein E knockout (*ApoE*$^{-/-}$*CD137*$^{-/-}$) mice and CD137 deficient low density lipoprotein receptor knockout (*Ldlr*$^{-/-}$*CD137*$^{-/-}$) mice. We found that CD137 is a significant atherosclerosis promoting factor mainly expressed on T cells infiltrated into atherosclerotic plaque lesions. Moreover, our finding showed that deficiency of CD137 strongly attenuates formation of atherosclerotic plaque lesion in *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, which implicates CD137 as a potential therapeutic target for atherosclerosis disease control.

Atherosclerotic plaques contain blood-borne inflammatory and immune cells such as macrophages and T cells, as well as vascular cells. Formation of atherosclerotic plaque lesions are characterized by inflammation, lipid accumulation, cell death, and fibrosis. Adaptive immunity, in particular T cells, is highly involved in atherosclerosis. When the naive T cell encounters antigen-presenting cell such as macrophages and dendritic cells that presents an antigenic peptide including oxidized low density lipoprotein (oxLDL), T cells undergo activation and become effector T cells secreting Th1 cytokine such as interferon gamma (IFN-γ), which promotes the development of atherosclerosis. Enhanced T cell activation by co-stimulatory receptors significantly increases inflammatory cytokine release by monocyte/macrophages, further exacerbating inflammation and promoting atherosclerosis. Here, we studied functional mechanisms of CD137 in atherosclerosis. We found that CD137 functionally induces activation of T cell by generation of IFN-γ. In turn, IFN-γ induces monocytes/macrophages and endothelial cells to augment pro-inflammatory cytokine production through a positive feedback mechanism, thereby facilitating formation of atherosclerotic plaque.

Over time, mature plaques advance to a vulnerable plaque, being more prone to rupture, causing subsequent atherothrombotic vascular disease such as myocardial infarction. Vulnerable plaques generally have a large necrotic core, and thin fibrous caps, attributable to the death of macrophages and vascular smooth muscle cells (VSMCs). Vulnerable plaques also contain elevated levels of proteases,

including matrix metalloproteinases (MMPs), which might degrade the extracellular matrix and weaken fibrous caps. Our previous results suggested that CD137/CD137L signaling facilitates atherosclerosis. However, there is no report whether CD137 signaling affects plaque stability of advanced atheroma or not. To answer this question, we tried to elucidate whether CD137 can induce advanced plaque phenotype in atherosclerotic model mice. To solve the mechanism of CD137 in plaque stability, we focused on T cells, macrophages, and VSMCs, which are major cells involved in plaque stability. We found CD137 expression in these cells in atherosclerotic plaque lesion. Next, we investigated the functional mechanisms and signaling pathways of CD137 on these cells. Finally, we tried to confirm whether the *in vivo* activation of CD137 signaling using agonistic anti-CD137 mAb can exacerbate plaque stability in plaque of high fat diet fed *Ldlr*$^{-/-}$ mice. These observations could support the role of CD137 in stability of advanced atherosclerotic plaque.

In summary, we show that 1) CD137 is a significant atherosclerosis-promoting factor mainly expressed on T cells infiltrated into atherosclerotic plaque lesions. Deficiency of CD137 reduces atherosclerosis in both normal chow diet fed *ApoE*$^{-/-}$ and high fat diet fed *Ldlr*$^{-/-}$ mice. 2) CD137 signaling induces activation of T cell by generation of IFN-γ, which leads to another phase of recruitment and activation of macrophages. The activated macrophages produce TNF-α and MCP-1, which cause endothelial CD137 expression. The endothelial CD137 signaling induces the production of MCP-1 and cell adhesion molecules, leading to

enhancement of leukocyte recruitment to the atherosclerotic plaque. Therefore, CD137 signaling facilitates atherosclerosis through a positive feedback mechanism to T cells, macrophages, and endothelial cells. 3) In advanced atheroma, we found that CD137 induces advanced atherosclerotic plaque phenotype exhibiting enhanced plaque necrosis, decreased collagen and VSMCs content, and enhanced macrophage infiltration. CD137 facilitates T cell activation leading to the production of INF-γ, and increases macrophage derived MMP-9 through phosphorylation of p38 MAPK and ERK1/2 pathways. Moreover, activation of CD137 signaling inhibited Bcl-2, and then by up-regulated cleaved caspase-3, leads to apoptosis of VSMCs.

Key words : CD137, atherosclerosis, T cell, IFN-γ, monocytes/macrophages, endothelial cell, VSMC, plaque stability, MMP, apoptosis

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LIST OF ABBREVIATIONS

AAA; abdominal aortic aneurysm

APCs; antigen presenting cells

ApoE$^{-/-}$; apolipoprotein E knockout

CD137L; CD137 ligand

CD40L; CD40 ligand

CTLA-4; cytotoxic T lymphocyte antigen-4

DC; dendritic cell

ERK1/2; extracellular signal-regulated kinase1/2

HDL; high density lipoprotein

HSP60; heat-shock protein 60

ICAM-1; intercellular adhesion molecule-1

IDO; indoleamine 2,3-dioxygenase

IFN-γ; interferon-gamma

LAG-3; lymphocyte-activation gene-3

LDL; low density lipoprotein

Ldlr$^{-/-}$; low density lipoprotein receptor knockout

LPS; lipopolysaccharide

MAPK; mitogen-activated protein kinase

MCP-1; monocyte cheomoattractant protein-1

MCSF; macrophage colony-stimulating factor

MHCII; major histocompatibility complex II

MMPs; matrix metalloproteinases

MOMA; monocyte/macrophage

NF-κB; nuclear factor-κB

NK; natural killer cell

oxLDL; oxidized low density lipoprotein

PDGF; platelet derived growth factor

RBCs; red blood cells

SAPK/JNK; stress-activated protein kinases/Jun amino-terminal kinases

TCR; T cell receptor

T_{eff}; effector T cells

TGF-β; transforming growth factor-β

Th1; T helper 1

TIMPs; tissue inhibitors of metalloproteinases

TLRs; Toll-like receptors

TNF-α; tumor necrosis factor-alpha

T_{reg}; regulatory T cells

VCAM-1; vascular cell adhesion molecule-1

VSMCs; vascular smooth muscle cells

GENERAL INTRODUCTION

I. Atherosclerosis

1. Development and immunological features of atherosclerosis

Atherosclerosis is a disease of blood vessel wall characterized by thickening of the walls of arteries, a process that occurs slowly (Binder, 2002; Ross, 1999; Steinberg, 2002). Atherosclerotic is a chronic inflammatory disease that intense immunological pathways play an essential role in all stages of atherosclerosis (Hansson, 2011; Hansson, 2006c). Atherosclerotic plaque contains immune cells and vascular cells (endothelial cells and vascular smooth muscle cells), extracellular matrix, and cellular debris and lipids (Fig. 1). During the initial stage of plaque formation, lipids accumulate in intima where these lipids such as low density lipoprotein (LDL) are modified by oxidation or enzymes to form oxidized LDL (oxLDL), as well as a chronic inflammatory response to these modified lipids. Lipid-laden macrophages, known as foam cells are most prevalent cells in early atherosclerotic plaque lesions (fatty streak), but these plaques also contain T cells. Inflammatory cytokines produced by the accumulated immune cells including T cells and antigen presenting cells (APCs) such as macrophages and dendritic cells that presents an antigenic peptide including oxLDL affect the development of atherosclerosis (Hansson, 2002; Libby, 1991; Stemme, 1995). Over

time, fatty streak can progress into mature, advanced atherosclerotic plaque. Mature plaque generally has abundant accumulation of macrophages and T cells. These immune cells are under activation and produce more inflammatory cytokines such as INF-γ and tumor necrosis factor (TNF). When these plaque progress into more complex lesion, the lipid core region becomes a large necrotic core which is attributed by the death of macrophages and vascular smooth muscle cells (VSMCs) (Clarke and Bennett, 2006; Kockx, 1998; Tabas *et al*., 2010) and elevated levels of matrix metalloproteinases (MMPs), which degrade the extracellular matrix leading to weakening fibrous caps (Lusis, 2000; Newby, 2007). Moreover, mature plaques are more prone to rupture, causing atherothrombotic vascular disease such as myocardial infarction (Fig. 2).

2. T cells in atherosclerosis

2-1. T helper1 (Th1) cells

T cells are present at all stages of atherosclerotic plaque development (~10% of plaques), and have the important roles and their cytokines in the atherosclerotic process (Hansson, 2006b; Tedgui, 2006). The majority of T cells in mouse and human atherosclerotic plaque are $CD4^+$ T cells that have αβ T cell receptor (TCR) and have a T-helper 1 (Th1) phenotype, indicating activation in response to a limited set of local antigens (Hansson, 2006a). Th1 cells are characterized by their production of INF-γ, which activates macrophages, as well

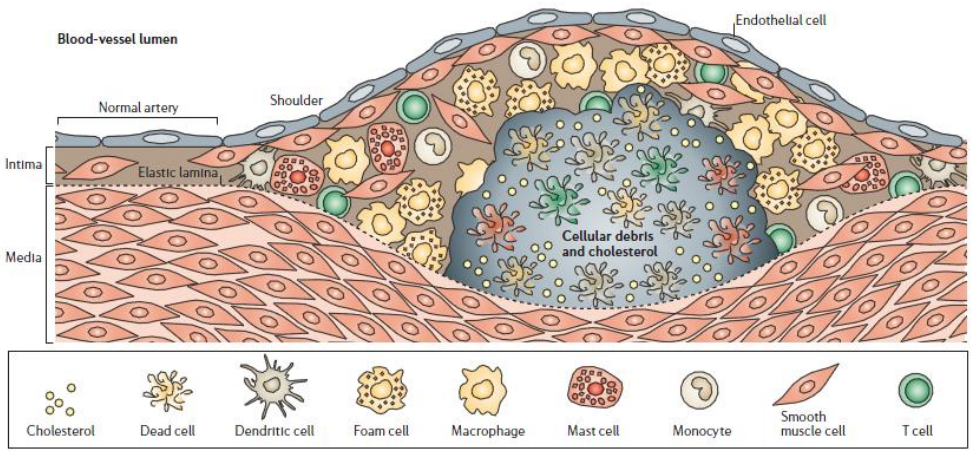
as other immune cells. Interaction of TCR on activated Th1 cells with major histocompatibility complex II (MHC II) binding antigen peptides delivered by APCs synergistically induces the bidirectional activation of both cell types (Bodmer, 2002; Locksley, 2001; Robertson, 2006). T cell activation is further enhanced by ligand binding to the co-stimulatory receptors CD40, CD137, and OX40, which are expressed on the cell surface. Enhanced T cell activation by ligand binding to co-stimulatory receptors significantly augments cytokine release by monocytes/macrophages, further exacerbating inflammation and promoting atherosclerosis (Kwon, 2003; Franc, 1998; Lee, 2006).

2-2. Regulatory T (T_{reg}) cells

As atherosclerosis is a chronic inflammatory disease and Th1 type cytokines dominate in mouse and human atherosclerotic plaques, immune homeostasis is crucial for protection and stabilization of atherosclerotic plaque. Two anti-inflammatory cytokines, interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), are counterbalancing cytokines for dampen disease activity. Several cell types can produce IL-10 and TGF-β, including platelets, M2 macrophages, endothelial cells, vascular smooth muscle cells, and regulatory T (T_{reg}) cells. $CD4^+CD25^+$ T_{reg} cells constitute 5-10% of peripheral $CD4^+$ T cells, which actively maintain immunological tolerance to self-antigens, control the development and progression of atherosclerosis (Sakaguchi *et al*., 2008; Tang and

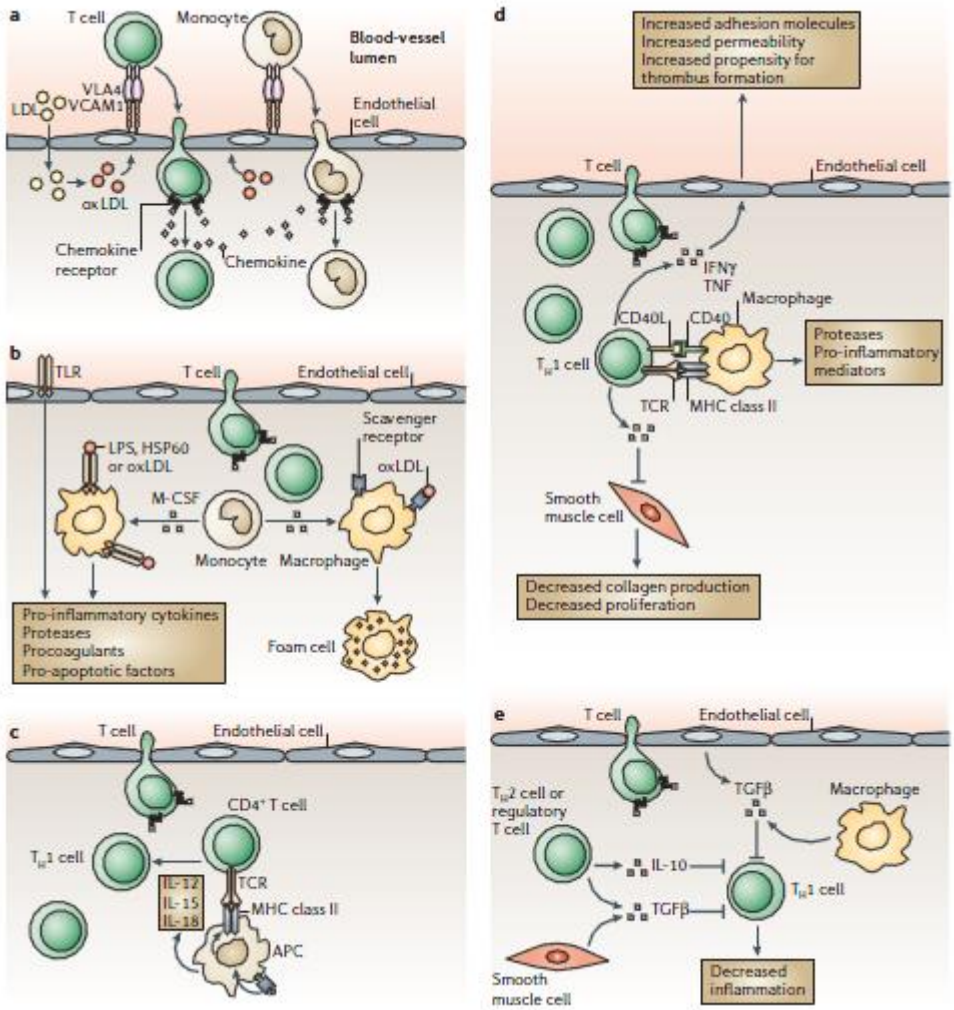
Bluestone, 2008; Vignali *et al*., 2008). T_{reg} cells actively suppress activation or effector function of effector T cells, either by direct effects on these T cells or through effects on APCs (Fig. 3). Foxp3, an X-linked transcription factor highly and specifically expressed in T_{reg} cells, is a lineage specification factor and has a critical role in their suppressive function (Fontenot *et al*., 2003; Zheng and Rudensky, 2007). $CD4^+CD25^+$ T_{reg} cells are produced in the thymus as a functionally mature subpopulation of T cells (natural T_{reg}, nT_{reg}) and can also be induced from naïve T cells in the periphery (adaptive T_{reg}). Deficiencies in the development or function of these cells are associated with severe autoimmune, inflammatory diseases (Mallat *et al*., 2007).

Many studies suggested that T_{reg} cells are important regulators in the development of atherosclerosis (Fig.4). Deficiency of T_{reg} cells increased atherosclerosis and plaque inflammation (Ait-Oufella *et al*., 2006; Gotsman *et al*., 2006). The anti-inflammatory cytokines produced from T_{reg} cells, IL-10 and TGF-β, have been shown to attenuate the development of atherosclerosis (Mallat *et al*., 1999; Mallat *et al*., 2001).



Nat Rev Immunol **6**, 508-519 (2006)

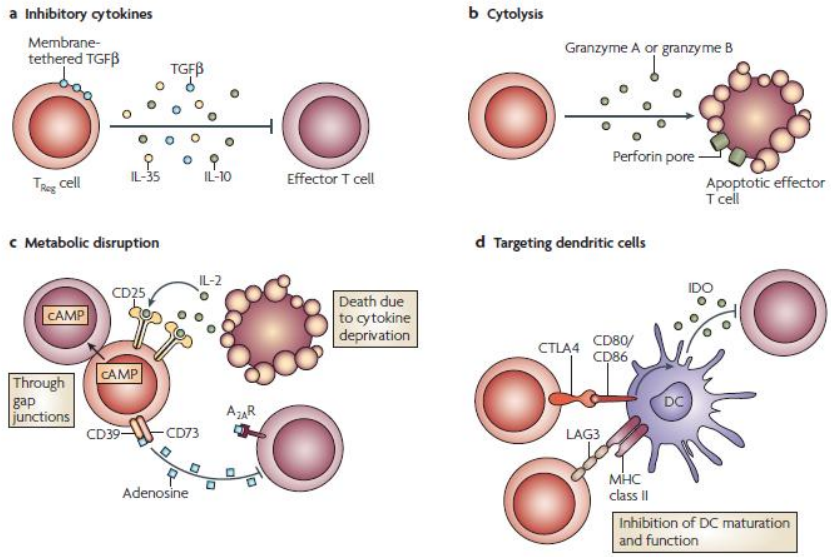
Figure 1. Immune components in atherosclerotic plaques. The atherosclerotic plaque has a core of lipids, including esterified cholesterol and cholesterol crystals, and debris from dead cells. Immune cells including macrophages, T cells and mast cells populate the plaque, and are frequently in an activated state. They produce cytokines, proteases, prothrombotic molecules and vasoactive substances, all of which can affect plaque inflammation and vascular function.



Nat Rev Immunol **6**, 508-519 (2006)

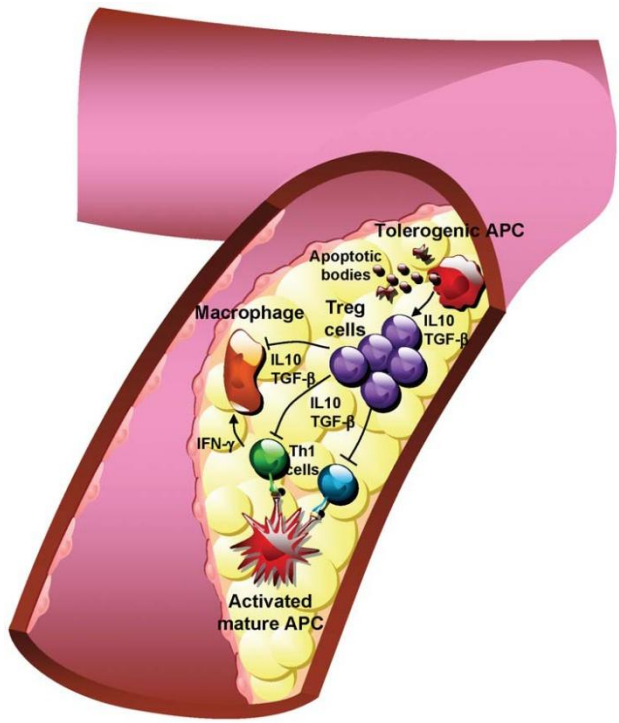
Figure 2. Recruitment and activation of immune cells in atherosclerotic plaques. a: LDL diffuses from the blood into the innermost layer of the artery

(intima), where LDL is modified to form molecules such as oxLDL. These active lipids are released and induce endothelial cells to express leukocyte adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1). **b**: Monocytes differentiate into macrophages in response to local macrophage colony-stimulating factor (MCSF) and other stimuli. Expression of many pattern-recognition receptors increases, including scavenger receptors and Toll-like receptors (TLRs). Scavenger receptors mediate macrophage uptake of oxLDL particles, which leads to intracellular cholesterol accumulation and the formation of foam cells. TLRs bind lipopolysaccharide (LPS), heat-shock protein 60 (HSP60), oxLDL and other ligands, which instigates the production of many pro-inflammatory molecules by macrophages. **c:** T cells undergo activation after interacting with APCs, such as macrophages or dendritic cells, both of which process and present local antigens including oxLDL, HSP60 and possibly components of local microorganisms. A Th1 cell-dominated response ensues, possibly owing to the local production of interleukin-12 (IL-12), IL-18 and other cytokines. **d:** Th1 cells produce inflammatory cytokines including IFN-γ and TNF and express co-stimulatory molecule such as CD40 ligand (CD40L). These messengers prompt macrophage activation, production of proteases and other pro-inflammatory mediators, activate endothelial cells, increase adhesion molecule expression. **e:** Plaque inflammation might be attenuated in response to the anti-inflammatory cytokines IL-10 and transforming growth factor-β (TGF-β), which are produced by several cell types including regulatory T cells, macrophages, and for TGF-β, also vascular cells and platelets.



Nat Rev Immunol **8**, 523-532 (2008)

Figure 3. Fundamental mechanisms used by T_{reg} cells. a: Inhibitory cytokines include IL-10, IL‑35 and TGF-β. **b:** Cytolysis includes granzyme A- and granzyme B-dependent and perforin-dependent killing mechanisms. **c:** Metabolic disruption includes high-affinity CD25 (also known as IL-2 receptor α)-dependent cytokine deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine receptor 2A (A2AR)-mediated immunosuppression. **d:** Targeting DCs includes mechanisms that modulate DC maturation and/or function such as lymphocyte-activation gene 3 (LAG3; also known as CD223)-MHC-II-mediated suppression of DC maturation, and cytotoxic T lymphocyte antigen4 (CTLA4)-CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs.



Trends Cardiovasc Med **17**, 113-118 (2007)

Figure 4. Pathogenic and regulatory immunity in atherosclerosis. Pathogenic and regulatory T cell immunity develops in response to antigen presentation. Candidate antigens such as oxLDL may induce T-helper pathogenic responses. Maturation of APC is necessary for T-cell priming. Distinct subsets of APCs, called "tolerogenic" cells, induce the differentiation of the T_{reg} subset. T_{reg} cells migrate to the periphery to suppress local immune responses. Other T_{reg} cells of the Tr1 type may not express Foxp3 and are mainly induced in the periphery in response to antigen stimulation. Phagocytosis of apoptotic cells may favor the development of tolerogenic APC, leading to a regulatory immune response.

II. Co-stimulatory molecules and receptors

As shown in above, T cells are initially activated by the interaction of TCR and MHC binding antigen peptides delivered by APCs. The second signal for T cell activation is generated by co-stimulatory molecules, which are expressed on the APC and bind to counter-part receptors on the T cells. Co-stimulatory pathways are necessary for T cell activation, because many studies suggested that their absence can lead to functional inactivation of the T cell, and cause death of T cell by apoptosis. There are two families of co-stimulatory molecules, B7 and tumor necrosis factor superfamily (TNFSF) (Fig. 5). In this study, we discussed CD137 and its ligand CD137L , belong to TNFSF group.

1. CD137 and CD137L

CD137 (also known as TNFRSF9 or 4-1BB) is a 30 kDa type I transmembrane glycoprotein and is a well-known T cell co-stimulatory molecule. Expression of CD137 is activation dependent. CD137 is not detected (< 3%) on resting T cells or naïve T cells. However, CD137 is up-regulated when T cells are activated. CD137 has been reported to be expressed on activated $CD4^+$ and $CD8^+$ T cells, natural killer (NK) cells, NKT cells, and $CD4^+CD25^+$ T_{reg} cells. CD137 is also expressed on myeloid cells, such as monocytes, neutrophils, mast cells, eosinophils, and dendritic cells (Chikara, 1999; Croft, 2003; Walter, 1997). In vascular cells including endothelial cells and vascular smooth muscle cells, CD137 is expressed

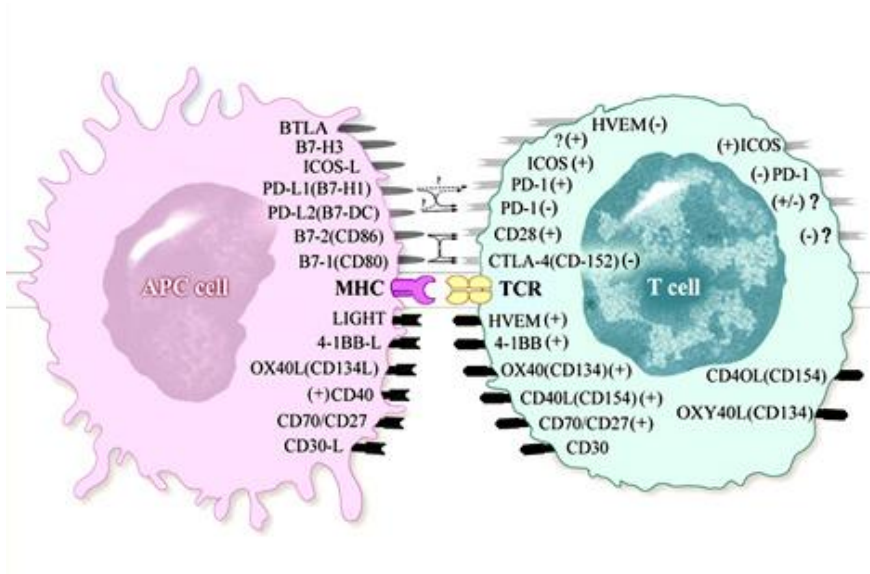
in an activation dependent manner. CD137L, a ligand for CD137, is a type II membrane glycoprotein of the TNF superfamily expressed on APCs such as monocytes, macrophages, dendritic cells, and activated B cells.

In general, TNFRSF members are initiators of inflammatory diseases. To define the role of CD137 in the immune system and immune-related diseases, CD137 deficient mouse appears to be useful. CD137 deficient mice have been demonstrated to show enhanced T cell response but normal T cell development (Kwon, 2002; Lee, 2005). Vinay and colleagues also showed that CD137 deficient mice have a reduced number of natural killer cells and natural killer T cells, which leads to resistance to lipopolysaccharide-induced shock syndrome (Vinay, 2004). In dendritic cell study, CD137 is a crucial survival factor (Choi *et al*., 2009) and controls regulatory activity through promoting production of retinal dehydrogenase (Lee *et al*., 2012b). Therefore, CD137 appears to have various roles in the immune system.

2. CD137 and CD137L in atherosclerosis

Olofsson and colleagues clearly showed CD137 expression in human atherosclerotic plaques (Olofsson *et al*., 2008). They also showed that CD137 was expressed on T cells and endothelial cells in human atherosclerotic plaque lesions. *In vitro*, CD137 was inducible on vascular cells including endothelial cells and vascular smooth muscle cells. Treatment with an agonistic CD137 antibody

promotes development of plaque inflammation in a mouse atherosclerosis model. However, although agonistic CD137 antibody has been used to understand the function of CD137 in several previous studies, Niu and colleagues showed that mice treated with anti-CD137 antibody have several immune abnormalities, which indicates that an agonistic antibody may not properly reveal the function of CD137 in atherosclerosis (Niu, 2007). Thus, another animal model system is needed to understand the role and underlying mechanism of CD137 in atherosclerosis.



Front Biosci **13**, 2120-2139 (2008)

Figure 5. Co-stimulatory molecules and their ligands. Depicted are co-stimulatory molecules belong to two main families, the CD28-B7 family located within the top half and the TNF-TNFR family located in the bottom half. This repertoire of co-stimulatory pathways contains both immune stimulatory and inhibitory specific pathways as well as some ambiguous pathways with the possibility of doing both.

III. Advanced, vulnerable Plaque

During the progression of atherosclerosis, lipid and leukocytes accumulate in intima, causing activation of the immune system. Inflammatory cytokines produced by the accumulated immune cells including T cells and APCs affect the development of atherosclerosis. Over time, mature plaque develops into vulnerable plaque, being more prone to rupture, causing subsequent atherothrombotic vascular disease such as myocardial infarction. Therefore, lesion growth in advanced plaques differs significantly from early lesions.

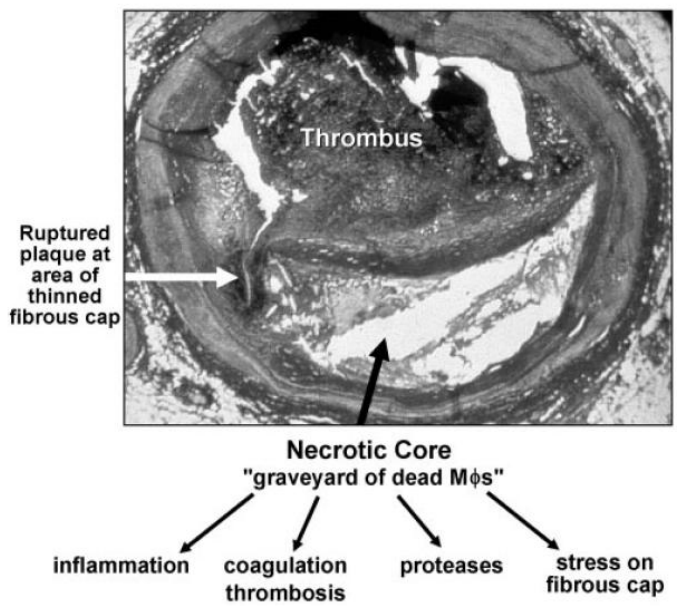
1. Pathological features of vulnerable plaque

Vulnerable plaque generally has a large necrotic core which is attributed by the death of macrophages and vascular smooth muscle cells (VSMCs) (Clarke and Bennett, 2006; Kockx, 1998; Tabas *et al*., 2010). A necrotic core characterizes plaque rupture with overlying thin fibrous cap infiltrated by macrophages and lymphocytes. Extracellular matrix including collagen fibres normally confers biochemical stability on the fibrous cap of the atherosclerotic plaque. Also, vulnerable plaque has elevated levels of matrix metalloproteinases (MMPs), which might degrade the extracellular matrix leading to weakening fibrous caps (Lusis, 2000; Newby, 2007). These proteolytic enzymes released from macrophages in atherosclerotic plaque exacerbate matrix degradation responsible for lesion instability (Moreno *et al*., 1994; Thorp and Tabas, 2009). Previous mouse studies

suggested that levels of MMP-9 increased during atherosclerosis, and MMP-9 expression in the carotid tissue is dependent predominantly on bone marrow derived macrophages (Choi, 2005). The overexpression of MMP-9 in macrophages induced vulnerable features of ruptured plaques (Gough, 2006) (Fig. 6). In advanced vulnerable plaque, pro-inflammatory cytokine IFN-γ secreted from activated T cells (Th1) inhibits the synthesis of new collagens by VSMCs (Amento *et al*., 1991). IFN-γ can also inhibit the proliferation and differentiation of VSMCs, leading to decreased collagen production (Hansson, 1989) (Fig. 7).

2. TNFRSF and vulnerable plaque

Ligation of CD40 expressed on macrophages and CD40L expressed on T cells causes the overproduction of matrix degrading proteases including MMP-1, 8, and 13 that induce collagen breakdown (Dollery and Libby, 2006). A recent mouse study identified that CD40 is involved in atherosclerotic plaque stability (Lutgens *et al*., 2010). Deficiency of CD40 reduced atherosclerosis and developed a stable atherosclerotic plaque phenotype by inducing plaque fibrosis and the M2 macrophage phenotype. However, deficiency of CD40 did not affect apoptosis of cells in atherosclerotic plaque lesions.



J Leukoc Biol **86**, 1089-1095 (2009)

Figure 6. Cross-section of an advanced human coronary plaque showing a large necrotic core, plaque rupture, and luminal thrombosis.

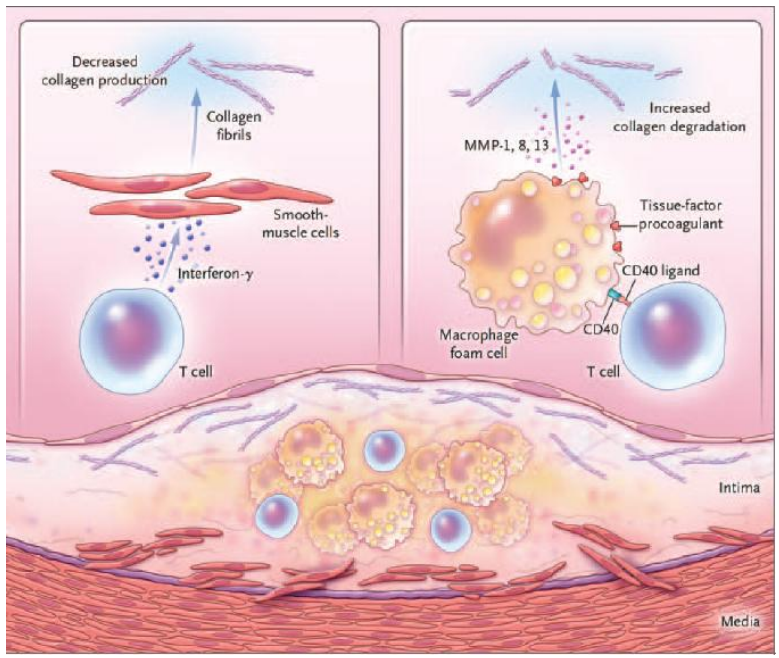


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Figure 7. Inflammatory pathways predisposing coronary arteries to rupture and thrombosis. Advanced atherosclerotic plaque shows the central lipid core that contains macrophage foam cells and T cells. The intima and media also contain VSMCs, which are the source of arterial collagen. Activated T cells (Th1 subtype) secrete cytokine IFN-γ, which inhibits the production of the new, interstitial collagen that is required to repair and maintain the plaque's protective fibrous cap. The T cells can also activate the macrophages in the intimal lesion by expressing CD40 ligand (CD154), which engages its cognate receptor (CD40) on the phagocyte. This inflammatory signaling causes overproduction of interstitial collagenases (MMP-1, 8, and 13) that facilitate collagen breakdown. CD40 ligation also causes macrophages to overproduce tissue-factor procoagulant.

PURPOSE OF THIS STUDY

The tumor necrosis factor receptor superfamily, which includes CD40, LIGHT, and OX40, plays important roles in atherosclerosis. CD137, a member of the tumor necrosis factor receptor superfamily, has been reported to be expressed in human atherosclerotic lesions. However, limited information is available on the precise role of CD137 in atherosclerosis and the effects of blocking CD137/CD137 ligand signaling on lesion formation. Moreover, the role of CD137 in atherosclerotic plaque stability and its possible molecular and cellular mechanisms are largely unknown. In this study, we found that CD137/CD137 ligand signaling plays multiple roles in the pathogenesis of atherosclerosis. In advanced plaque, the activation of CD137 signaling decreased plaque stability in advanced atherosclerotic plaque via the combined effects on T cells, macrophages, and VSMCs.

PART I

We generated *ApoE*$^{-/-}$*CD137*$^{-/-}$ and *Ldlr*$^{-/-}$*CD137*$^{-/-}$ mice to study the roles of CD137 in the pathogenesis of atherosclerosis. Initially, we examined whether CD137 and its ligand, CD137L are expressed in mouse atherosclerotic lesion sites. Next, we compared the formation of atherosclerotic plaque in the aorta of *ApoE*$^{-/-}$

background mice (fed a normal chow diet) and *Ldlr*$^{-/-}$ background mice (fed a high fat diet).

PART II

In atherosclerotic plaque, T helper 1 (Th1) cells release cytokines, such as IFN-γ and this cytokine activates macrophages and vascular cells, facilitates inflammation, and participates in cellular immunity. To find cellular mechanisms of atherosclerotic plaque formation regulated by CD137 signaling, we examined whether T cells that express CD137 facilitate cytokines such as IFN-γ in atherosclerotic plaque. Next, we investigated the significance of CD137 expression on monocyte/macrophage migration under inflammatory conditions. Accordingly, we examined that reverse CD137L signaling activated by CD137 might increase the production of pro-inflammatory molecules in atherosclerotic lesion sites.

PART III

In PART I and II, we found that CD137/CD137 ligand signaling promotes the atherosclerotic lesion formation (pathogenesis of atherosclerosis). Therefore, we could raise a possibility that CD137 is a potent factor for plaque instability, late stage of atherosclerosis. In this part, we hypothesized that CD137 signaling can affect plaque inflammation, MMP expression, and cell apoptosis, which are key

events in advanced atheroma. To gain a better understanding on the role of CD137 signaling in plaque stability, we focused on T cells, macrophages, and VSMCs, which are major cells involved in plaque stability. Accordingly, we confirmed that these cells express CD137 in advanced plaque lesion. Next, we investigated the functional mechanisms and signaling pathways of CD137 on these cells. Finally, we performed *in vivo* experiments to validate our hypothesis.

PART I

The Roles of CD137/CD137L Signaling in the Pathogenesis of Atherosclerosis

Abstract

The tumor necrosis factor receptor superfamily (TNFRSF), which includes CD40, LIGHT, and OX40, plays important roles in the development of atherosclerosis. CD137 (4-1BB), a member of the TNFRSF, is an activation-induced T cell co-stimulatory molecule. Signaling via CD137 up-regulates survival genes, enhances cell division, induces cytokine production, and prevents activation-induced cell death in T cells. CD137 has been reported to be expressed in human atherosclerotic plaque lesions. However, limited information is available on the precise role of CD137 in the development of atherosclerosis using mice models. To study the roles of CD137 in atherogenesis, we generated CD137 deficient apolipoprotein E knockout (*ApoE*$^{-/-}$*CD137*$^{-/-}$) mice and CD137 deficient low density lipoprotein receptor knockout (*Ldlr*$^{-/-}$*CD137*$^{-/-}$) mice. We found that CD137 is a significant atherosclerosis promoting factor mainly expressed on T cells infiltrated into atherosclerotic plaque lesions. Moreover, our finding showed that deficiency of CD137 strongly attenuates formation of atherosclerotic plaque lesion in *ApoE*$^{-/-}$and *Ldlr*$^{-/-}$ mice, which implicates CD137 as a potential therapeutic target for atherosclerosis disease control.

Key words : CD137, atherosclerosis, T cell

Introduction

Atherosclerosis is an inflammatory disease of the arterial wall that involves both innate and adaptive T cell driven inflammatory responses (Hansson, 2011; Hansson, 2006c). Accumulating evidence on human disease suggests that auto-antigens are involved in the promotion of atherosclerosis. In T cells isolated from fresh human plaques, oxLDL is a major auto-antigen that triggers an immune response that involves activation of T cells and subsequent stimulation of local cytokine release by monocytes/macrophages, such as monocyte chemoattractant protein-1 (MCP-1) (Ait-Oufella *et al*., 2006; Hansson and Libby, 2006). T cell activation is further enhanced by ligand binding to the co-stimulatory receptors.

CD137 (also known as TNFRSF9 or 4-1BB) is a 30 kDa type I transmembrane glycoprotein and is a well-known T cell co-stimulatory molecule. Expression of CD137 is activation dependent. CD137 is not detected (< 3%) on resting T cells or naïve T cells. However, CD137 is up-regulated when T cells are activated. CD137 has been reported to be expressed on activated $CD4^+$ and $CD8^+$ T cells, natural killer (NK) cells, NKT cells, and $CD4^+CD25^+$ T_{reg} cells. CD137 is also expressed on myeloid cells, such as monocytes, neutrophils, mast cells, eosinophils and dendritic cells (Chikara, 1999; Croft, 2003; Shuford, 1997). In vascular cells including endothelial cells and vascular smooth muscle cells, CD137 is expressed in an activation dependent manner. CD137L, a ligand for CD137, is a

type II membrane glycoprotein of the TNF superfamily expressed on APCs such as monocytes, macrophages, dendritic cells, and activated B cells.

Previous study showed CD137 expression in human atherosclerotic plaques (Olofsson *et al*., 2008). They also showed that CD137 was expressed on T cells and endothelial cells in human atherosclerotic plaque lesions. *In vitro,* CD137 was inducible on vascular cells including endothelial cells and vascular smooth muscle cells. Treatment with an agonistic CD137 antibody promotes development of plaque inflammation in a mouse atherosclerosis model. However, although agonistic CD137 antibody has been used to understand the function of CD137 in several previous studies, Niu and colleagues showed that mice treated with anti-CD137 antibody have several immune abnormalities, which indicates that an agonistic antibody may not properly reveal the function of CD137 in atherosclerosis (Niu, 2007). Thus, another animal model system is needed to understand the role and underlying mechanism of CD137 in atherosclerosis. In this study, we examined the contribution of CD137 to atherosclerosis in 2 well-established mouse models of atherosclerosis using *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice. Our findings show that deficiency of CD137 strongly attenuates atherosclerotic lesion formation.

Materials and methods

1. Experimental animals

CD137 knockout (*CD137*$^{-/-}$) mice, generated by embryonic stem cell homologous recombination, was kindly donated from Dr. BS Kwon (Ulsan University). These mice have no abnormality in the weight of spleen and thymus or other hematologic parameters. We cross-bred these mice with *ApoE* knockout (*ApoE*$^{-/-}$) or *Ldlr* knockout (*Ldlr*$^{-/-}$) mice to generate *ApoE*$^{-/-}$*CD137*$^{-/-}$ or *Ldlr*$^{-/-}$ *CD137*$^{-/-}$ mice. We used 66 week old normal chow diet fed *ApoE*$^{-/-}$, *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice. 8 week old *Ldlr*$^{-/-}$ male mice were fed a high-fat diet (Research diet, 0.15% cholesterol, 20% fat) for 16 weeks. All strains are C57BL/6 congenic lines backcrossed more than 10 times with C57BL/6J. All animal experiments were approved by the Institutional Animal Care and Use Committees (IAUCC) of Ewha Womans University and followed National Research Council Guidelines. C57BL/6J mouse housed under a 12 hours light/dark cycle, at 23 ± 2℃, 55 ± 10% humidity, were used.

2. Identification of mouse genotype

a) Mouse tail DNA extraction

Cut the mouse tail about 1 cm and put into 1.5 ml micro-centrifuge tube.

Add 10 µl of proteinase K, 290 µl of tail lysis buffer. Incubate overnight at 55℃ water bath. Then add 180 µl of 6 M saturated NaCl and mix well by shaking by repeated inversion. Centrifuge 13,000 rpm at 4℃ for 10 min. Pipet 400 µl of supernatant into a fresh 1.5 ml micro-centrifuge tube. Avoid transferring the white precipitate. Then add 420 µl of ice-cold iso-prophanol, mix by inversion until a white thready material is visible (DNA). Centrifuge 13,000 rpm at 4℃ for 20 min. Pipet off the supernatant and add 1 ml of ice-cold 70% ethanol and centrifuge 13,000 rpm at 4℃ for 10 min. Pipet off the supernatant, being careful not to disturb the pellet. Drain tube onto a kimwipe, air dry for 20 - 30 min. Dissolve the pellet in 20 µl of distilled water. Let dissolve overnight at 4℃**.** Finally, measure OD 260/280 with ND-1000 spectrophotometer (Thermo scientific, USA).

b) Polymerase chain reaction (PCR) analysis

PCR was performed from 1 µg total DNA using PCR kit (Bioneer, Accupower^{TM} PCR premix, Korea). Primers used for PCR were CD137-forward: 5’ AGT GTC CTG TGC ATG TGA C 3’, reverse: 5’ AGT TAT CAC AGG AGT TCT GC 3’. Ldlr-forward: 5’ AGG TGA GAT GAC AGG AGA TC 3’, reverse: 5’ ACC CCA AGA CGT GCT CCC AGG ATG A 3’, neo: 5’ CGC AGT GCT CCT CAT CTG ACT TGT 3’. ApoE-forward: 5'-GA ACT GAC GTG AGT GTC CA-3', reverse: 5'-GTT CCA GAA GTT GAG AAG C-3', neo: 5’- GCT TCC TCG TGC TTT ACG GTA-3'. PCR reaction was carried out to amplify these genes and

signals were detected by BiPS UV detector (Gendix, Korea).

3. Analysis of atherosclerotic plaque in mice

After mice were euthanatized, the right atrium was removed and hearts and aortas were perfused with phosphate-buffered saline (PBS) through the left ventricule. Hearts were embedded in OCT compound (Sakura, USA) and frozen on dry ice. Aortas were dissected from the proximal ascending aorta to the bifurcation of the iliac artery, and had adventitial fat removed. For *en face* analysis, aortas were opened longitudinally, pined onto flat black silicone plate and fixed 10% neutral buffered formaldehyde in PBS overnight. Fixed aortas were stained with oil red O for 4 hours and washed with PBS then digitally photographed at a fixed magnification. Total aortic area and lesion areas were calculated using Axio Vision (Carl Zeiss, Germany). For analyzing aortic sinus plaque lesion, cryosection slides were performed. Each section was stained with oil red O overnight and digitized.

4. Immunohistochemistry

All human materials for this study were approved by the Ethics Committees of Ewha Womans University. We performed immunohistochemical and immunofluorescence staining on 10 μm thick cryosection slides (mouse) and 3-4 μm thick paraffin-embedded sections (human). Both section types were prepared

from formalin-fixed tissue. For mouse tissue staining, the following primary antibodies were used: goat anti-CD137 (R&D Systems, USA), biotinylated rat anti-CD137L (ebioscience, USA), rat anti-CD3-FITC (BD Pharmingen, USA), rat anti-CD68-FITC (Ab Serotec, UK), After incubation with primary antibodies, except fluorescein-labeled antibody, chicken anti-goat, anti-rabbit Alexa488, 594 (Invitrogen, USA) antibodies were applied in the second step to visualize the antigen. For CD137L staining, the tyramide signal amplification kit (Invitrogen, USA) was used to generate high-density labeling. The following antibodies were used for human tissue staining: goat anti-CD137 (Santa Cruz, USA), goat anti-CD137L (Santa Cruz, USA), rat anti-CD3-PE (BD Pharmingen, USA), mouse anti-CD68 (Ab Serotec, UK). After incubation with the primary antibodies, except fluorescein-labeled antibody, chicken anti-goat, anti-rabbit Alexa488, 594 (Invitrogen, USA) antibodies and fluorescein avidin D (Vector, USA) were used in the second step to visualize the antigen. DAPI stain was employed to counterstain for fluorescence. Negative control tissues were prepared in a similar manner described using rat, goat, and mouse IgG isotype control antibodies (Santa Cruz, USA). The digital images of immunostaining were analyzed using LSM Image Examiner (Carl Zeiss, Germany).

5. Statistical analysis

Results were analyzed with Wilcoxon rank sum test for comparing 2

groups, or Kruskal-Wallis test followed by Wilcoxon rank sum test for multiple comparisons if more groups were compared with a control group. For the matched experiments, the results were analyzed with Wilcoxon signed rank test.

Results

1. Expression of CD137 and CD137L in T cells and macrophages in mouse atherosclerotic plaque lesions

In this study, to investigate the role of CD137 in the pathogenesis, we generated *ApoE*$^{-/-}$*CD137*$^{-/-}$ and *Ldlr*$^{-/-}$*CD137*$^{-/-}$ mice. To confirm knockout before all experiments were performed, mouse tail DNA was extracted and performed PCR (Fig. I-1A). Immune cells including macrophages, T cells populate the atherosclerotic plaque, and are frequently in an activated state. Also, because CD137 and CD137L have been reported to be highly expressed in activated T cells and macrophages, we initially examined in mouse atherosclerotic lesion sites. Immunohistochemical analysis of atherosclerotic plaque from the aortic sinus of *Ldlr*$^{-/-}$ mice fed a high fat diet showed that CD137 and CD137L was indeed expressed in CD3^{+} T cells and CD68^{+} macrophages, respectively (Fig. I-1B and C). Importantly, we detected ligation of CD137 and CD137L as well as interaction of T cells and macrophages (Fig. I-1D).

A

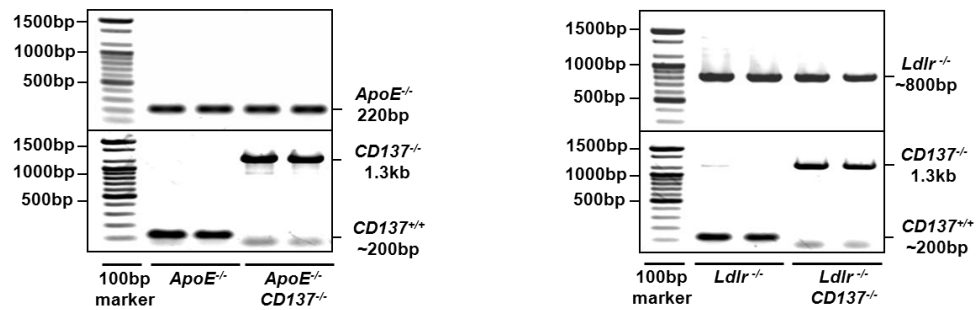


Figure I-1. Expression of CD137 and CD137L in mouse atherosclerotic plaque lesions. A: PCR genotyping of *ApoE*$^{-/-}$*CD137*$^{-/-}$, *Ldlr*$^{-/-}$*CD137*$^{-/-}$ mice.

B

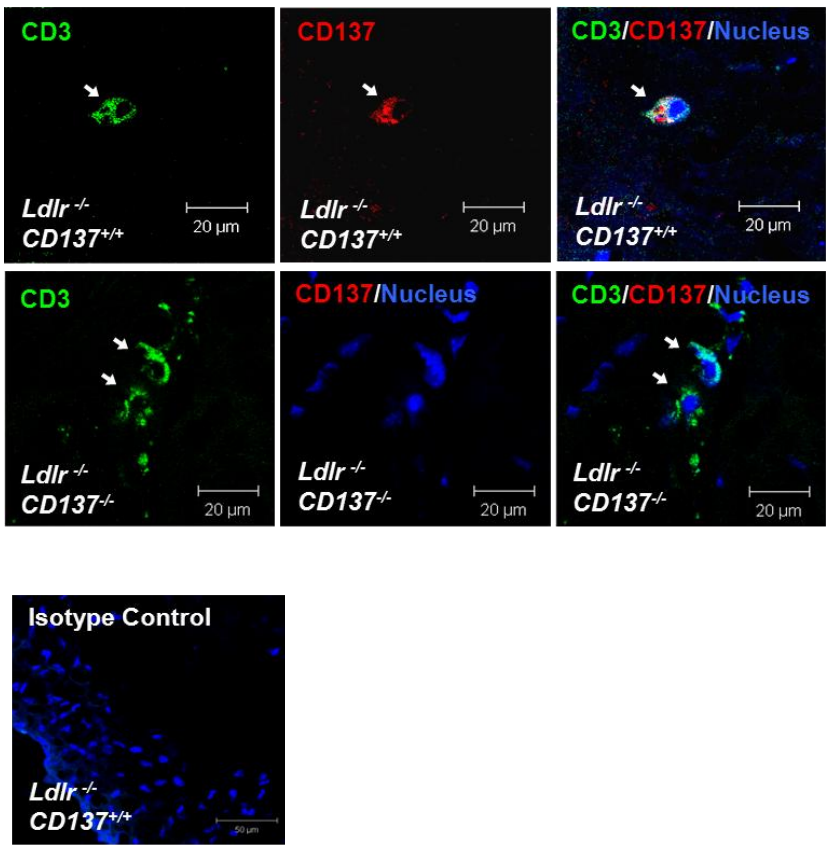


Figure I-1. Continued. B: Expression of CD137 in mouse atherosclerotic plaque. Representative images of CD137 expressing $CD3^+$ T cells in atherosclerotic plaque of *Ldlr*$^{-/-}$ mice (white arrow heads in upper panel). Atherosclerotic plaques from *Ldlr*$^{-/-}$ *CD137*$^{-/-}$ mice were not stained by anti-CD137 antibody (middle panel). Both CD3 and CD137 were not stained by anti-rat IgG and anti-rabbit IgG (isotype control, lower panel).

C

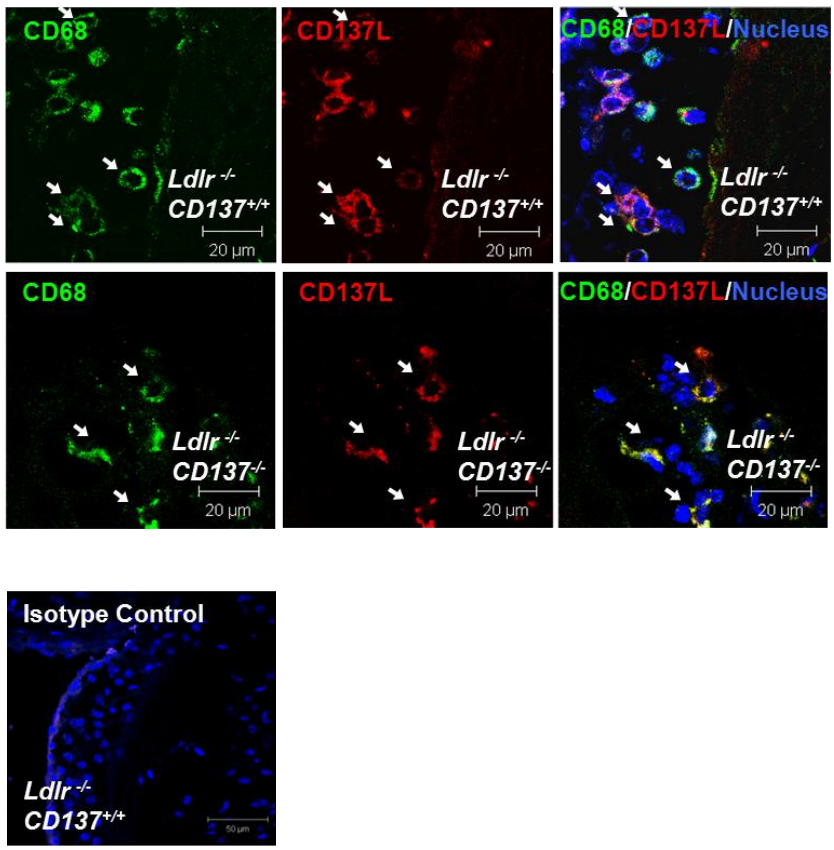


Figure I-1. Continued. C: Expression of CD137L in mouse atherosclerotic plaque. Representative images of CD137L expressing $CD68^{+}$ macrophages in atherosclerotic plaque of *Ldlr*$^{-/-}$ mice (white arrow heads in upper panel) and *Ldlr*$^{-/-}$ *CD137*$^{-/-}$ mice (white arrow heads middle panel). Both CD68 and CD137L were not stained by anti-rat IgG and anti-rabbit IgG (isotype control, lower panel).

D

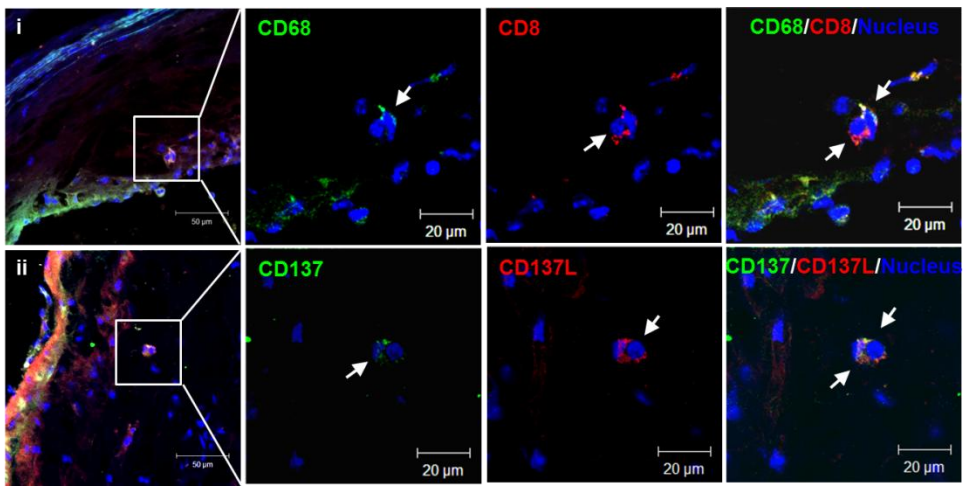


Figure I-1. Continued. D: Interaction of $CD68^+$ macrophages and $CD8^+$ T cells (white arrow heads in upper panel). Enlargement of area is indicated by white opened box in (i). Ligation of CD137 and CD137L is shown (white arrow heads in lower panel). Enlargement of area is indicated by white opened box in (ii).

2. Deficiency of CD137 reduces atherosclerotic plaque

As CD137 is expressed in T cells at atherosclerotic lesion sites (Fig. 1), we hypothesized that deficiency of CD137 may lead to reduced atherosclerotic plaque formation in mice by attenuating the activation of T cells. Initially, we compared formation of atherosclerotic plaque in *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice fed a normal chow diet for 66 weeks. Compared with *ApoE*$^{-/-}$ mice, atherosclerotic plaque in the aortic artery and descending aorta was decreased significantly in *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice (Fig. I-2A). Also, we measured atherosclerotic plaque formation in high fat diet fed mice. In *Ldlr*$^{-/-}$ background mice, atherosclerotic plaque in the aortic artery and descending aorta was reduced significantly even in *Ldlr*$^{-/-}$*CD137*$^{-/-}$ mice (Fig. I-2B). However, there were no significant differences in blood lipid profiles (Table I), and body weight (Table II) between experimental groups. These results suggested that CD137 on T cells is critical factor for atherosclerotic plaque formation.

A

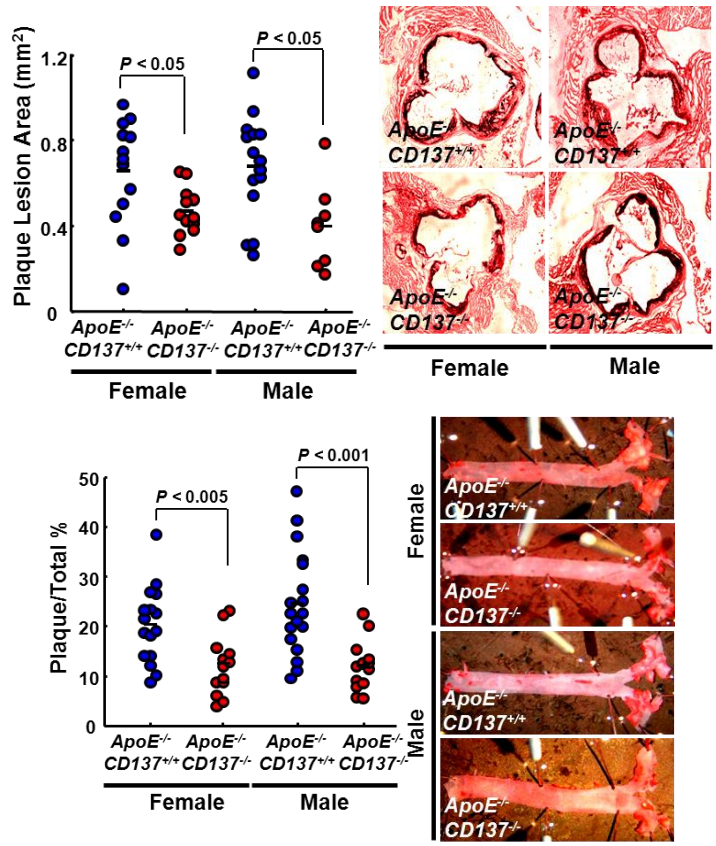


Figure I-2. Deficiency of CD137 reduces atherosclerotic plaque. A: Oil red O stained *ApoE*$^{-/-}$and *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice fed a normal chow diet for 66 weeks. Deficiency of CD137 reduced atherosclerotic plaque lesion in the aortic artery (upper panel, n=14 for *ApoE*$^{-/-}$ female mice, n=13 for *ApoE*$^{-/-}$*CD137*$^{-/-}$ female mice, n=15 for *ApoE*$^{-/-}$ male mice, n=8 for *ApoE*$^{-/-}$*CD137*$^{-/-}$ male mice) and descending aorta (lower panel, n=16 for *ApoE*$^{-/-}$ female mice, n=17 for *ApoE*$^{-/-}$*CD137*$^{-/-}$ female mice, n=18 for *ApoE*$^{-/-}$ male mice, n=13 for *ApoE*$^{-/-}$*CD137*$^{-/-}$ male mice).

B

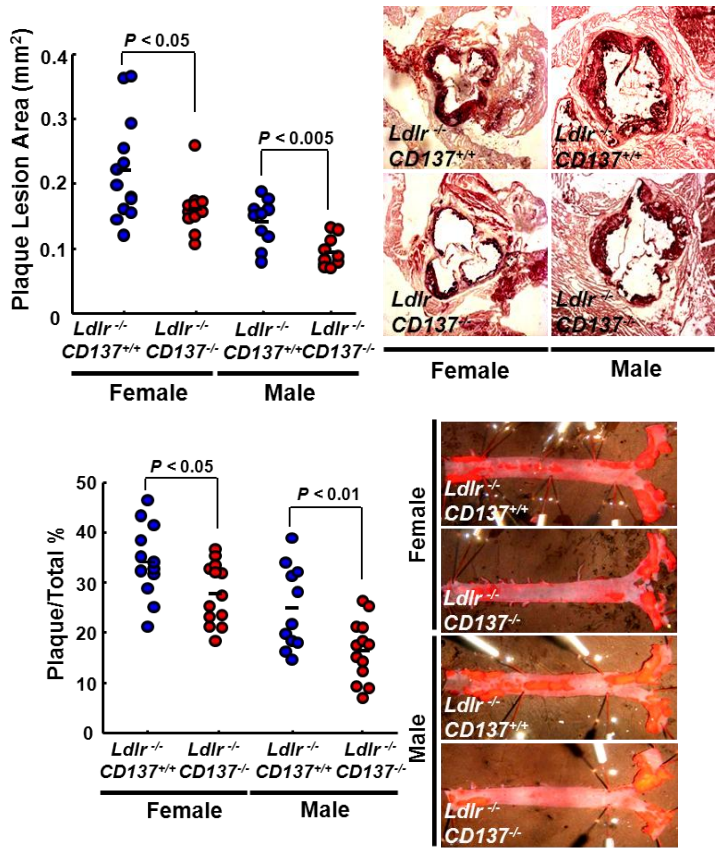


Figure I-2. Continued. B: Oil red O stained *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*CD137*$^{-/-}$ mice fed a high fat diet for 18 weeks. Deficiency of CD137 reduced atherosclerotic plaque lesion in the aortic artery (upper panel, n=13 for *Ldlr*$^{-/-}$ female mice, n=11 for *Ldlr*$^{-/-}$*CD137*$^{-/-}$ female mice, n=10 for *Ldlr*$^{-/-}$ male mice, n=10 for *Ldlr*$^{-/-}$*CD137*$^{-/-}$ male mice) and descending aorta (lower panel, n=13 for *Ldlr*$^{-/-}$ female mice, n=13 for *Ldlr*$^{-/-}$*CD137*$^{-/-}$ female mice, n=11 for *Ldlr*$^{-/-}$ male mice, n=13 for *Ldlr*$^{-/-}$*CD137*$^{-/-}$ male mice). Representative specimens from each group are shown.

Table I. Plasma total cholesterol, triglyceride, HDL, and LDL levels (mg/dL) of each experimental mice.

Diet	Sex	Groups	Total cholesterol	Triglyceride	HDL	LDL
High fat diet	Male	*Ldlr*$^{-/-}$*CD137*$^{+/+}$	1244.5±282.5	128.5±50.7	76.3±25.1	537.2±167.6
		Ldlr$^{-/-}$*CD137*$^{-/-}$	1108.2±165.7	95.5±45.2	57.0±20.0	442.3±97.2
		P value	0.27	0.21	0.10	0.21
	Female	*Ldlr*$^{-/-}$*CD137*$^{+/+}$	1217.2±247.4	125.2±81.9	77.4±20.4	540.4±112.5
		Ldlr$^{-/-}$*CD137*$^{-/-}$	1163.0±172.1	97.2±31.5	63.5±26.2	495.3±125.5
		P value	0.34	0.23	0.18	0.28
Chow diet	Female	*ApoE*$^{-/-}$*CD137*$^{+/+}$	498.2±184.6	44±8.4	16±4	243.8±94.0
		ApoE$^{-/-}$*CD137*$^{-/-}$	504.4±164.2	42.6±13.2	16.5±3.7	249.2±82.2
		P value	0.94	0.78	0.78	0.89
	Male	*ApoE*$^{-/-}$*CD137*$^{+/+}$	515.4±151.2	55.8±27.7	36.5±9.8	293.7±85.8
		ApoE$^{-/-}$*CD137*$^{-/-}$	481.5±72.6	82.5±47.9	45.5±10	261.1±41.7
		P value	0.62	0.16	0.09	0.40

Table II. Body weights of each experimental mice.

Group	Sex	Diet	Body weight (g)
Ldlr$^{-/-}$*CD137*$^{+/+}$	Male	High Fat	29.2±3.8
Ldlr$^{-/-}$*CD137*$^{-/-}$			27.5±0.9
Ldlr$^{-/-}$*CD137*$^{+/+}$	Female		25.4±1.0
Ldlr$^{-/-}$*CD137*$^{-/-}$			23.5±2.0
ApoE$^{-/-}$*CD137*$^{+/+}$	Male	Normal Chow	46.0±3.6
ApoE$^{-/-}$*CD137*$^{-/-}$			43.1±9.0
ApoE$^{-/-}$*CD137*$^{+/+}$	Female		43.1±5.0
ApoE$^{-/-}$*CD137*$^{-/-}$			39.5±6.3

Discussion

Atherosclerosis is a disease of blood vessel wall characterized by thickening of the walls of arteries, a process that occurs slowly. Atherosclerotic is a chronic inflammatory disease that intense immunological pathways play an essential role in all stages of atherosclerosis (Hansson, 2011; Hansson, 2006c). Previously, several TNF receptor superfamily including CD40, LIGHT and OX40 have been reported to be involved in atherosclerosis (Franc, 1998; Lee, 2006). Recently, it has been reported that CD137 is also expressed in human atherosclerosis, and the stimulation of CD137 signaling increases atherosclerotic lesion in mice (Olofsson *et al*., 2008). However, exact role of CD137 in atherosclerosis was still unknown. Therefore, in this study, to elucidate the role of CD137 in atherosclerosis, we established CD137 deficient atherogenic mice.

Initially, we confirmed CD137 and CD137L expression on activated T cells and macrophages in mouse atherosclerotic plaques. Multiple studies have demonstrated that T cells influence atherosclerotic plaque formation and progression of atherosclerosis (Daugherty, 1997; Dansky, 1997; Reardon *et al*., 2001). For instance, atherosclerosis-prone *ApoE*$^{-/-}$ mice display slower progression of atherosclerosis if the Rag gene, which is required for lymphocyte development, is also lost (*ApoE*$^{-/-}$*Rag*$^{-/-}$) when fed a normal chow diet. Those results indicate loss of Rag, which results in lymphocyte deficiency, reduces atherosclerosis formation.

However, a change to a high-fat diet eliminated this reducing effect. Because CD137 was expressed on T cells at atherosclerotic lesion sites (Fig. I-1B), we hypothesized that loss of CD137 may lead to reduced atherosclerotic plaque formation in mice by reducing the activation of T cells. Initially, we analyzed blood lipid including plasma total cholesterol, triglyceride, HDL, and LDL levels of each experimental mice. There were no significant differences in blood lipid profiles between experimental groups (Table I). Also, CD137 deficiency did not affect body weight between experimental groups (Table II). Next, we analyzed atherosclerotic lesion formation. We found that CD137 deficiency significantly reduced the formation of atherosclerotic plaques in aortic artery by an *en face* view and aortic sinus by oil red O staining. These data collectively suggest that CD137 function may extend beyond T cells for atherosclerotic plaque formation.

PART II

Regulatory Mechanisms by CD137/CD137L Signaling

Abstract

Atherosclerotic plaques contain blood-borne inflammatory and immune cells such as macrophages and T cells, as well as vascular cells. Formation of atherosclerotic plaque lesions are characterized by inflammation, lipid accumulation, cell death and fibrosis. Adaptive immunity, in particular T cells, is highly involved in atherosclerosis. When the naive T cell encounters antigen-presenting cell such as macrophages and dendritic cells that presents an antigenic peptide including oxLDL, T cells undergo activation and become effector T cells secreting Th1 cytokine such as IFN-γ, which promotes the development of atherosclerosis. Enhanced T cell activation by co-stimulatory receptors significantly increases inflammatory cytokine release by monocyte/macrophages, further exacerbating inflammation and promoting atherosclerosis. Here, we studied functional mechanism of CD137 in atherosclerosis. We found that CD137 functionally induces activation of T cell activation by generation of IFN-γ. In turn, IFN-γ induces monocytes/macrophages and endothelial cells to augment pro-inflammatory cytokine production through a positive feedback mechanism, thereby facilitating formation of atherosclerotic plaque.

Key words : CD137, IFN-γ, monocytes/macrophages, endothelial cell

Introduction

T cells are present at all stages of atherosclerotic plaque development (~10% of plaques), and have the important roles and their cytokines in the atherosclerotic process. The majority of T cells in mouse and human atherosclerotic plaque are $CD4^+$ T cells that have αβ T cell receptor (TCR) and have a T-helper 1 (Th1) phenotype, indicating activation in response to a limited set of local antigens (Hansson, 2006a). Th1 cells are characterized by their production of INF-γ, which activates macrophages, as well as other immune cells. Interaction of TCR on activated Th1 cells with major histocompatibility complex II (MHC II) binding antigen peptides delivered by APCs synergistically induces the bidirectional activation of both cell types (Bodmer, 2002; Locksley, 2001; Robertson, 2006).

T cell activation is further enhanced by ligand binding to the co-stimulatory receptors CD40, CD137, and OX40, which are expressed on the cell surface. Enhanced T cell activation by ligand binding to co-stimulatory receptors significantly augments cytokine release by monocytes/macrophages, further exacerbating inflammation and promoting atherosclerosis (Kwon, 2003; Franc, 1998; Lee, 2006). Moreover, recent studies show that CD137 is also expressed on blood vessel walls during inflammation and tumorigenesis, thereby mediates the migration of monocytes/macrophages (Drenkard *et al*., 2007; Broll, 2001). Many studies demonstrate that TNF receptors induce reverse signaling through

interaction with their ligands (Croft, 2003). Reverse signaling through the ligands offers the advantage of rapid feedback and fine-tuning of biological responses. However, limited information is available on the signaling mechanisms of CD137L induced by CD137.

In this PART II, we examined functional mechanisms of CD137 on both T cells and endothelial cells. Next, we investigated the significance of CD137 signaling on monocytes/macrophages migration under inflammatory conditions. Finally, we confirmed reverse signaling through CD137L activation by CD137 in macrophages and inflammation sites of atherosclerosis. Our finding showed that activated T cells by CD137 produce more IFN-γ, which leads to recruitment and activation of macrophages produce more inflammatory cytokines. Moreover, endothelial CD137 induces the production of MCP-1 and cell adhesion molecules. Thus, CD137/CD137L signaling facilitates atherosclerosis its combined actions on T cells, macrophages, and endothelial cells.

Materials and Methods

1. Immunohistochemistry

All human materials for this study were approved by the Ethics Committees of Ewha Womans University. We performed immunohistochemical and immunofluorescence staining on 10 μm thick cryosection slides (mouse) and 3-4 μm thick paraffin-embedded sections (human). Both section types were prepared from formalin-fixed tissue. For mouse tissue staining, the following primary antibodies were used: goat anti-CD137 (R&D Systems, USA), rat anti-CD4 (BD Pharmingen, USA), rat anti-CD8 (BD Pharmingen, USA), goat anti-CD31 (Santa Cruz, USA), rat anti-mouse macrophage (MOMA-2, Ab Serotec, UK), rat anti-IFN-γ-APC (BD Pharmingen, USA), rabbit anti-TNF-α (Santa Cruz, USA), and rabbit anti-MCP-1 (Santa Cruz, USA). After incubation with primary antibodies, except fluorescein-labeled antibody, chicken anti-goat, anti-rabbit Alexa488, 594 (Invitrogen, USA) antibodies were applied in the second step to visualize the antigen. For CD137L staining, the tyramide signal amplification kit (Invitrogen, USA) was used to generate high-density labeling. The following antibodies were used for human tissue staining: goat anti-CD137 (Santa Cruz, USA), goat anti-CD137L (Santa Cruz, USA), rat anti-CD3-PE (BD Pharmingen, USA), mouse anti-CD4-biotin (Ab Serotec, UK), mouse anti-CD8-biotin (Ab Serotec, UK), mouse anti-CD68 (Ab Serotec, UK), and rat anti-IFN-γ-PE (Biolegend, USA). After

incubation with the primary antibodies, except fluorescein-labeled antibody, chicken anti-goat, anti-rabbit Alexa488, 594 (Invitrogen, USA) antibodies and fluorescein avidin D (Vector, USA) were used in the second step to visualize the antigen. DAPI stain was employed to counterstain for fluorescence. Negative control tissues were prepared in a similar manner described using rat, goat, and mouse IgG isotype control antibodies (Santa Cruz, USA). The digital images of immunostaining were analyzed using LSM Image Examiner (Carl Zeiss, Germany).

2. Analysis of intracellular T cell cytokine production

Mice were sacrificed and spleen was removed to FACS buffer (PBS containing 2% FBS). Then spleen was smashed in 70 μm cell strainer (BD Falcon, USA). Debris was removed and red blood cells (RBCs) were lysed by RBC lysis buffer (ebioscience, USA). Splenocytes were washed with FACS buffer twice and the same number of cells (approximately 5 x 10^5 cells/ml) was divided into 5 ml FACS tube with DMEM containing 10% FBS, penicillin/streptomycin and L-glutamine. Anti-CD28 antibody (final concentration: 0.1 μg/ml) and brefeldin A (final concentration: 5 nM) was added into each tube. For positive control, calcium ionophore (final concentration: 500 ng/ml) and PMA (final concentration: 50 ng/ml) was added. As an antigen, oxLDL was treated to activate T cells via antigen presentation by APC cells (final concentration: 5 μg/ml). After 5 hours incubation, cells were centrifuged and collected into v-bottom 96 well plate. Cells were

incubated with FcR blocker (Ab Serotec, UK) diluted in FACS buffer (1 : 100 dilution) for 30 min at 4℃. Then cells were washed with FACS buffer twice. For detecting surface molecules, anti-CD3-FITC, anti-CD4-PE, anti-CD8-PerCP, and anti-CD4-APC antibodies were diluted in FACS buffer (1 : 200 dilution) and then added into each well. After 40 min incubation with mild shaking, cells were washed with FACS buffer then fixed and permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen, USA) for 10 min at 4℃**.** After incubation, cells were washed with Cytowash buffer (BD Pharmingen, USA) twice and intracellular staining was performed with anti-IFN-γ-APC antibody (BD Pharmingen, USA) diluted in Cytowash buffer (1 : 200 dilution) for 30 min at room temperature. After incubation, cells were washed and resuspended in FACS buffer. After labeling with FITC, PE, PerCP or APC-conjugated antibody directed against CD3, CD4, CD8, and IFN-γ, the surface expressions of these molecules or intracellular production of cytokine were analyzed by FACS Calibur II flow cytometer (BD science, USA).

3. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Harvested tissues and cells were washed twice with PBS. Total RNA was extracted using TRIzol (MRC, TR118, UK). The first strand was synthesized using oligo-dT and a cDNA synthesis kit, according to the manufacturer's protocols. For real-time PCR analysis, Taqman probe was added to the reactions. Experiments were performed with the ABI7300 real-time PCR system. For each analysis, two

independent PCR reactions were performed per sample. Beta-actin was used as the internal control for each gene. PCR was performed using Taqman master mix (Applied Biosystems, USA), specific primers, and samples using the following cycling parameters: 94℃ for 30 seconds, 60℃ for 30 seconds, and 72℃ for 30 seconds for the number of recommended cycles.

4. Macrophage migration assay

Migration of peritoneal macrophages from C57BL/6J was assayed using Corning 3421 (Corning, USA) in a 24-well plate housing a collagen coated polycarbonate filter with 5.0 μm pores, according to the manufacturer's protocols. Lower chambers were filled with 600 μl of medium obtained from cultured primary MAECs stimulated with TNF-α (20 ng/ml). Filter membranes were loaded with 1.4 x 10^5 cells in 100 μl RPMI 1640 medium (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). After incubation for 12 hours at 37℃, migrated cells to the lower chambers were counted using a cell counter.

5. Cell culture and cytokine treatment

Peritoneal macrophages were isolated from the peritoneal lavage. Peritoneal macrophages were cultured in RPMI media supplemented with 10%

fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Macrophages were activated by 10 μg/ml of LPS. The primary culture of mouse aortic endothelial cells (MAEC) was prepared from thoracic aorta perfused with heparinized saline. After perfusion, the thoracic aorta was opened and cut into several pieces. The aorta was placed in matrigel (BD Biosciences, USA) and maintained in EGM-2 medium. After 7 days, the aorta was removed and cultured MAEC in matrigel up to 14 days. MAECs were isolated by treatment with dispase (BD Biosciences, USA). Cells were maintained up to passage 1, and used at the passage 2 stage for experiments.

6. Aorta organ culture

After euthanasia, hearts and aortas were perfused with sterilized PBS. Adventitial fat was removed carefully, and aortas dissected from the proximal ascending aorta to the thoracic artery. Isolated aortas were longitudinally divided into half. Each aorta was placed in a 48-well plate to which 200 μl of complete DMEM (containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 100 μg/ml L-glutamine) was added. One set was stimulated with 5 μg of recombinant CD137-Fc chimera (R&D systems, USA) and the other with 5 μg of IgG from human serum (Sigma Aldrich, USA) as a control. After incubation at 37℃, 5% CO_2 for 13 hours, aortas were removed to 5 ml round bottom tubes containing 500 μl of RNA TRIzol reagent for the preparation of RNA.

Total RNA was extracted from each aorta, and used for cDNA synthesis. Real-time RT PCR was performed with probes for VCAM-1, ICAM-1, MCP-1, and the expression levels of mRNA monitored. Each experiment was performed three times.

7. Bone marrow transplantation

Total bone marrow (BM) cells were taken from the femurs and tibias of *CD137*$^{+/+}$ and *CD137*$^{-/-}$ mice. And 1 x 10^6 BM cells from each group were transferred to lethally irradiated (10 Gy) *Ldlr*$^{-/-}$ mice or *Ldlr*$^{-/-}$*CD137*$^{-/-}$ mice. After 4 weeks to allow the BM reconstitution to donor cells, the mice were started to be fed high fat diet for 8 weeks. Next, the atherosclerotic lesions formed in aortic sinus were measured.

8. Statistical analysis

Results were analyzed with Wilcoxon rank sum test for comparing 2 groups, or Kruskal-Wallis test followed by Wilcoxon rank sum test for multiple comparisons if more groups were compared with a control group. For the matched experiments, the results were analyzed with Wilcoxon signed rank test.

Results

1. Deficiency of CD137 reduced IFN-γ production in atherosclerotic mice

In atherosclerotic plaque, T cells undergo activation after interacting with antigen presenting cells such as macrophages, dendritic cells, both of which process and present local antigens such as oxLDL. Co-stimulatory receptors on T cells mediate T cell activation and induce cytokine secretion in immune responses. Th1 cells release cytokines, such as IFN-γ and this cytokine activates macrophages and vascular cells, facilitates inflammation, and participates in cellular immunity. We examined whether T cells that express CD137 facilitate cytokines such as IFN-γ in atherosclerotic plaque. Immunohistochemical analysis showed that $CD4^+$ T and $CD8^+$ T cells expressing CD137 release IFN-γ in atherosclerotic plaque (Fig. II-1A). These findings were also observed in human atherosclerotic plaque (Fig. II-1B and 1C). Accordingly, to determine whether CD137 contributes to IFN-γ production by T cells, we examined intracellular IFN-γ levels in activated T cells by oxLDL treatment. IFN-γ production by splenic T cells induced by oxLDL was decreased significantly in CD137 deficient mice than CD137 intact mice (Fig. II-1D), which indicates that CD137 facilitates T cell activated inflammatory response.

A

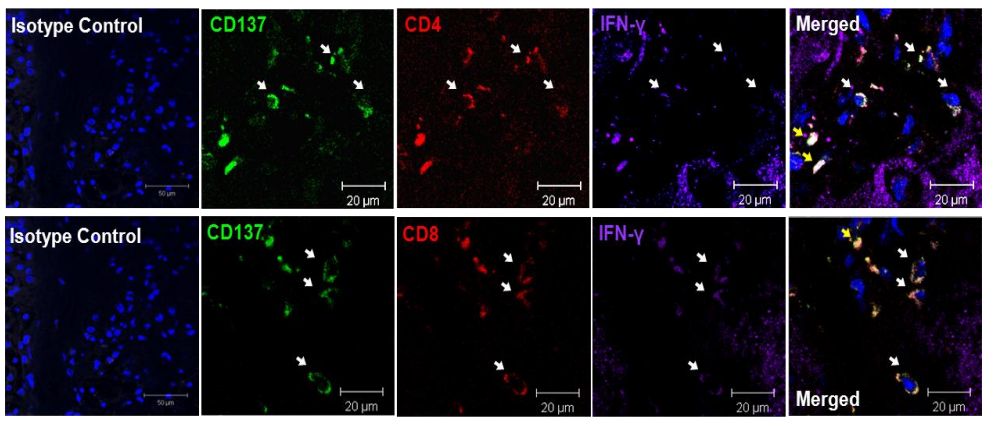


Figure II-1. Activated T cells expressing CD137 produce IFN-γ in atherosclerotic plaque. A: IFN-γ production by CD137 expressing $CD4^+$ and $CD8^+$ T cells. In upper panel, white arrowheads indicate $CD4^+$ T cells (red) expressing CD137 (green) and IFN-γ (violet). In lower panel, white arrowheads indicate $CD8^+$ T cells (red) expressing CD137 (green) and IFN-γ (violet). Yellow arrowheads signify red blood cells. No expression was detected when using isotype antibody.

B

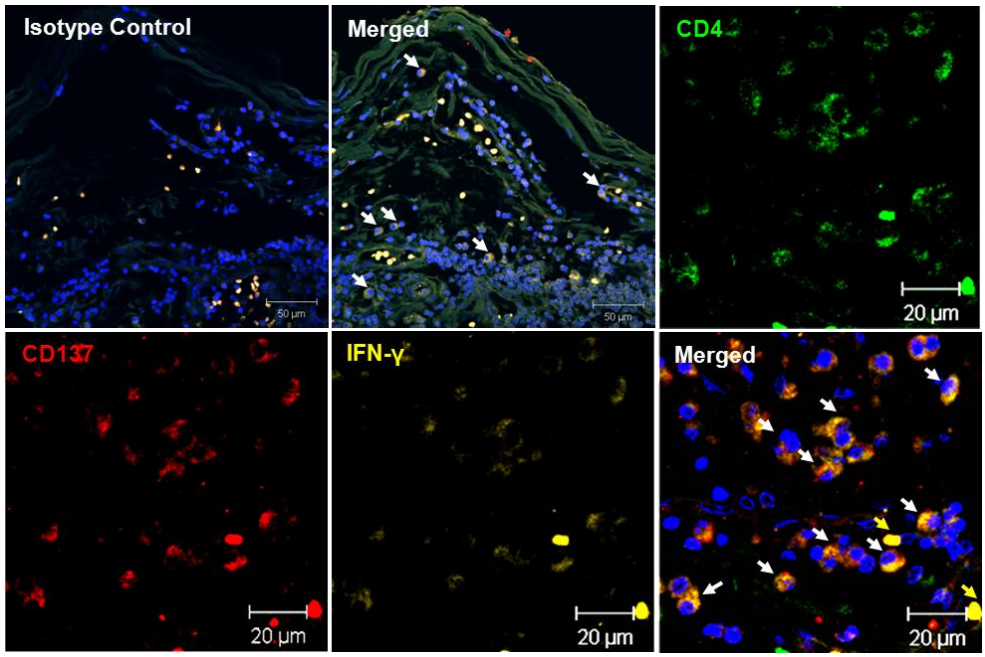


Figure II-1. Continued. **B:** $CD4^+$ T cells expressing CD137 produce pro-inflammatory cytokine IFN-γ in human atherosclerotic plaque. White arrows indicate the IFN-γ production in CD137 expressing $CD4^+$ T cells. No expression was detected when used IgG isotype control antibody. CD4 (green), CD137 (red), IFN-γ (yellow). Yellow arrows indicate red blood cells (RBCs).

C

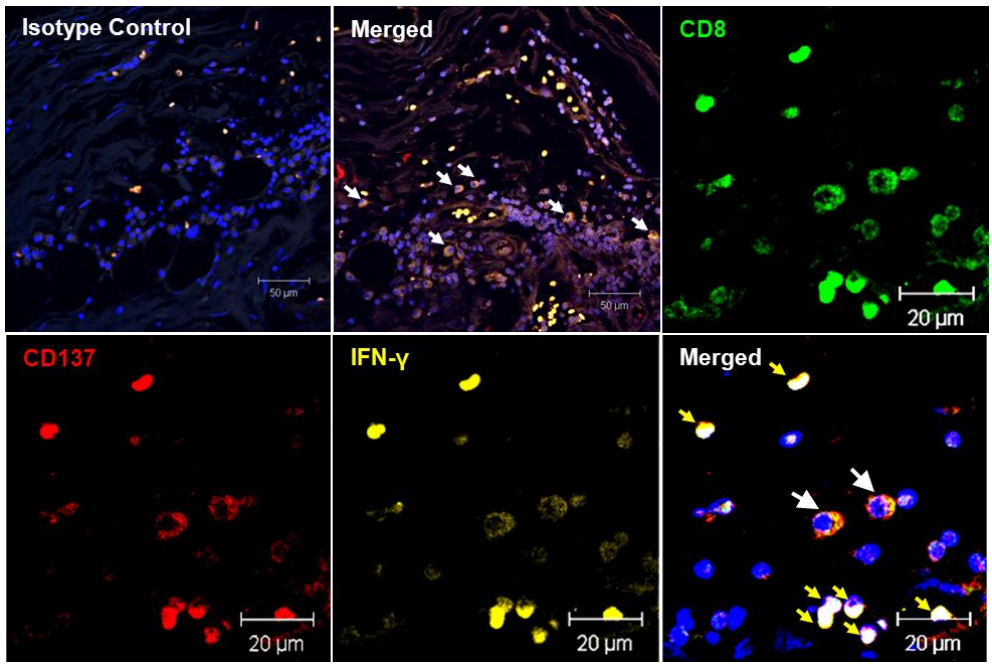


Figure II-1. Continued. **C:** $CD8^+$ T cells expressing CD137 produce pro-inflammatory cytokine IFN-γ in human atherosclerotic plaque. White arrows indicate the IFN-γ production in CD137 expressing $CD8^+$ T cells. No expression was detected when used IgG isotype control antibody. CD8 (green), CD137 (red), IFN-γ (yellow). Yellow arrows indicate red blood cells (RBCs).

D

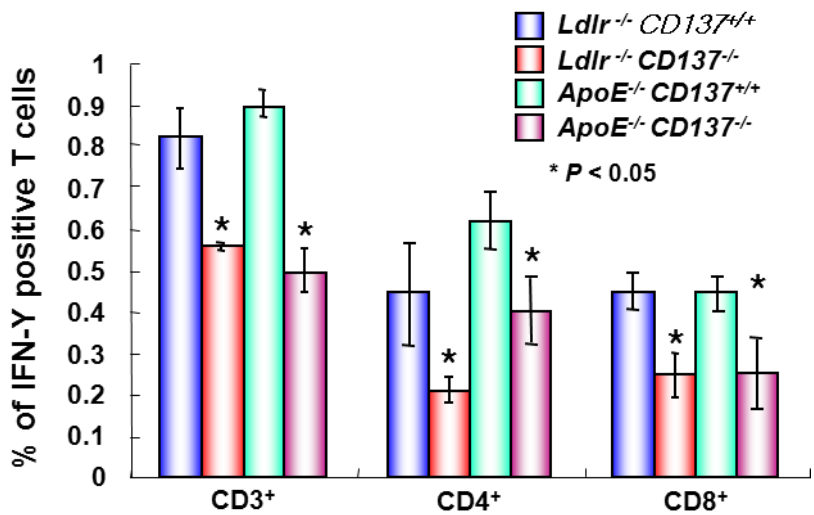


Figure II-1. Continued. D: Attenuated T cell response to oxLDL in *CD137*$^{-/-}$ mice. Splenocytes from 16 week old *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice and *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*CD137*$^{-/-}$ mice fed high fat diet for 8 weeks were treated with oxLDL (5μg/mL). Percentages of IFN-γ producing T cells were analyzed with FACS Calibur flow cytometry.

2. CD137 signaling enhances the production of pro-inflammatory molecules in endothelial cells

Recent studies show that CD137 is induced in endothelial cells during inflammation and tumorigenesis mediates the migration of monocytes/macrophages (Drenkard *et al*., 2007; Broll, 2001). In this study, we observed CD137 expression on endothelial cells in atherosclerotic plaque, whereas normal endothelium did not express CD137 (Fig. II-2A). Next, we investigated the significance of CD137 expression on monocyte/macrophage migration under inflammatory conditions. First, primary endothelial cells were treated with TNF-α, and we noted CD137 expression was increased significantly, which suggests that various inflammatory stimuli may induce CD137 expression in endothelial cell (Fig. II-2B). We next determined the outcome of increased CD137 expression under inflammation condition. Endothelial cells isolated from *CD137*$^{+/+}$ and *CD137*$^{-/-}$ mice were stimulated with TNF-α for 24 hours. And then cells were cultured on anti-CD137 agonistic antibody pre-coated plates to activate CD137 signaling. We found that stimulation of CD137 signaling up-regulated the expression of adhesion molecules (vascular cell adhesion molecule-1, VCAM-1, and intercellular adhesion molecule-1, ICAM-1) and chemo-attractants, including MCP-1 and IL-6, which are known to be important for leukocyte migration into atherosclerotic plaque (Fig. II-2C). These changes were absent in *CD137*$^{-/-}$ endothelial cells. In addition, conditioned culture medium from *CD137*$^{+/+}$

endothelial cells stimulated with anti-CD137 agonistic antibody was more effective at inducing macrophage migration than that from *CD137*$^{-/-}$ or *CD137*$^{+/+}$ endothelial cells not treated with an agonistic antibody (Fig. II-2D). These results indicate that endothelial CD137 activation induces infiltration of macrophages into atherosclerotic plaque by inducing the secretion of cytokines, including MCP-1 and IL-6, as well as up-regulation of cell adhesion molecules. Therefore, endothelial CD137 may mediate the recruitment of macrophages into atherosclerotic plaque by affecting cytokines production or cell adhesion molecules.

A

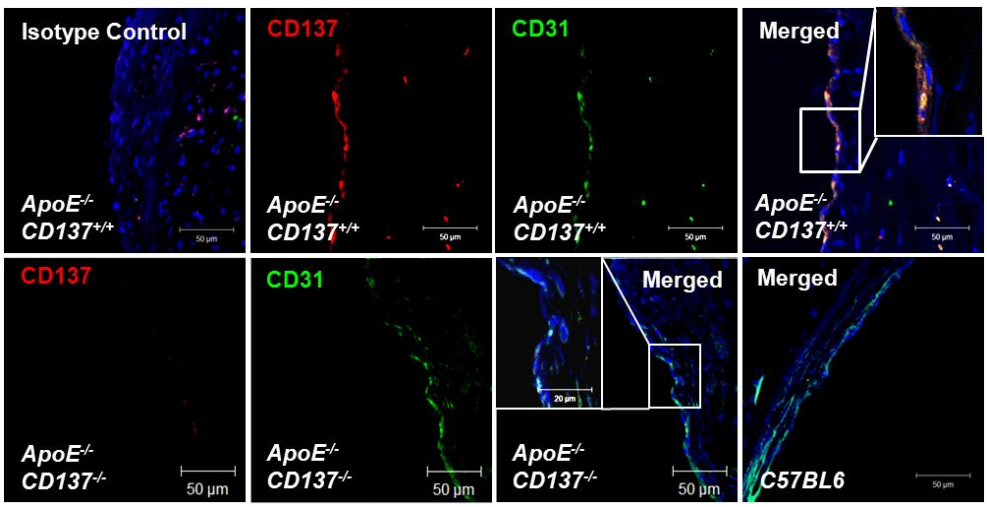


Figure II-2. Endothelial cells in mouse atherosclerotic plaque express CD137. **A:** CD137 expression in endothelial cells in atherosclerotic plaque. In atherosclerotic plaque of *ApoE*$^{-/-}$ mouse (in upper panel), double immunostained images for CD137 (red) and CD31 (green) were shown. DAPI was applied for nuclear staining. In lower panel, immunostaining for CD137 in aortas from *ApoE*$^{-/-}$ *CD137*$^{-/-}$ and C57BL/6J mice.

B

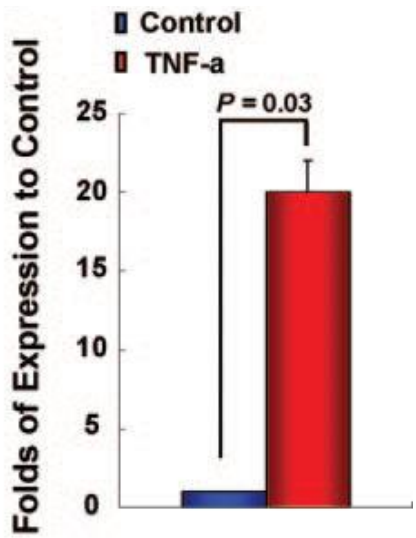


Figure II-2. Continued. B: Real-time reverse-transcription polymerase chain reaction analysis of CD137 expression in primary mouse endothelial cells induced by TNF-α treatment.

C

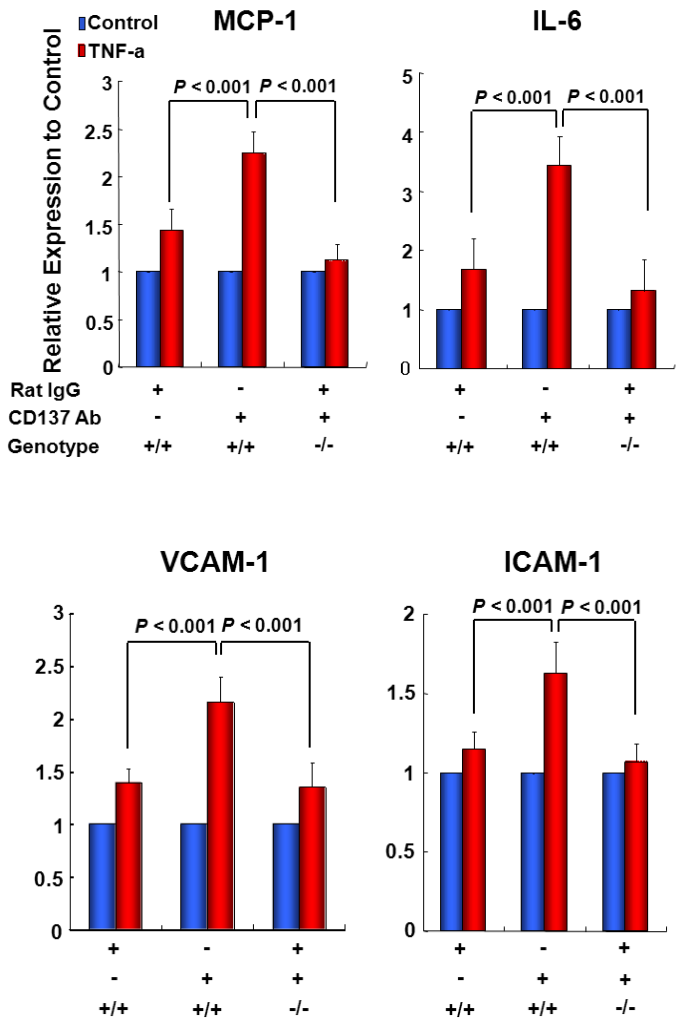


Figure II-2. Continued. C: Activation of CD137 signaling in endothelial cells increases production of pro-inflammatory molecules. Quantitative reverse-transcription polymerase chain reaction for mRNA levels of MCP-1, IL-6, VCAM-1, and ICAM-1. TNF-α treated or non-treated endothelial cells from C57BL/6 mice were incubated with agonistic anti-CD137 or isotype control antibody.

D

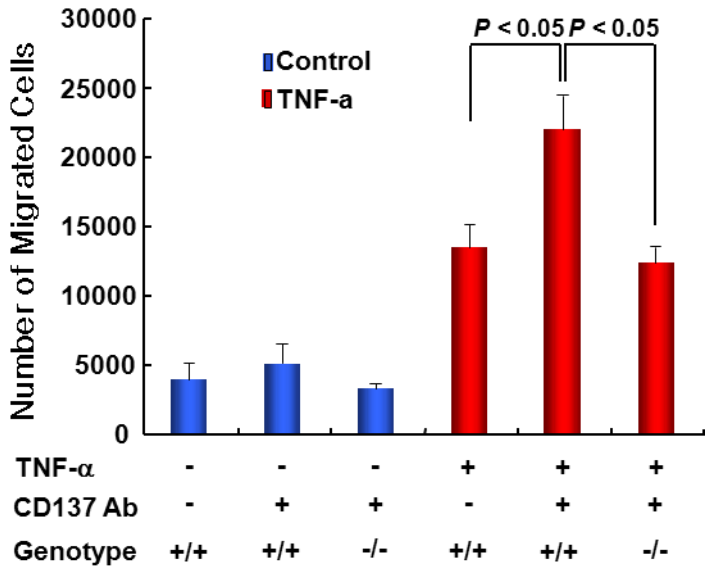


Figure II-2. Continued. D: Macrophage migration assay. *CD137*$^{+/+}$ and *CD137*$^{-/-}$ endothelial cells isolated from mice were cultured on 12-well plates coated with anti-CD137 agonistic antibody (5 μg/mL) and stimulated with TNF-α (10 ng/mL) for 24 hours. The conditioned media from the endothelial cell culture were transferred to trans-well plates, and peritoneal macrophages from C57BL/6 mice (10 weeks old) were added to trans-well inserts. After 12 hours of incubation at 37℃ and 5% CO_2, the cells that migrated from trans-well inserts to the plate were counted.

3. Reverse CD137L signaling activation by CD137 contributes to the production of MCP-1, and TNF-α in atherosclerotic plaque

Many studies demonstrate that TNF receptors induce reverse signaling through interactions with their ligands. Therefore, we hypothesized that CD137 might activate reverse CD137L signaling leading to increase the production of pro-inflammatory molecules in atherosclerotic plaque. First, to evaluate TNF-α and MCP-1 expression in atherosclerotic plaque, we performed immunostaining. Protein expression of TNF-α and MCP-1 in atherosclerotic plaque was markedly reduced in *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice (Fig. II-3A). Also, mRNA levels of both cytokines were reduced in the aortas of *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice than that of *ApoE*$^{-/-}$ mice (Fig. II-3B). Next, to test the effect of CD137L activation on TNF-α and MCP-1 in macrophages, we treated macrophages isolated from *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice with LPS in the presence of CD137-Fc protein. In LPS-activated peritoneal macrophages, treatment with CD137-Fc increased more MCP-1 and TNF-α secretion, whereas cytokine production was not changed by CD137-Fc treatment in non-activated peritoneal macrophages (Fig. II-3C and 3D). Next, we investigated the relative contribution of *CD137*$^{-/-}$ in immune and vascular cells to formation of atherosclerotic plaque using bone marrow transplantation experiments. After 8 weeks of a high-fat diet, bone marrow–reconstituted mice developed early atherosclerotic lesions (Fig. II-3E and 3F). When we analyzed the atherosclerotic plaque of each group, we found that the *Ldlr*$^{-/-}$ or *Ldlr*$^{-/-}$*CD137*$^{-/-}$ mice replaced with

CD137-deficient bone marrow had significantly fewer atherosclerotic plaque in the aortic artery. However, *Ldlr*$^{-/-}$*CD137*$^{-/-}$ mice replaced with wild type bone marrow did not have fewer atherosclerotic plaque than *Ldlr*$^{-/-}$ mice replaced with wild type bone marrow, which indicates that deficiency of vascular CD137 only is not sufficient to reduce atherosclerotic plaque formation. Thus, the present bone marrow transplantation experiment clearly shows that CD137 in immune cells has an important role in atherosclerotic lesion formation. Nevertheless, because leukocyte adhesion molecules and cytokine production levels were decreased significantly in CD137 deficient endothelium in the present *in vitro* and *ex vivo* experiments, further studies are needed to elucidate the exact role of endothelial CD137 at advanced stages of atherosclerosis. These results suggested that ligation of CD137 and CD137L can induce further activation of macrophages leading to enhanced production of TNF-α and MCP-1. Collectively, the mechanism involves a positive-feedback loop that includes CD137 signaling between T cells and monocytes/macrophages, which act to promote cytokine production, further increasing inflammation and atherosclerosis (Fig. II-3G).

A

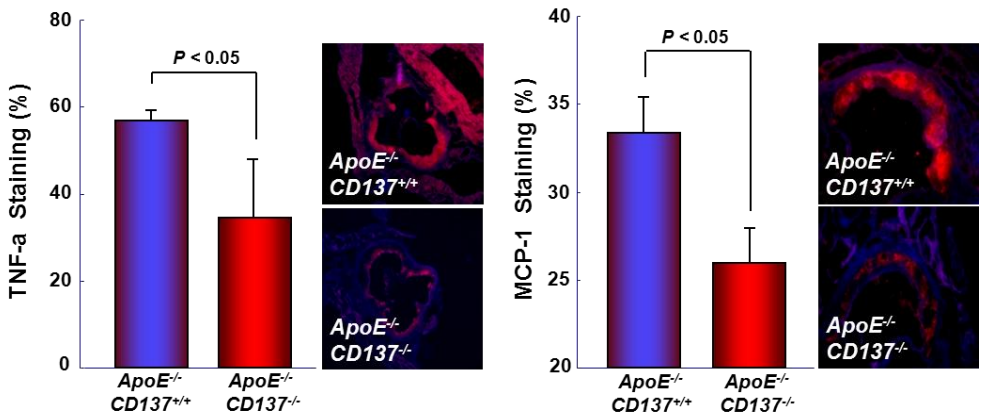


Figure II-3. Activation of reverse CD137L signaling by CD137 induces pro-inflammatory molecules. A: Immunostaining of TNF-α and MCP-1 in atherosclerotic plaque of *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice. Percentages of TNF-α, MCP-1 stained area per atherosclerotic plaque were shown.

B

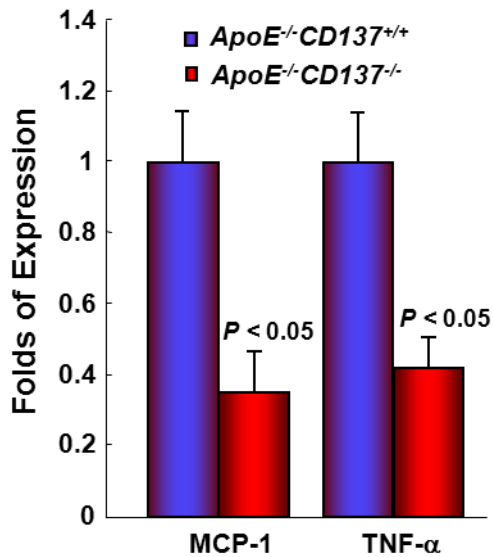


Figure II-3. Continued. B: Quantitative reverse transcriptase polymerase chain reaction for MCP-1 and TNF-α mRNA expression in the aorta of *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice.

C

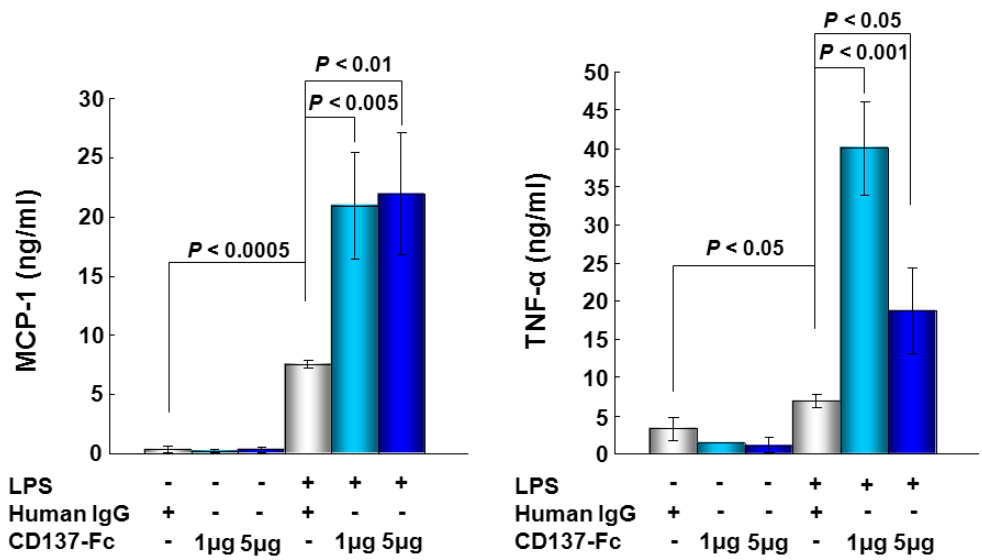


Figure II-3. Continued. C: Production of MCP-1 and TNF-α released from peritoneal macrophages stimulated by CD137-Fc. Peritoneal macrophages isolated from the peritoneal lavage were activated by 10 μg/ml of LPS. Released MCP-1 and TNF-α were measured by ELISA.

D

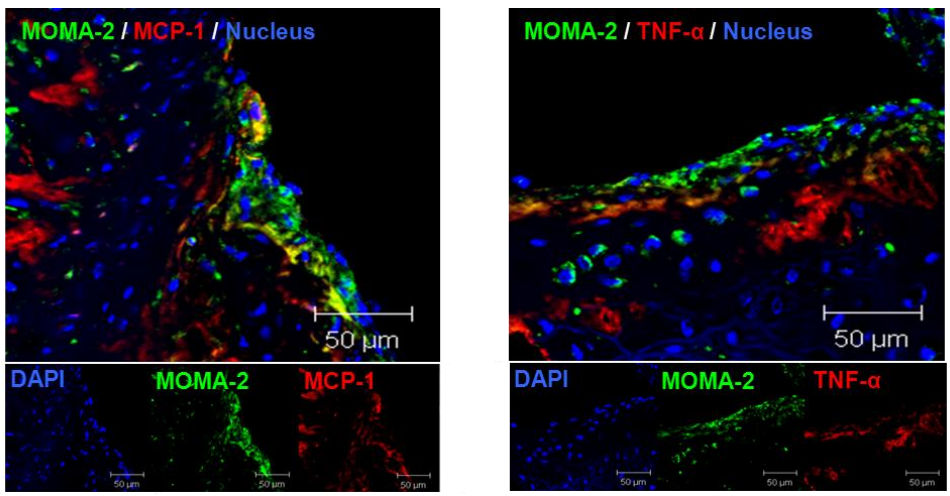


Figure II-3. Continued. D: Production of MCP-1 and TNF-α released from CD137-Fc treated atherosclerotic plaque. MOMA-2 positive macrophages produce MCP-1 (left) and TNF-α (right) in atherosclerotic plaque.

E

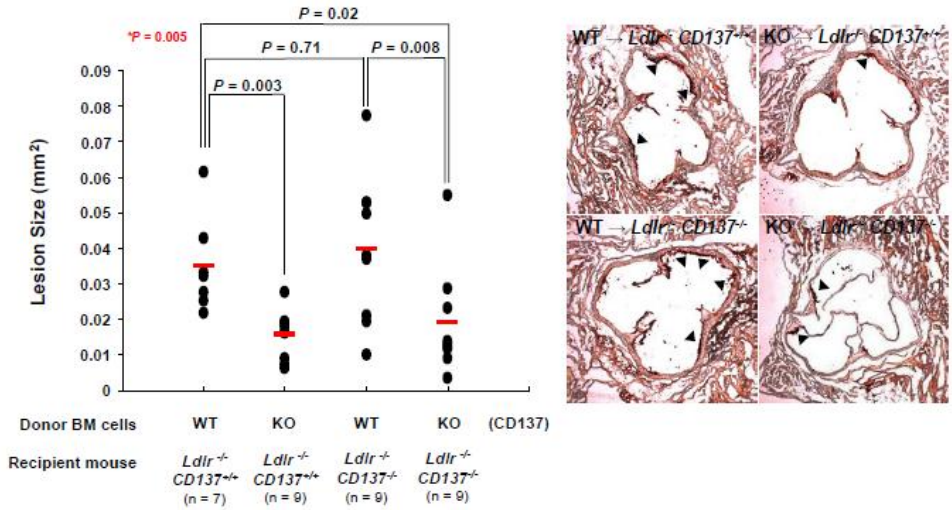


Figure II-3. Continued. E: Contribution of CD137 deficiency in immune cells and vascular cells. We performed reciprocal bone marrow transplantation (BMT) experiments using BM from *CD137*$^{-/-}$ mice into *Ldlr*$^{-/-}$ mice versus BM from *CD137*$^{+/+}$ into *Ldlr*$^{-/-}$ mice. Furthermore, WT or KO BM cells were injected into *Ldlr*$^{-/-}$*CD137*$^{-/-}$ mice. After recovery for 4 weeks to allow the BM reconstitution to donor cells, the mice were started to be fed high-fat diet for 8 weeks. Next, the atherosclerotic plaque lesions formed in the aortic artery were measured.

F

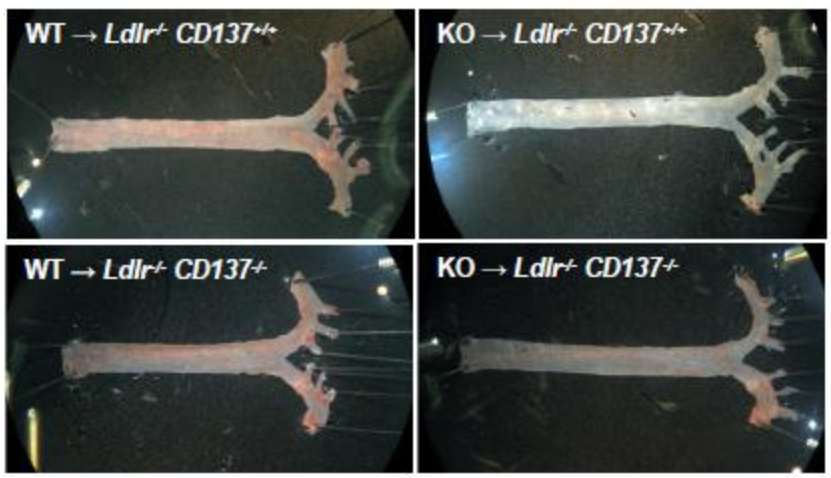


Figure II-3. Continued. F: Representative *en face* view of aortas from BM reconstituted mice stained with oil red O. The atherosclerotic plaques from all BMT groups fed high-fat diet for 8 weeks were early stage in aortic arch and descending aorta.

G

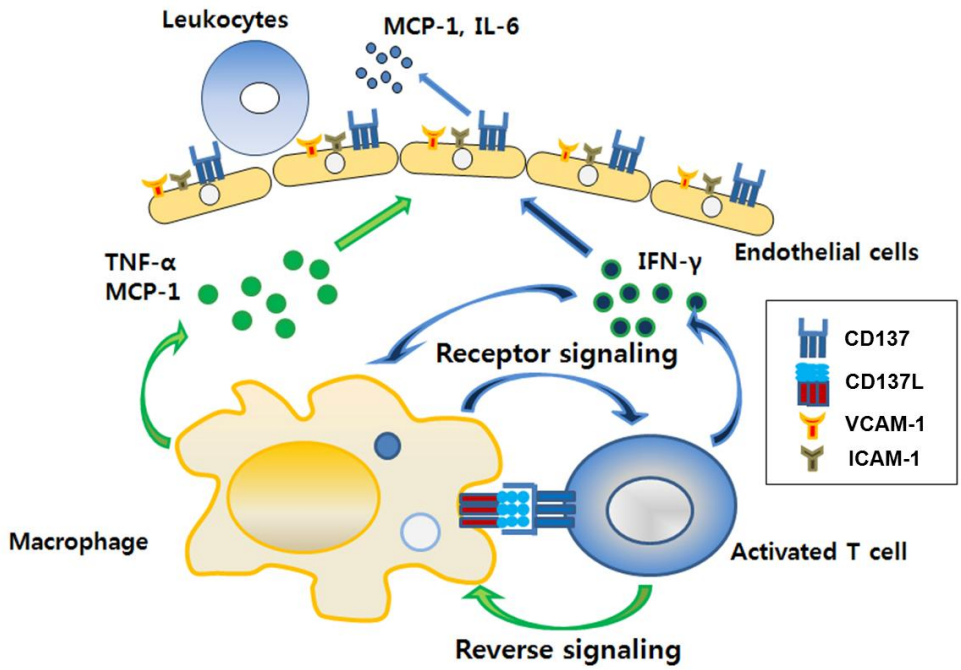


Figure II-3. Continued. G: Model for the roles of CD137/CD137L signaling in atherosclerosis. Ligation of CD137 and CD137L can induce further activation of macrophages and T cells through its bidirectional signaling. The activated T cells produce more IFN-γ, which leads to another phase of recruitment and activation of macrophages. The activated macrophages produce more cytokines, including TNF-α and MCP-1, which cause endothelial CD137 expression. The endothelial CD137 signaling induces the production of MCP-1 and cell adhesion molecules, leading to enhancement of leukocyte recruitment to the atherosclerotic plaque. Taken together, CD137/CD137L signaling facilitates atherosclerosis through its positive feedback mechanism related to T cells, macrophages, and endothelial cells.

Discussion

Here, using two different mouse atherosclerosis models, we have demonstrated that CD137/CD137L signaling contributes significantly to the formation of atherosclerotic lesions, and we present the cellular mechanisms underlying CD137 dependent atherosclerosis development. Initially, T cells with CD137 signaling displayed increased IFN-γ production. T cells lacking CD137 function displayed little response to the pathways, which led to attenuation of atherosclerosis in hyperlipidemic *Ldlr*$^{-/-}$ and *ApoE*$^{-/-}$ mice. Next, CD137 in endothelial cells plays a role in monocyte attachment to endothelium. We showed that endothelial cells in atherosclerotic plaques expressed CD137, and CD137 deficient endothelial cells were less effective at inducing monocyte recruitment and attachment. Finally, CD137 stimulated CD137L signaling in the lesion and macrophages, which led to an increase in pro-atherogenic molecules. The results of the present study indicate that CD137 plays significant roles in atherosclerotic lesion formation through combined actions in T cells, macrophages, and endothelial cells.

Clearly, T cells are an important immune component in atherosclerosis, and atherosclerotic lesions can be reduced by attenuating their activity. The present results show that CD137 stimulates T cell activity. In this study, we showed that CD137 deficiency decreases the $CD4^+$ and $CD8^+$ T cell responses to oxLDL *in*

vitro. CD137 is also expressed in vessel walls of tumors and inflammatory sites. Stimulation of CD137 signaling in endothelial cells increases monocyte migratory activity (Broll, 2001). Recent reports show that in human atherosclerosis, CD137 is expressed in endothelial cells. Activation of endothelial CD137 signaling with CD137L enhances the expression of cell adhesion molecules required for migration, including VCAM-1 and ICAM-1 (Olofsson *et al*., 2008). We confirmed that CD137 was expressed in the endothelial cells of mouse atherosclerotic lesions, and this expression was inducible by TNF-α stimulation. Furthermore, stimulation of CD137 signaling increased the levels of both cell adhesion molecules (VCAM-1 and ICAM-1) and MCP-1 and IL-6 in TNF-α treated endothelial cells. The inflammatory cytokines secreted after CD137 stimulation promote macrophage migration. Therefore, although the contribution of endothelial CD137 to atherosclerotic lesion formation in the present bone marrow transplantation experiment was minor, CD137 in endothelial cells may play a role in more advanced atherosclerosis development.

In the present study, to elucidate the roles of CD137L activation by its receptor, CD137, LPS-activated peritoneal macrophages were treated with CD137 fusion protein. CD137-Fc treatment enhanced the production of pro-inflammatory cytokines such as TNF-α and MCP-1 in the activated peritoneal macrophages. Conversely, the increases in area and mRNA expression levels for these molecules in the atherosclerotic region are decreased significantly in *CD137*$^{-/-}$ mice. Furthermore, we demonstrated that CD137 protein in atherosclerotic aortas in *ex*

vivo culture increases the mRNA expression of inflammatory molecules in lesion sites. These results strongly support the idea that reverse CD137L signaling contributes to atherosclerosis.

We suggest that CD137 not only regulates T cell activation as a co-stimulatory receptor but also mediates atherosclerosis via effects on endothelial cells and macrophages (Fig. II-3). Moreover, because CD137L is expressed in other cell types, including dendritic cells and activated B cells, CD137/ CD137L signaling may contribute to atherosclerosis in these cells. In conclusion, the results of the present study indicate that CD137 deficiency can reduce atherosclerosis, and blocking of CD137/CD137L signaling offers a valuable therapeutic target in atherosclerosis.

PART III

The Roles of CD137 Signaling in the Stability of Advanced Atherosclerotic Plaque

Abstract

Over time, mature plaques advance to a vulnerable plaque, being more prone to rupture, causing subsequent atherothrombotic vascular disease such as myocardial infarction. Vulnerable plaques generally have a large necrotic core, and thin fibrous caps, attributable to the death of macrophages and vascular smooth muscle cells (VSMCs). Vulnerable plaques also contain elevated levels of proteases, including matrix metalloproteinases (MMPs), which might degrade the extracellular matrix and weaken fibrous caps. Our previous results suggested that CD137/CD137L signaling facilitates atherosclerosis. However, there is no report whether CD137 signaling affects plaque stability of advanced atheroma or not. To answer this question, we tried to elucidate whether CD137 can induce advanced plaque phenotype in atherosclerotic model mice. To solve the mechanism of CD137 in plaque stability, we focused on T cells, macrophages, and VSMCs, which are major cells involved in plaque stability. We found CD137 expression in these cells in atherosclerotic plaque lesion. Next, we investigated the functional mechanisms and signaling pathways of CD137 on these cells. Finally, we tried to confirm whether the *in vivo* activation of CD137 signaling using agonistic anti-CD137 mAb can exacerbate plaque stability in plaque of high fat diet fed *Ldlr*$^{-/-}$ mice. These observations could support the role of CD137 in stability of advanced atherosclerotic plaque.

Key words : CD137, plaque stability, T cell, macrophage, VSMC, MMP, apoptosis

Introduction

During the progression of atherosclerosis, lipids and leukocytes accumulate in intima, causing activation of the immune system. Inflammatory cytokines produced by the accumulated immune cells including T cells and APCs affect the development of atherosclerosis. Over time, mature plaque develops into vulnerable plaque, being more prone to rupture, causing subsequent atherothrombotic vascular disease such as myocardial infarction. Vulnerable plaque generally has a large necrotic core which is attributed by the death of macrophages and vascular smooth muscle cells (VSMCs) (Clarke and Bennett, 2006; Kockx, 1998; Tabas *et al*., 2010) and elevated levels of matrix metalloproteinases (MMPs), which might degrade the extracellular matrix leading to weakening fibrous caps (Lusis, 2000; Newby, 2007).

Tumor necrosis factor receptor superfamily (TNFRSF) is a group of cytokine receptor which can bind with TNFs. Several members of TNFRSF, including CD40, CD134 (OX40), and CD137 have been shown to play important roles in atherosclerosis (Jeon *et al*., 2010; Lutgens *et al*., 2010; Olofsson *et al*., 2008; van Wanrooij *et al*., 2007). A recent study identified that CD40 is involved in atherosclerotic plaque stability (Lutgens *et al*., 2010). Deficiency of CD40 reduced atherosclerosis and developed a stable atherosclerotic plaque phenotype by inducing plaque fibrosis and the M2 macrophage phenotype. In addition,

deficiency of CD40 reduced proteolysis through decreased expression of MMP, but did not affect apoptosis of cells. CD137, another member of TNFRSF, is expressed in a variety of immune cells, including $CD4^+CD25^+$ regulatory T cells (T_{reg}), NK cells, neutrophils, resting monocytes, and dendritic cells (Choi *et al*., 2009; Croft, 2003; Lee *et al*., 2012b; Zheng, 2004). CD137 is also induced in vascular cells, such as VSMCs and endothelial cells under pro-inflammatory conditions (Drenkard *et al*., 2007). With regard to the formation of atherosclerotic plaque lesions, several studies reported the critical role of CD137. CD137 is expressed in human atherosclerotic plaques and promotes the development of plaque inflammation (Olofsson *et al*., 2008). In a mouse atherosclerosis model, we have previously reported that the deficiency of CD137 induced a reduction in atherosclerotic plaque lesions (Jeon *et al.*, 2010). However, the mechanisms and signaling pathways which govern the role of CD137 in plaque stability of advanced atheroma are unknown.

Considering the detrimental role of TNFR in atherosclerotic plaque stability which has been shown in CD40 deficient mouse (Lutgens *et al.*, 2010), we could raise a possibility that CD137 is a potent factor for plaque instability. In this study, we hypothesized that CD137 signaling can affect pro-inflammatory cytokine production, MMP expression, and cell apoptosis, which are key events in advanced atheroma. To gain a better understanding on the role of CD137 signaling in plaque stability, we focused on three cell types, T cells, macrophages, and VSMCs, which are major cells involved in plaque stability. In the present study, we confirmed

CD137 expression in these cells in advanced plaque lesion. Next, we investigated the functional mechanisms and signaling pathways of CD137 on these cells. Finally, we performed *in vivo* experiments to validate our hypothesis.

Materials and Methods

1. Experimental animals

CD137$^{-/-}$ mice were crossed with *ApoE*$^{-/-}$ mice. All strains were C57BL/6 congenic lines backcrossed more than 10 times with C57BL/6J. We used 66 week old normal chow diet fed *ApoE*$^{-/-}$, *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice. Also, we used *Ldlr*$^{-/-}$ male mice 8 weeks of age were fed a high-fat diet (Research diet, 0.15% cholesterol, 20% fat) for 16 weeks. For *in vivo* activation of CD137, *Ldlr*$^{-/-}$ mice were injected i.p. with 50 μg rat IgG_{2a} (control) or 50 μg agonistic anti-CD137 mAb (clone 3E1) once a week for 16 weeks. All mice were housed under a 12 h light/dark cycle, at 23 ± 2℃, 55 ± 10% humidity, and were bred in a pathogen-free facility in accordance with the Institutional Animal Care and Use Committees (IACUC) of Ewha Womans University.

2. Aortic single cell, splenocytes, lymph node cells preparation and flow cytometry

Aortic single cell was prepared using previous methods (Choi *et al*., 2011). Briefly, after careful removal of the perivascular fat, and using microscissors under a dissecting microscope, single cell suspensions from whole aortic segments were prepared by incubation with an enzyme mixture containing 675 U/ml collagenase I,

187.5 U/ml collagenase XI, 90 U/ml hyaluronidase, and 90 U/ml DNase (all Sigma Aldrich, USA) in Hank's balanced salt solution with calcium and magnesium for 90 min at 37℃ with gentle shaking. Spleen and skin draining lymph node single cell suspensions were prepared by filtration using a 70 μm cell strainer (BD Falcon). After blocking Fc receptors with CD16/32 mAb, cells were stained with fluorochrome-conjugated mAbs to cell surface markers CD45 and CD11b. Then cells were fixed, permeabilized and intracellular CD4 was stained. All antibodies and reagents were from ebioscience (USA). We used a FACS Calibur II flow cytometer and analyzed the data with Cell Quest Pro (BD Biosciences, USA).

3. Analysis of atherosclerosis in mice

Mice were euthanized with carbon dioxide inhalation, hearts and aortas were perfused with phosphate-buffered saline (PBS) through the left ventricle. Hearts were embedded in OCT and were frozen. Aortas were dissected from the proximal ascending aorta to the bifurcation of the iliac artery, and adventitial fat was removed. For *en face* analysis, aortas were split longitudinally, pinned onto flat black silicone plates, and fixed in 10% buffered neutral formaldehyde in PBS overnight. Fixed aortas were stained with oil red O for 4 h, washed with PBS briefly, and digitally photographed at a fixed magnification. Total aortic areas and lesion areas were calculated using Axio Vision (Carl Zeiss, Germany). For analyzing aortic sinus plaque lesions, cryosection slides were used. Each slide was

stained with oil red O overnight and digitized. Plasma lipid levels were measured using an automatic blood chemical analyzer (Hitachi, Japan).

4. Immunohistochemistry for atherosclerotic plaque stability of aortic root

5 μm thick cryosection slides of aortic root were used. After fixation in 10% buffered formaldehyde, slides were labeled with unconjugated primary Abs against VSMC (SMα-actin, Abcam, UK), macrophage (CD68, Ab Serotec, UK), apoptotic cell (*in situ* apoptosis detection kit, Trevigen, USA), anti-CD137 (3E1) Abs followed by Alexa 488, 594 conjugated Abs (Invitrogen, USA) as secondary Abs to visualize the antigen. In addition, Hematoxylin & Eosin (Sigma Aldrich, USA) and picrosirius red staining (Sigma Aldrich, USA) were performed for plaque necrosis and collagen deposition. The digital images of immunostaining were visualized by LSM 510 meta confocal microscopy (Carl Zeiss, Germany) and morphometric studies were performed using Axiovision AC software (Carl Zeiss, Germany).

5. Preparation of T_{reg} cells and i*n vitro* proliferation assay of T_{eff} cells.

$CD4^+CD25^+$ T_{reg} cells was separated from the spleens from the spleens for T_{reg} by immunomagnetic selection using the regulatory T-cell isolation kit from Miltenyi Biotech (Auburn, CA, USA). $CD4^+CD25^-$ fraction was also separated for the responder cells (T_{eff}), while the $CD4^-$ fraction was used for antigen-presenting cells (APCs) after irradiation. Ten thousand CFSE-labeled T_{eff} cells were cocultured

with10^5 APCs in the presence of 10^4 T_{reg} cells. All these cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) in round-bottomed 96-well plates by stimulating with various concentrations of anti-CD3e Ab (eBiosciences).

6. *In vitro* analysis of intracellular IFN-γ in activated T cells

Splenocytes (2 x 10^6 cells/well) were activated *in vitro* with 48-well plate bound anti-CD3 Ab (BD Biosciences, 1 μg/ml) and control rat IgG (Sigma Aldrich) in DMEM containing media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) in the presence of brefeldin A (Sigma Aldrich, 1 μg/ml). After 5 hour post-stimulation, the cells were harvested, washed, and stained for intracellular IFN-γ using anti-IFN-γ, anti-CD4, anti-CD8 Abs (all BD Biosciences). We used a FACS Calibur II flow cytometer and analyzed the data with CellQuest Pro (BD Biosciences).

7. Cell culture and cytokine treatment

Peritoneal macrophages were isolated from the peritoneal lavage and were cultured in RPMI1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). To determine CD137 expression on peritoneal macrophages, cells were stimulated with 50 μg/ml oxLDL for 24 h. For

the induction of MMP-9 expression, cells were incubated for 3, 24 h. Mouse aortic vascular smooth muscle (MOVAS-1) cell line obtained from American Type Culture Collection (ATCC) was maintained in DMEM containing 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). To determine CD137 expression, MOVAS-1 cell line was stimulated with cytokine mix (25 ng/ml IL-1β, 25 ng/ml IFN-γ and 25 ng/ml TNF-α) for 24, 36 h. To induce apoptosis of MOVAS-1 cells, they were treated with a cytokine mix in the presence of 5 μg/ml agonistic anti- CD137 mAb, or the same dose of rat IgG (control) for 72 h.

8. Quantitative real-time polymerase chain reaction

Total RNA was extracted with Trizol reagent (MRC, TR118), and cDNA was synthesized with Superscript III First-strand synthesis system (Invitrogen). Quantitative real-time PCR (SYBRgreen) assays were performed using a 7700 sequence detector (Applied Biosystems) with mouse CD137, MMP-9, GAPDH, and β-actin primers. Expression was normalized to that of house keeping gene expression. Primer sequences were as follows: CD137 (5'-AGT GTC CTG TGC ATG TGA-3'/ 5'-AGT TAT CAC AGG AGT TCT GC-3'); MMP-9 (5'-GAG CTG TGC GTC TTC CCC CTT C-3'/ 5'-GGA ATG ATC TAA GCC CAG TGC-3') GAPDH (5'-TGG AGG GAC TTA TGG TAG GAG T-3'/ 5'-GCC TGC TTC ACC ACC TTC TTG-3'); β-actin (5'-ACG GCC AGG TCA TCA CTA TTG-3'/ 5'-

CAC AGG ATT CCA TAC CCA AGA AG-3').

9. Flow cytometry analysis for CD137 expression

To analyze CD137 expression, aortic cells, peritoneal macrophages and MOVAS-1 cells treated as indicated were incubated with Alexa 647 labeled anti-CD137 (3E1) mAb for 30 minutes at 4℃. Data were collected using a FACS Calibur II flow cytometer, and were analyzed with CellQuest Pro (BD Biosciences).

10. Matrix metalloproteinase (MMP) activity

To evaluate MMP activities in atherosclerotic plaque, we performed *in situ* zymography. Freshly cut 5 μm thick cryosection slides of aortic sinus were incubated with a green fluorogenic gelatin substrate (DQ gelatin, Molecular Probes) according to manufacturer's protocol. We detected proteolytic MMP activity by LSM 510 meta confocal microscopy (Carl Zeiss, Germany) and MMP activity was measured using Axiovision AC software (Carl Zeiss, Germany). In double immunostained images, MMP labeled tissue section slides were fixed in 10% buffered formaldehyde, after which macrophages and VSMCs were stained with anti-CD68 and anti-SMα-actin antibodies, followed by Alexa 594 conjugated Abs (Invitrogen, USA). MMP activities in peritoneal macrophage conditioned medium were examined by gelatin zymography. Remaining cell lysates were used in

immuno-blot assay of MMP-9. We subjected the same loading volume of conditioned medium to electrophoresis in SDS-PAGE gels containing 10 mg/ml gelatin (Sigma Aldrich, USA). We incubated the gels for 16 h at 37℃ in zymography buffer (50 mmol Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$ and 1 μM $ZnCl_2$) and then the gels were stained with coomassie brilliant blue.

11. Immuno-blot for MMP, cell signaling pathways and cell apoptosis

Cell lysates from peritoneal macrophages and MOVAS-1 cells were subjected to 8% SDS-PAGE electrophoresis. Separated proteins were transferred onto PVDF membranes (Bio-rad, USA) followed by blocking for 1 h at room temperature in 5% skim milk. The primary Abs were anti-MMP-9 Ab (Oncogene, USA), anti- phospho p38, anti-p38, anti-phospho ERK1/2, anti-ERK1/2, anti-phospho SAPK/JNK, anti-SAPK/JNK Abs, anti-caspase-3 Ab, and anti-cleaved caspase-3 Ab (Cell Signaling, USA). Expression was normalized to housekeeping gene expression with anti-β-tubulin Ab (Sigma Aldrich, USA) or anti-GAPDH Ab (Santa Cruz, USA). Transferred proteins were visualized with enhanced chemiluminescence reagents (Ab Frontier, Korea). The band intensity of each protein was measured by Axiovision AC software (Carl Zeiss, Germany).

12. Peritoneal macrophages survival assay

The isolated peritoneal macrophages were cultured in 6-well plates at a density of 1 x 10^6 cells/ml and were treated with 50 μg/ml oxLDL in the presence of 5 μg/ml agonistic anti-CD137 mAb and the same dose of rat IgG for 24 h. The survival rate of the macrophages was determined by measuring cell viability using propidium iodide (PI) staining (BD Biosciences, USA).

13. Statistical analysis

All data displayed in the text and figures are expressed as mean ± SEM. The *n* numbers are indicated for each experiment in the figure legends. Comparison between two groups was analyzed with Wilcoxon rank sum test or Kruskal-Wallis test followed by Wilcoxon rank sum test for multiple comparisons. For the matched experiment, the results were analyzed with Wilcoxon signed rank sum test. Statistical significance was determined for P values < 0.05.

Results

1. CD137 induces advanced atherosclerotic plaque phenotype

Our initial finding (PART I and II) was that atherosclerotic plaque areas in the aortic arch and descending aorta were decreased significantly in normal chow diet *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice at 66 weeks of age. Recently, we performed flow cytometry and observed that the presence of CD137 increased the number of $CD45^+$ leukocytes, $CD11b^+$ macrophages and $CD4^+$ T_{eff} (effector T) cells in the aortas of mice (Fig. III-1A through 1D). The number of $Foxp3^+$ T_{reg} (regulatory T) cells, which suppress T_{eff} cells and actively maintain immunological tolerance, was unaffected. However, balanced ratio of T_{reg} cells and T_{eff} cells was increased significantly in *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice because of increased number of T_{eff} cells (Fig. III-1E, F). Next, to unequivocally elucidate the involvement of CD137 in atherosclerotic plaque stability, we performed immunohistochemistry and analyzed atherosclerotic plaque phenotype. Initially, we compared the atherosclerotic plaque in *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice. Compared with *ApoE*$^{-/-}$ mice, atherosclerotic plaque in the aortic artery was reduced in *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice (Fig. III-2A). Also, the necrotic core area of plaque of *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice was decreased significantly compared with *ApoE*$^{-/-}$ mice (Fig. III-2B). This was associated with an increase in the relative content of collagen (Fig. III-2C), as well as an increase in the migrated VSMCs (Fig. III-2D), and an decrease in macrophage infiltration (Fig.

III-2E). These plaque phenotypes followed the same patterns of occurrence as *ApoE*$^{-/-}$, *ApoE*$^{-/-}$*CD137*$^{-/-}$ female mice (Fig. III-3A through 3D). Taken together, these results indicate that CD137 is associated with advanced atherosclerotic plaque phenotype: exhibiting enhanced plaque necrosis, decreased collagen and VSMCs content, and enhanced macrophage infiltration.

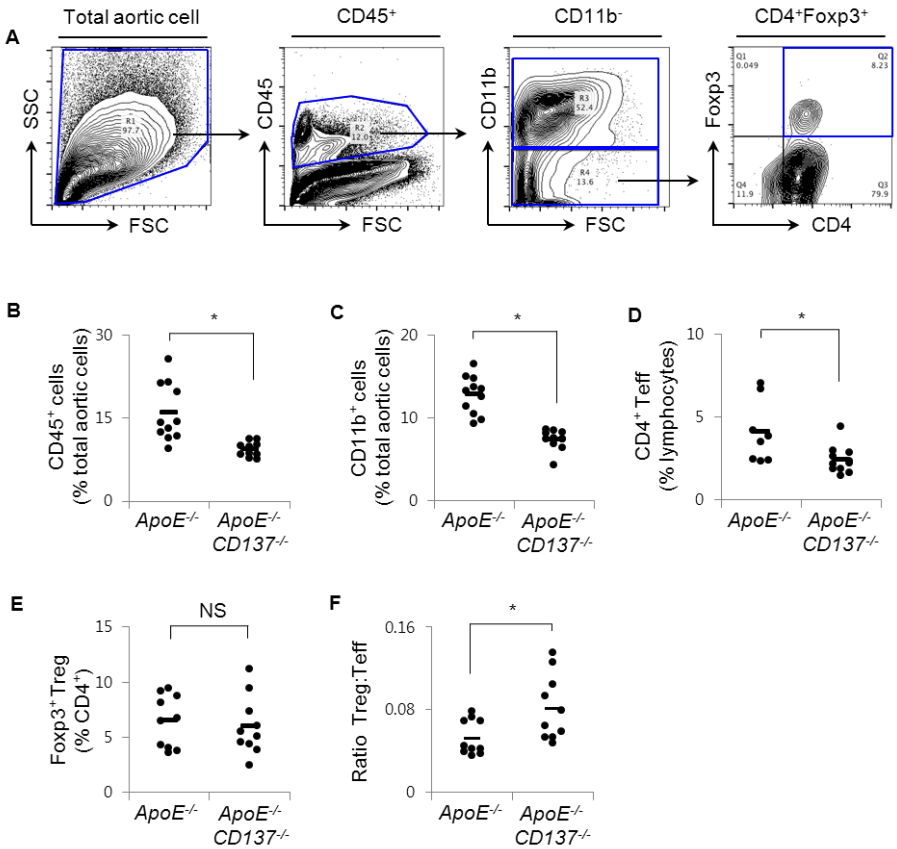


Figure III-1. CD137 deficiency affects migration of leukocytes. Flow cytometry for analysis of aortic leukocytes in enzyme digested whole aorta (from aortic sinus to femoral) from 66 week old chow diet fed *ApoE*$^{-/-}$, *ApoE*$^{-/-}$*CD137*$^{-/-}$ male mice. **A:** Gating strategy. Cell suspensions were stained with anti-CD45, anti-CD11b, anti-CD4, and anti-Foxp3 mAbs. Percentages of **B:** CD45^{+} leukocytes, **C:** CD11b^{+} macrophages, **D:** CD4^{+} T_{eff} cells, and **E:** Foxp3^{+} T_{reg} cells are shown. **F:** Ratio of T_{reg} cells and T_{eff} cells. n = 11 for *ApoE*$^{-/-}$, n = 10 for *ApoE*$^{-/-}$*CD137*$^{-/-}$. Circles represent individual mice; black lines represent the mean. *P < 0.05. NS, not significant

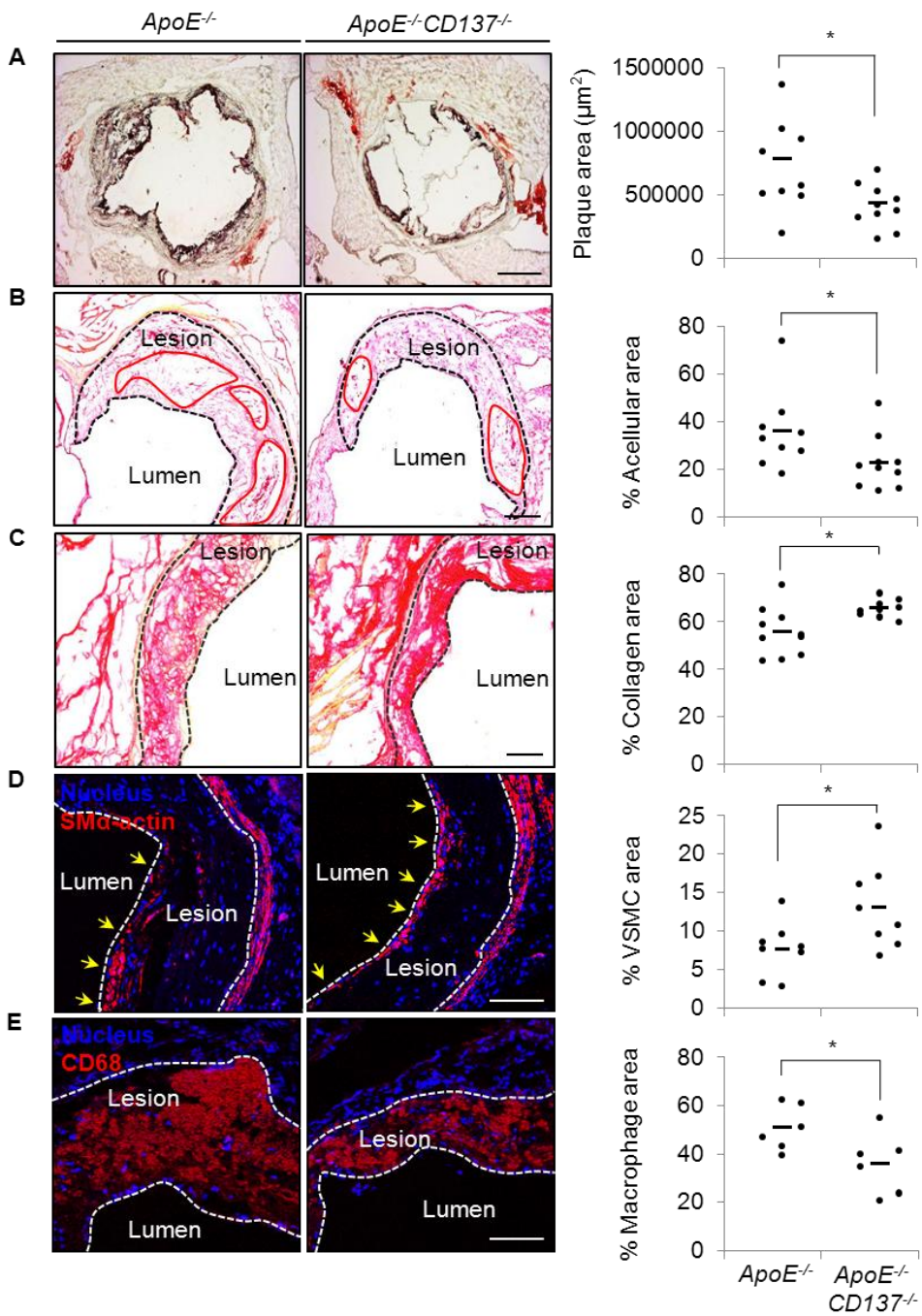

ApoE-/-
ApoE-/-CD137-/-
A
B
C
D
E
Lesion
Lumen
Nucleus
SMα-actin
CD68
Plaque area (μm2)
1500000
1000000
500000
0
% Acellular area
% Collagen area
% VSMC area
% Macrophage area
80
60
40
20
25
15
10
5
*
ApoE-/-
ApoE-/-
CD137-/-

Figure III-2. CD137 induces advanced atherosclerotic plaque phenotype. Representative photomicrographs and analysis of atherosclerotic plaque phenotypes in the aortic root of *ApoE*$^{-/-}$, *ApoE*$^{-/-}$*CD137*$^{-/-}$ male mice at 66 weeks of age on a normal chow diet. **A:** Oil red O staining for atherosclerotic plaque (n = 9 for *ApoE*$^{-/-}$, n = 10 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). **B:** Hematoxylin & Eosin staining for acellular regions (red lined area, n = 9 for *ApoE*$^{-/-}$, n = 10 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). **C:** Picrosirius red staining for collagen (n = 10 for *ApoE*$^{-/-}$, n = 9 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). **D:** Smooth muscle α-actin (SMα-actin) immunostaining for detecting migrated VSMCs into intima (yellow arrows, n = 8 for *ApoE*$^{-/-}$, n = 8 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). **E:** CD68 immunostaining for detecting infiltrated macrophages (n = 6 for *ApoE*$^{-/-}$, n = 6 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). Data are means ± SEM. Scale bars for **A, B** and **C**, 200 μm; **C** and **D**, 100 μm. Circles represent individual mice; black lines represent the mean. *$P < 0.05$.

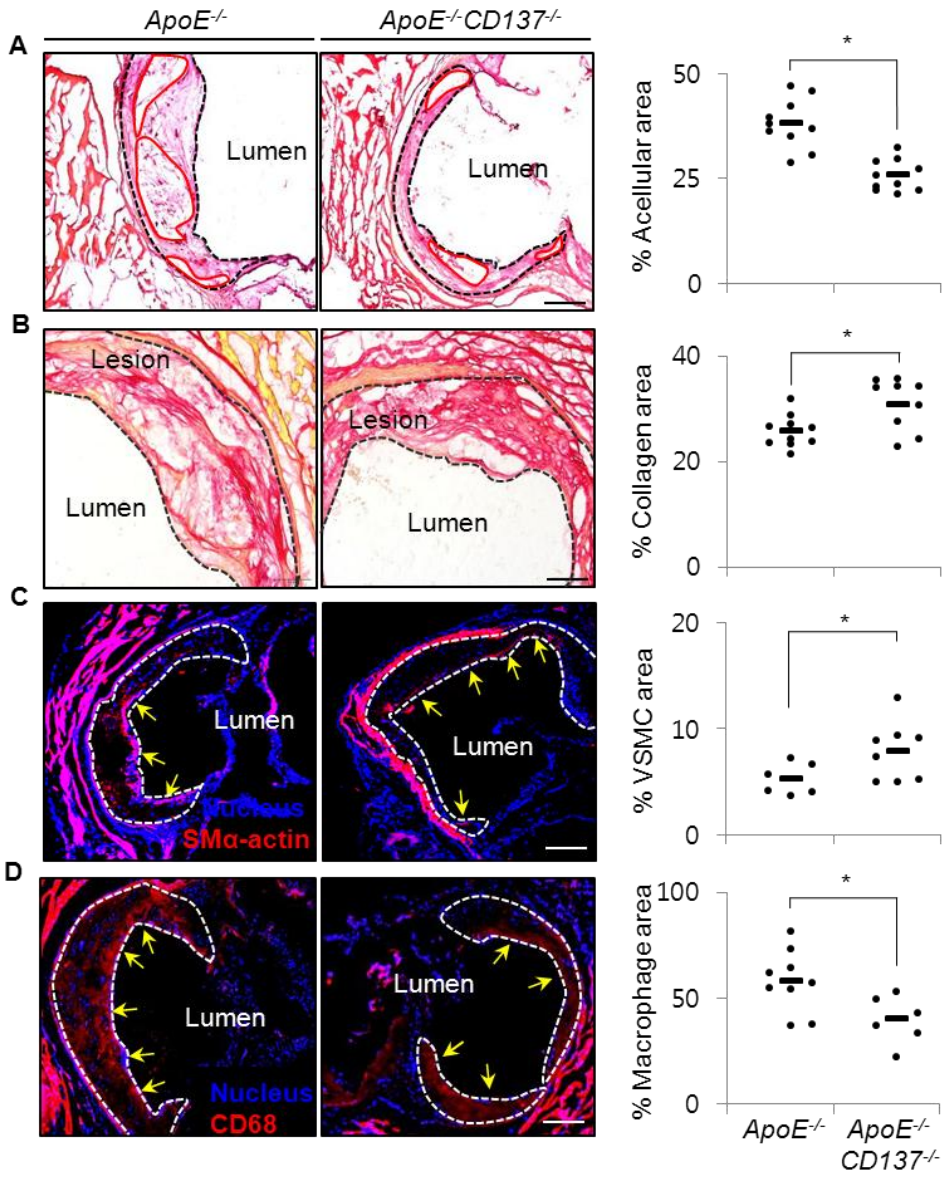

ApoE-/-
ApoE-/-CD137-/-
A
Lumen
Lumen
B
Lesion
Lumen
Lesion
Lumen
C
Lumen
Lumen
Nucleus
SMα-actin
D
Lumen
Lumen
Nucleus
CD68
% Acellular area
50
25
0
*
% Collagen area
40
20
0
*
% VSMC area
20
10
0
*
% Macrophage area
100
50
0
*
ApoE-/-
ApoE-/-
CD137-/-

Figure III-3. CD137 induces advanced atherosclerotic plaque phenotype. Representative photomicrographs and analysis of atherosclerotic plaque phenotypes in the aortic root of *ApoE*$^{-/-}$, *ApoE*$^{-/-}$*CD137*$^{-/-}$ female mice at 66 weeks of age on a normal chow diet. **A:** Hematoxylin & Eosin staining for acellular regions (red lined area, n = 10 for *ApoE*$^{-/-}$, n = 10 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). **B:** Picrosirius red staining for collagen (n = 10 for *ApoE*$^{-/-}$, n = 9 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). **C:** Smooth muscle α-actin (SMα-actin) immunostaining for detecting migrated VSMCs into intima (yellow arrows, n = 6 for *ApoE*$^{-/-}$, n = 8 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). **D:** CD68 immunostaining for detecting infiltrated macrophages (n = 9 for *ApoE*$^{-/-}$, n = 6 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). Data are means ± SEM. Scale bars for **A, C** and **D**, 200 μm; **B**, 100 μm. Circles represent individual mice; black lines represent the mean. *P < 0.05.

2. CD137 leads to enhanced expression of IFN-γ in T_{eff} cells.

Co-stimulatory receptors on T cells mediate T cell activation and induce cytokine secretion in immune responses. After activation, Th1 T_{eff} cells release cytokines, such as IFN-γ and TNF-α. These Th1 cytokines activate macrophages and vascular cells, facilitate inflammation, and participate in cellular immunity. In previous study, CD137 facilitates effector T cell activated inflammatory response via increased IFN-γ production. In case of T_{reg} cells, CD137 has been reported to be one of the target genes of the T_{reg} cell specific master transcription factor Foxp3 (Marson *et al*., 2007) and to be up-regulated after polyclonal activation of murine T_{reg} cell (McHugh, 2002). Because CD137 is not involved in the development of T_{reg} cells as shown in Fig. II-1E, we investigated further CD137 effect on functional activity of T_{reg} cells. In co-culture system of APCs, T_{eff} cells, and T_{reg} cells, there was no effect of CD137 on suppression of $CD137^{+/+}$ and $CD137^{-/-}$ T_{reg} cells (Fig. III-4A). Therefore, we could conclude that CD137 has no effect on the development and suppressive function of T_{reg} cells. So, we investigated CD137 effect on other T cell type, T_{eff} cells. IFN-γ production by activated $CD4^+$ and $CD8^+$ T_{eff} cells was decreased significantly in $CD137^{-/-}$ mice than $CD137^{+/+}$ mice (Fig. III-4B and 4C). Accordingly, to determine whether CD137 expresses and contributes to IFN-γ production under atherosclerotic environment, we examined CD137 expression in aortic T cells and IFN-γ levels in the aorta, skin draining lymph nodes, and spleen of $ApoE^{-/-}$ and $ApoE^{-/-}CD137^{-/-}$ mice. There was highly

elevated CD137 expression in aortic T cells from *ApoE*$^{-/-}$ mice (Fig. III-4D). Moreover, mRNA expression of IFN-γ was increased significantly in the aorta, skin draining lymph nodes, and spleen of *ApoE*$^{-/-}$ mice compared to *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice (Fig. III-4E). These results indicate that CD137 facilitates T cell activation leading to the production of INF-γ, and these CD137 dependent effects continue during atherosclerosis.

A

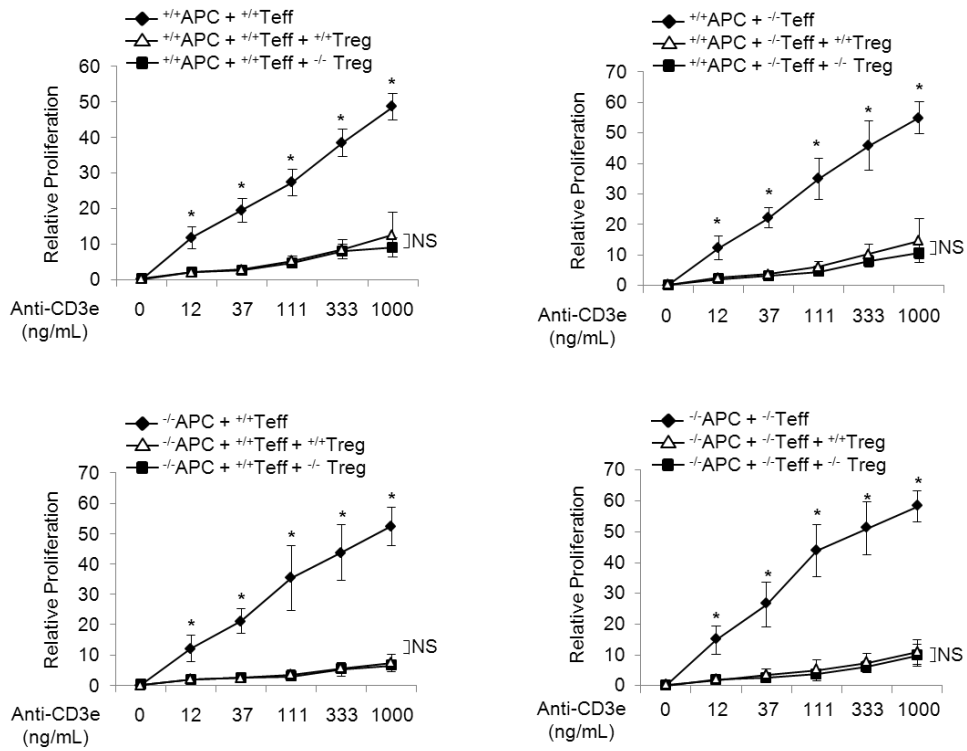
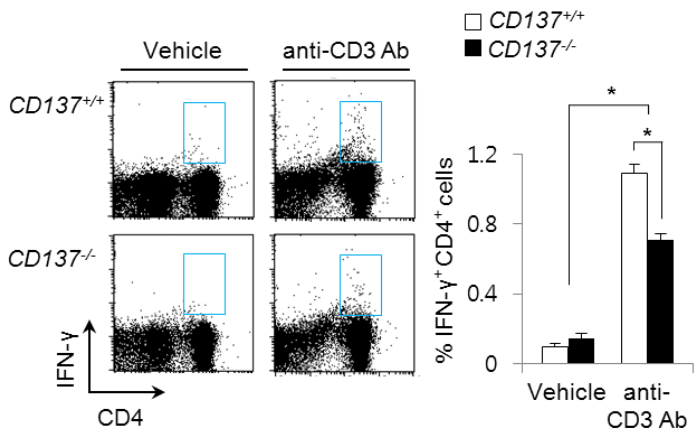


Figure III-4. CD137 leads to enhanced expression of IFN-γ in T_{eff} cells. A: Suppressive function of T_{reg} cells is independent of CD137 signaling. CFSE labeled T_{eff} cells were co-cultured with APCs and T_{reg} cells isolated from spleen of *CD137*$^{+/+}$ and *CD137*$^{-/-}$ mice. And then cells were activated with anti-CD3e Ab in dose dependent manner. Percentages of CFSE^{low} cells show relative proliferation of T_{eff} cells. **P* < 0.05. NS, not significant.

B



C

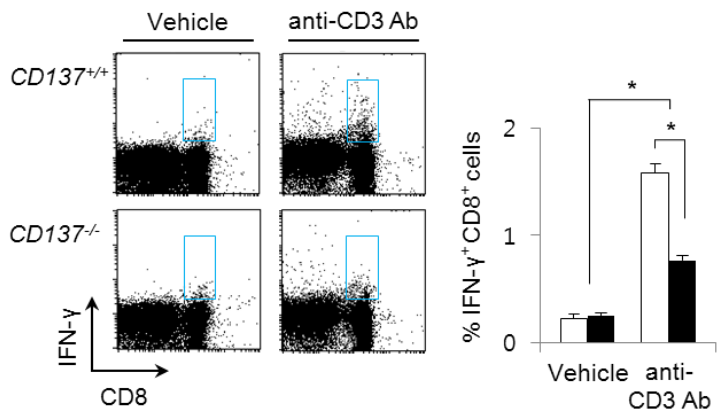


Figure III-4. Continued. B, C: Deficiency of CD137 attenuated IFN-γ production in T cells. *CD137*$^{+/+}$ and *CD137*$^{-/-}$ splenocytes were cultured with anti-CD3 Ab, rat IgG (vehicle control) and brefeldin A for 5 hr (n = 5 spleens/strain). Flow cytometry analysis showed the percentages of IFN-γ positive CD4^{+} (**B**) and CD8^{+} (**C**) T cells delineated by the boxed region. *$P < 0.05$.

D

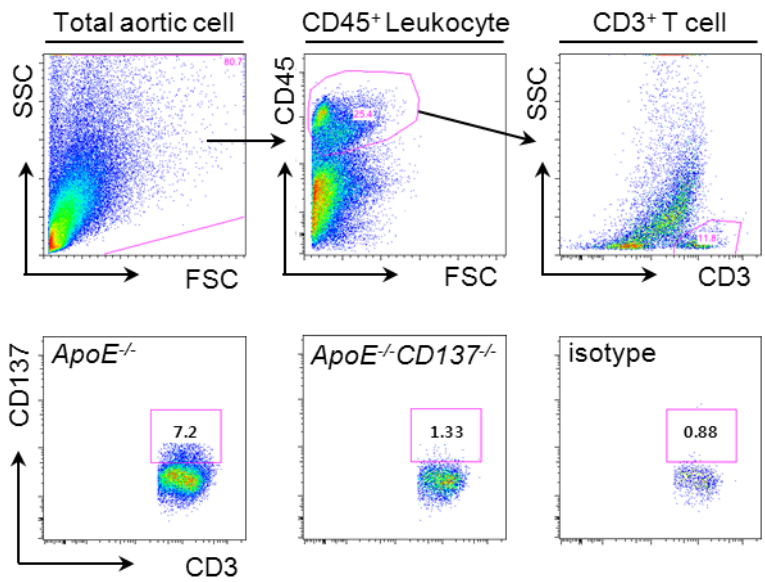


Figure III-4. Continued. D: Flow cytometry for analysis of CD137 expression in the whole aorta (from aortic sinus to femoral aorta) from 66 week old chow diet fed *ApoE*$^{-/-}$, *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice. Single aortic cell suspensions were prepared by digestion of aorta with a mixture of collagenase I and XI, hyaluronidase, and DNase I (aortic enzyme mixture). Cell suspensions were stained with anti-CD45, anti-CD3, and anti-CD137 mAbs. Percentages of CD137 positive CD3^{+} T cells delineated by the boxed region.

E

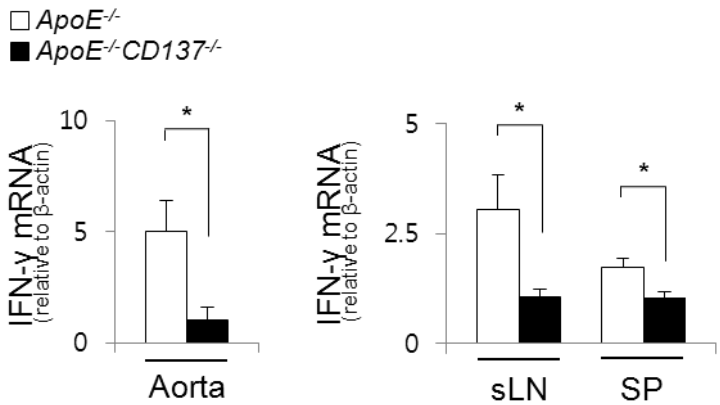


Figure III-4. Continued. E: CD137 increases IFN-γ production *in vivo*. Quantitative reverse-transcription polymerase chain reaction for mRNA levels of IFN-γ in the aorta, skin draining lymph nodes, and spleen in 66 week old *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice (n=5 mice/strain). Data was normalized to housekeeping gene β-actin and presented as fold change. *$P < 0.05$.

3. CD137 is expressed in macrophages and VSMCs

Next, to investigate whether CD137 is expressed in macrophages and VSMCs, which are functional cells involved in atherosclerotic plaque stability, we performed immunohistochemistry of advanced atherosclerotic plaque of normal chow diet fed *ApoE*$^{-/-}$ mice. We observed that CD137 is expressed in infiltrated macrophages (Fig. III-5A), VSMCs in fibrous cap (Fig. III-5B), and migrated VSMCs (Fig. III-5C) into atherosclerotic plaque lesions. However, medial VSMCs do not express CD137. Accordingly, we examined *in vitro* CD137 expression on the surface of macrophages and VSMCs induced by atherogenic factors. In macrophages, high levels of CD137 were expressed and activated with oxLDL resulting in strong CD137 mRNA (Fig. III-5D), and protein expression (Fig. III-5E). Also, levels of CD137 mRNA (Fig. III-5F) and protein (Fig. III-5G) were increased significantly on the surface of VSMCs treated with cytokine mixture (IFN-γ, TNF-α, and IL-1β). Taken together, these results suggested that CD137 expression is dependent upon atherogenic and inflammatory stimuli, and imply possibilities that CD137 would be involved in functional activity in both macrophages and VSMCs.

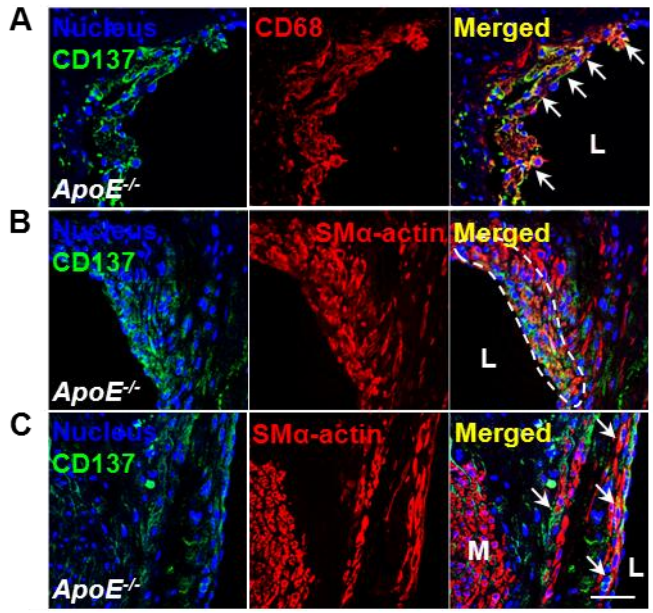
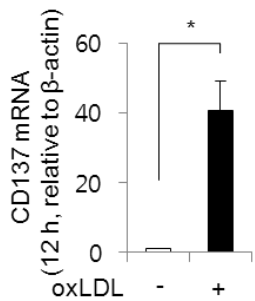


Figure III-5. CD137 is expressed in macrophages and VSMCs. A-C: Representative images for CD137 expression in infiltrated macrophages and intimal VSMCs in advanced atherosclerotic plaque of *ApoE*$^{-/-}$ mice. **A:** CD137 expression in macrophages. White arrows indicate CD68^{+} macrophages expressing CD137. **B-C:** CD137 expression in VSMCs. CD137 is expressed in VSMCs in fibrous caps (**B,** white dotted line area) and migrated VSMCs (**C,** white arrows) in plaque of *ApoE*$^{-/-}$ mice. Scale bars for **A-C**, 50 μm.

D



E

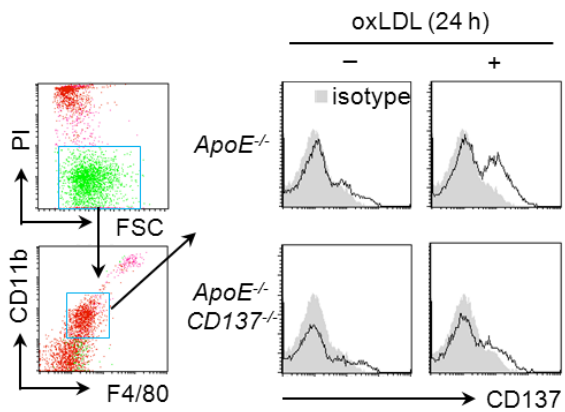
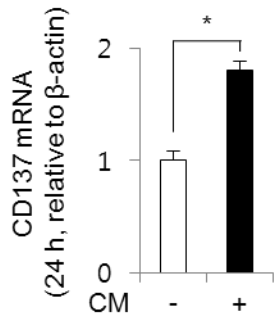


Figure III-5. Continued. D-E: PBS-elicited peritoneal macrophages from *ApoE*$^{-/-}$ mice were stimulated with 50 μg/ml oxLDL for 12, 24 h. **D:** Levels of CD137 mRNA were analyzed by real-time quantitative polymerase chain reaction. Data was normalized to housekeeping gene β-actin and presented as fold change. **E:** Levels of CD137 protein in non-apoptotic (PI negative), F4/80, CD11b double positive cells were determined by flow cytometry. Staining with isotype control Ab is shown (gray histogram). *$P < 0.05$.

F



G

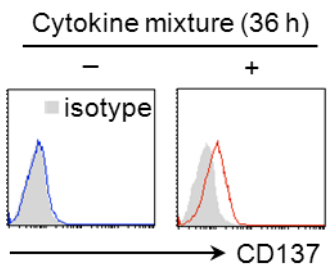


Figure III-5. Continued. F-G: MOVAS-1 cells were treated with a mixture of IFN-γ, TNF-α, and IL-1β at 25 ng/ml (referred to as cytokine mix, CM) for 24, 36 h. **F:** Levels of CD137 mRNA were analyzed by real-time quantitative polymerase chain reaction. Data was normalized to housekeeping gene β-actin and presented as fold change compared with unstimulated samples. **G:** Expression of CD137 protein was determined by flow cytometry. Staining with isotype control Ab is shown (gray histogram). *$P < 0.05$.

4. CD137 signaling increases oxLDL dependent macrophage MMP-9

Previous mouse studies suggested that levels of MMP-9 increased during atherosclerosis, and MMP-9 expression in the carotid tissue is dependent predominantly on bone marrow derived macrophages (Choi, 2005). Also, the overexpression of MMP-9 in macrophages induced vulnerable features of ruptured plaques (Gough, 2006). So, we investigated whether MMP-9 expression is involved in advanced atherosclerotic plaque lesions of *ApoE*$^{-/-}$ mice. Immunohistochemical staining revealed that high levels of MMP-9 were expressed in the area of accumulated macrophages in atherosclerotic plaque (Fig. III-6A). Because oxLDL was a strong stimuli for CD137 expression on peritoneal macrophages (Fig. III-5D and 5E), we stimulated *CD137*$^{+/+}$ peritoneal macrophages with oxLDL to induce MMP-9 along with agonistic anti-CD137 mAb to activate CD137 signaling. We found that basal levels of MMP-9 mRNA were low in the absence of oxLDL. However, higher levels of MMP-9 mRNA were induced significantly in oxLDL treated *ApoE*$^{-/-}$ macrophages. Moreover, activation of CD137 signaling induced a stronger increase in mRNA expression of MMP-9 in the presence of oxLDL (Fig. III-6B). Next, to investigate whether MMP-9 expression is dependent on CD137, we examined *in vitro* CD137 dependent MMP-9 expression in macrophages of *CD137*$^{+/+}$ and *CD137*$^{-/-}$ mice. After isolation of macrophages, we treated them with oxLDL. Basal expression of MMP-9 was low in macrophages of both genotypes. However, after oxLDL treatment, MMP-9

expression was increased significantly in *CD137*$^{+/+}$ macrophages and this increase was attenuated in oxLDL treated *CD137*$^{-/-}$ macrophage (Fig. III-6C). We also performed gelatin zymography. MMP-9 activity showed no significant difference in conditioned medium obtained from control *CD137*$^{+/+}$ and *CD137*$^{-/-}$ macrophages. However, MMP-9 activity was much stronger in conditioned medium from oxLDL treated *CD137*$^{+/+}$ macrophages compared with control *CD137*$^{+/+}$ macrophages. Furthermore, MMP-9 activity in conditioned medium obtained from oxLDL treated *CD137*$^{-/-}$ macrophages was significantly decreased compared to that of *CD137*$^{+/+}$ macrophages (Fig. III-6D). As MMP-9 expression is mediated by p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase1/2 (ERK1/2) pathways (Arai *et al*., 2003; Lo *et al*., 2013), leading to nuclear factor (NF-κB) activation (Lee *et al*., 2012a; Mukhopadhyay *et al*., 2012), we aimed to determine whether CD137 signaling could enhance MMP-9 expression. Based on these observations, we investigated the molecular mechanisms for CD137 mediated MMP-9 expression. We found that the phosphorylation of p38 MAPK and ERK1/2 was increased markedly in *CD137*$^{+/+}$ macrophages stimulated with oxLDL in the presence of agonistic anti-CD137 mAb (Fig. III-6E). By contrast, there were no phosphorylated changes among the other MAPK family, stress-activated protein kinases/Jun amino-terminal kinases (SAPK/JNK). Therefore, these results demonstrated that CD137 signaling increases oxLDL dependent macrophage MMP-9, and activation of CD137 signaling followed by phosphorylation of p38 MAPK and ERK1/2 pathways, induced an enhanced expression of MMP-9.

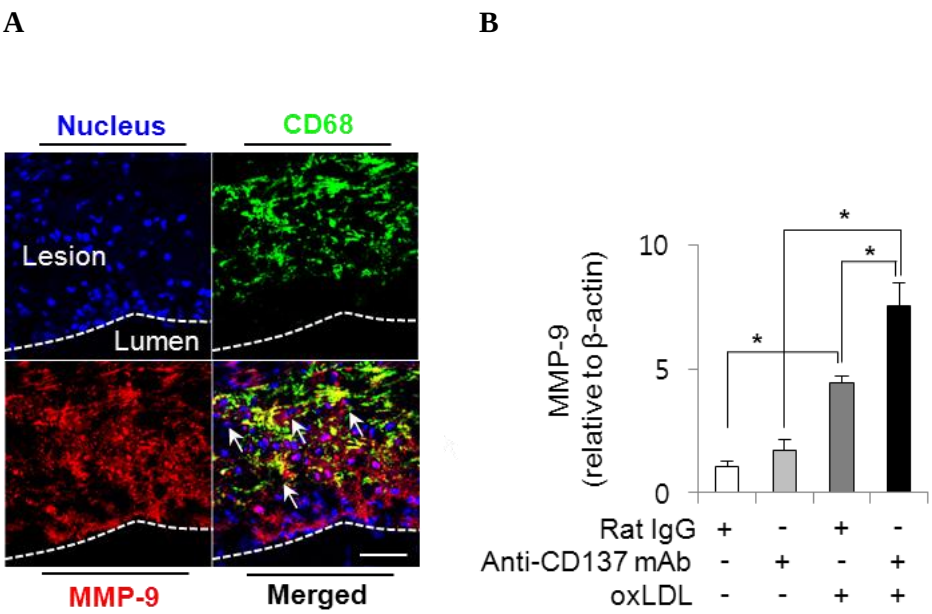


Figure III-6. OxLDL induced macrophage MMP-9 is increased via activation of CD137 signaling. A: Representative images for MMP-9 expression in infiltrated macrophages in atherosclerotic plaque of *ApoE*$^{-/-}$ mice. The white arrows indicate MMP-9 expressing macrophages. Scale bar, 50 μm; **B:** PBS-elicited peritoneal macrophages from *ApoE*$^{-/-}$ mice were stimulated with 50 μg/ml oxLDL for 30 min. Levels of MMP-9 mRNA were analyzed by real-time quantitative polymerase chain reaction. Data was normalized to housekeeping gene β-actin and presented as fold change. Data are means ± SEM from three independent experiments. *$P < 0.05$.

C

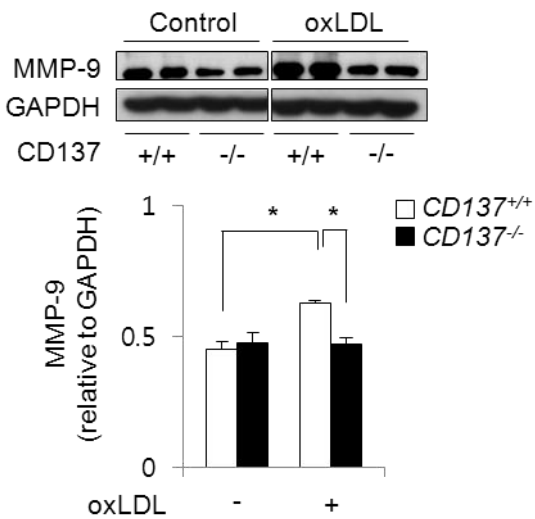


Figure III-6. Continued. C-D: PBS-elicited peritoneal macrophages from *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice were stimulated with 50 μg/ml oxLDL in conditioned medium for 24 h. **C:** Immnuo-blot of MMP-9 expression in cell lysate of the indicated genotypes. Band intensity of MMP-9 relative to GAPDH was analyzed. Data are means ± SEM from three independent experiments. *$P < 0.05$.

D

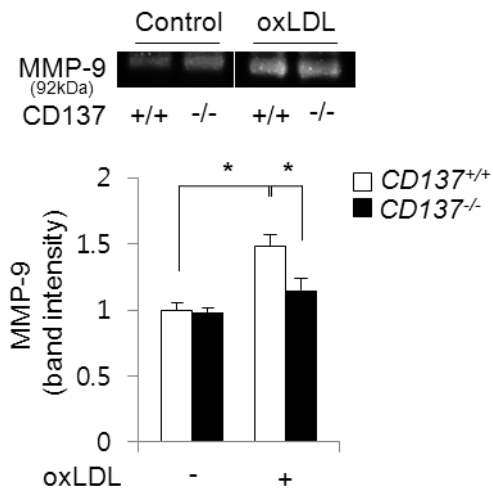


Figure III-6. Continued. D: Gelatin zymography of conditioned medium of the indicated genotypes. Band intensity of MMP-9 was analyzed. Data are means ± SEM from three independent experiments. *$P < 0.05$.

E

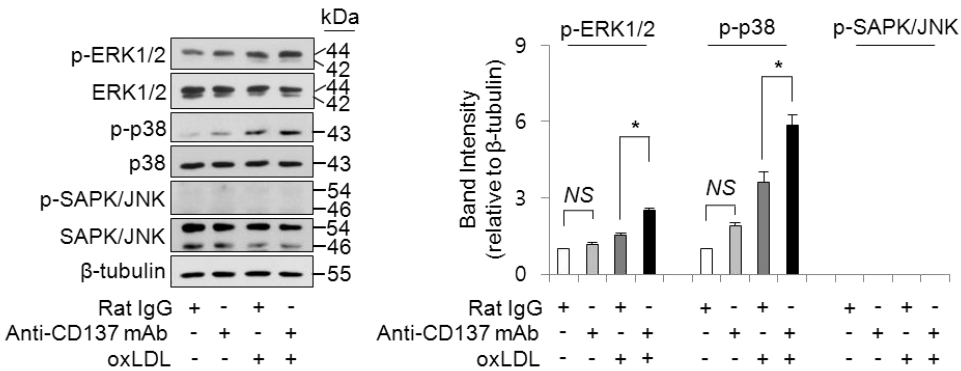


Figure III-6. Continued. E: PBS-elicited peritoneal macrophages from *ApoE*$^{-/-}$ mice were stimulated with 50 μg/ml oxLDL for 30 min in the presence of 5 μg/ml agonistic anti-CD137 mAb or 5 μg/ml rat IgG. Activities of p38 MAPK, SAPK/JNK and ERK1/2 were examined by immuno-blot analysis using phospho-specific Abs. The total protein levels of p38 MAPK, SAPK/JNK and ERK1/2 were also measured by band intensity normalized to β-tubulin. Data are means ± SEM from three independent experiments. *$P < 0.05$. NS, not significant.

5. CD137 signaling activates MMP expression

To assess the *in vivo* effects of CD137 signaling on MMP expression, we performed gelatin zymography for MMP activity in the aortas of *ApoE*$^{-/-}$, *ApoE*$^{-/-}$ *CD137*$^{-/-}$ mice. *In situ* gelatin zymography showed that levels of active MMP in advanced atherosclerotic plaque were higher in *ApoE*$^{-/-}$ mice than in *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice (Fig. III-7A). Next, to confirm which cell type is prominent in expressing MMPs, we performed immunohistochemistry. We observed that most active MMP expression was prominent in macrophage-rich regions in the atherosclerotic plaque of both mice groups (Fig. III-7B). Importantly, active MMP in macrophages in atherosclerotic plaque was higher significantly in *ApoE*$^{-/-}$ mice than *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice. In contrast, migrated intimal VSMCs did not induce active MMPs, but medial VSMCs expressed a small amount of active MMPs (Fig. III-7C and 7D). Next, we quantified lesional MMP-9 and MMP-2 expression in atherosclerotic plaque lesions of both mice groups. Abundant MMP-9 was increased highly in atherosclerotic plaque of *ApoE*$^{-/-}$ mice compared to that of *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice (Fig. III-7E). MMP-2, another gelatinase, was less expressed in atherosclerotic plaque lesions of both mice groups. Taken together, these findings suggested that CD137 is crucial for the expression and activation of MMPs, which are predominantly derived from macrophages in atherosclerotic plaque lesions. Moreover, we confirmed that increased lesional MMP-9 expression is regulated by CD137.

A

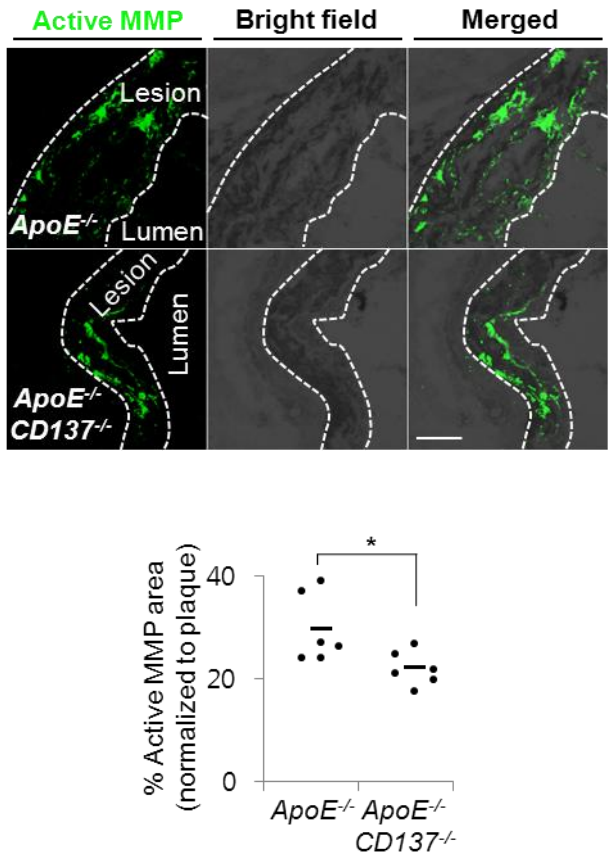


Figure III-7. CD137 signaling activates MMP expression. A: *In situ* zymography for gelatinase activity. Representative images for active MMP expression in atherosclerotic plaque in the aortic root of *ApoE*$^{-/-}$ (upper panel), *ApoE*$^{-/-}$*CD137*$^{-/-}$ (lower panel) mice. Percentage of active MMP stained area per plaque was shown (n = 6 for *ApoE*$^{-/-}$, n = 6 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). *P < 0.05.

B

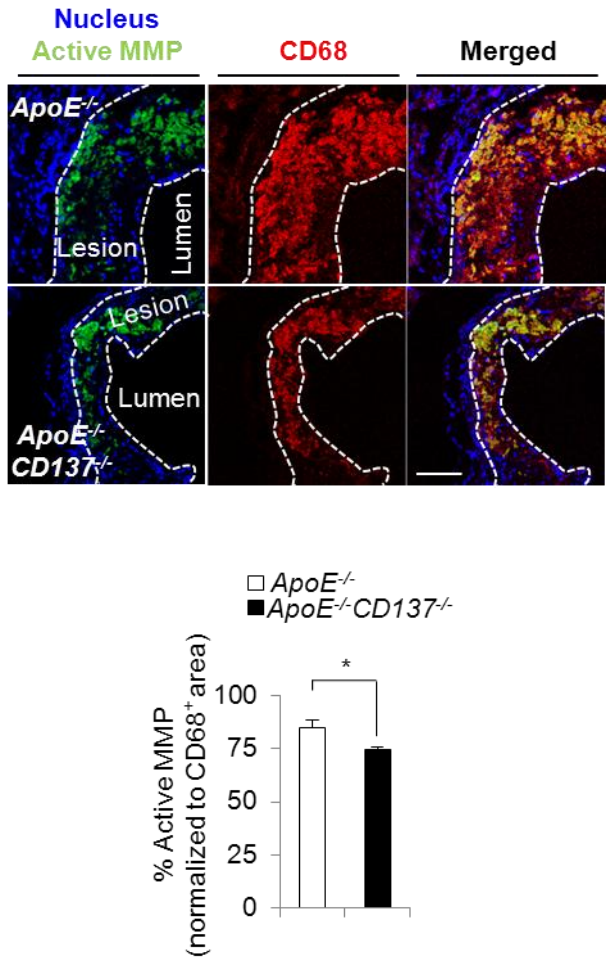
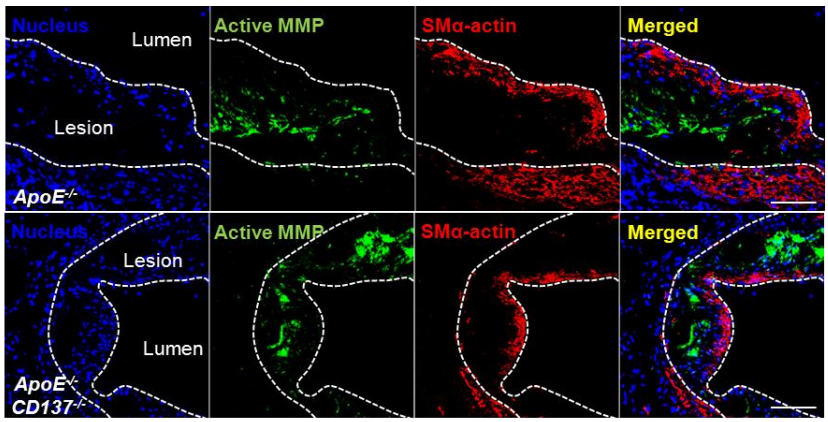


Figure III-7. Continued. B: Active MMP expression on infiltrated macrophages in advanced atherosclerotic plaque. After performing *in situ* zymography, tissues were fixed, and macrophages were labeled with anti-CD68 Ab. Percentage of active MMP stained area per CD68 positive macrophages was shown (n = 8 for *ApoE*$^{-/-}$, n = 8 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). *P < 0.05.

C



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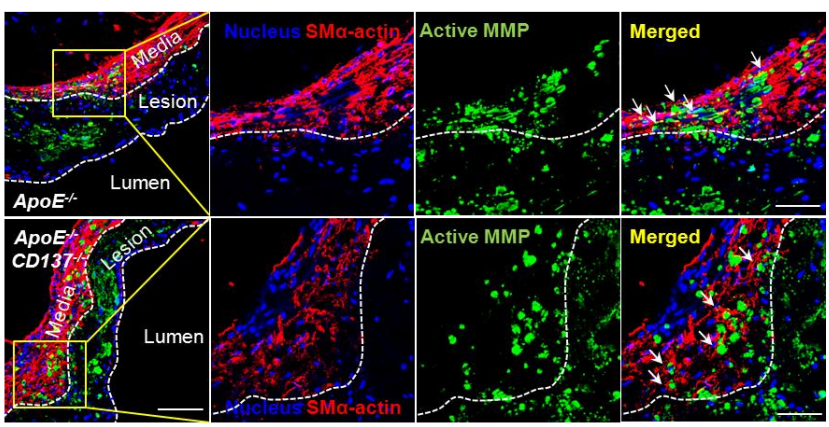

Figure III-7. Continued. C, D: C, In atherosclerotic plaque lesion, migrated intimal VSMCs did not express active MMPs in both mice groups. **D,** In both mice groups, medial VSMCs expressed a little amounts of active MMPs (yellow lined box area in left panel, white arrows indicate active MMPs expressing medial VSMCs at enlarged images of yellow lined box area). Scale bars for **C**, 100 μm; **D,** left panel of **D**, 100 μm; middle and right panel of **D**, 50 μm.

E

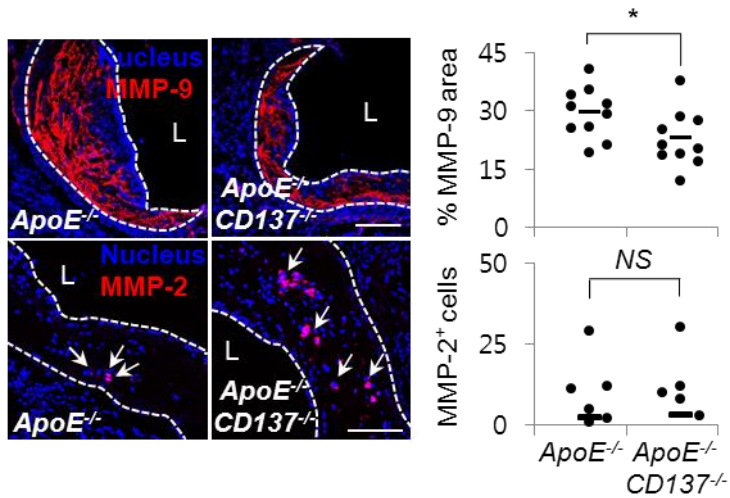

Figure III-7. Continued. E: MMP-9 and MMP-2 expression in atherosclerotic plaque (MMP-9; n = 10 for *ApoE*$^{-/-}$, n = 10 for *ApoE*$^{-/-}$*CD137*$^{-/-}$, MMP-2; n = 6 for *ApoE*$^{-/-}$, n = 5 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). Circles represent individual mice; black lines represent the mean. Scale bars, 100 μm. *P < 0.05. NS, not significant.

6. CD137 signaling induces apoptosis of VSMCs

In advanced plaque lesions, a fibrous cap consists of endothelial cells and migrated VSMCs. In terms of CD137 signaling on endothelial cells, we have recently shown that CD137 was expressed in endothelial cells within inflammation sites of atherosclerotic plaque lesions. Also, CD137 signaling enhances the production of pro-inflammatory molecules in endothelial cells (Jeon *et al*., 2010). So, we investigated the role of CD137 on another non-hematopoietic cell type, VSMC. It has been suggested that VSMC apoptosis induces plaque inflammation (Clarke *et al.*, 2009), as well as further features of plaque vulnerability in atherosclerosis (Clarke *et al*., 2006). In CD137 signaling in VSMCs, a previous human study showed that human aortic VSMC expressed CD137 and VSMC proliferation was decreased in response to CD137 signaling. To address the functional effect of CD137 signaling in VSMCs in a mouse model, we first performed a TUNEL assay to detect apoptotic VSMCs in atherosclerotic plaque (Fig. III-8A). Compared with *ApoE*$^{-/-}$*CD137*$^{-/-}$, TUNEL positive VSMCs in a fibrous cap were increased in the atherosclerotic plaque of *ApoE*$^{-/-}$. Since caspase-3 cascade is a key marker of apoptosis, we measured the levels of caspase-3 cascade in VSMCs *in vitro* (Fig. III-8B). Caspase-3 was detected in VSMCs, and the expression levels were comparable in the presence or absence of agonistic anti-CD137 mAb. However, the expression level of cleaved caspase-3, the activated form of caspase-3, was increased significantly by treatment of cytokines combined

with agonistic anti-CD137 mAb. Also, immunostaining showed a CD137 activation dependent increase in intracellular cleaved caspase-3 (Fig. III-8C). As Bcl-2 is a well-known anti-apoptotic gene which prevents cell death by inhibition of caspase activation (Finucane, 1999; Zhou *et al*., 2011), we measured the cellular level of Bcl-2. Treatment of cytokines combined with agonistic anti-CD137 mAb significantly decreased the expression of Bcl-2 proteins in VSMCs (Fig. III-8D). Oxidized lipids or lipoproteins induced macrophage apoptosis and endoplasmic reticulum stress activated the apoptotic pathway in advanced lesional macrophages (Seimon and Tabas, 2008; Timmins *et al*., 2009). In our system, we found that resident apoptotic cells were increased in atherosclerotic plaque of *ApoE*$^{-/-}$ mice compared to that of *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice (Fig. III-8E). However, we could not determine any difference of apoptotic macrophages in the plaque of *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice (data not shown). Consistent with *in vivo* results, *in vitro* culture of peritoneal macrophages of both mice treated with oxLDL in the presence of agonistic anti-CD137 mAb revealed that apoptosis of macrophages was not increased by activation of CD137 signaling (Fig. III-8F). It is well known that, TGF-β production has a beneficial role in the vascular stability of VSMCs (Amento *et al*., 1991; Bobik, 2006). However, no significant difference in TGF-β production via CD137 activation was observed in our experiment (Fig. III-8G). An increased production of TGF-β was only observed upon treatment with PDGF-BB. Taken together, these results suggest that the activation of CD137 signaling inhibited Bcl-2, and then by up-regulated cleaved caspase-3, leads to apoptosis of VSMCs.

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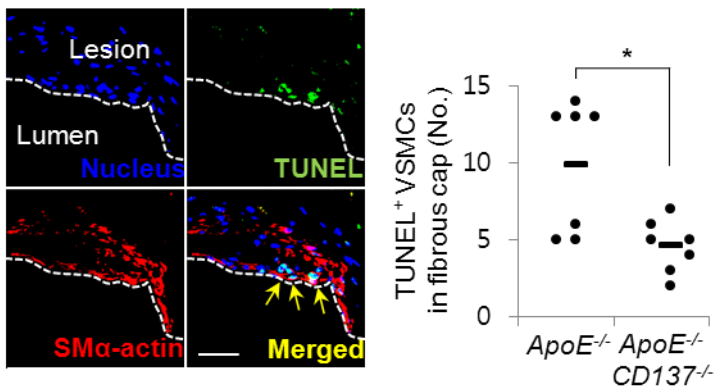

Figure III-8. Activation of CD137 signaling induces apoptosis of VSMCs. A: Representative images for apoptosis on VSMCs in atherosclerotic plaque. Yellow arrows indicate TUNEL positive apoptotic VSMCs. *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice at 66 weeks of age on a normal chow diet were used (n = 7 for *ApoE*$^{-/-}$, n = 7 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). Circles represent individual mice; black lines represent the mean. Scale bar, 50 μm. *P < 0.05.

B

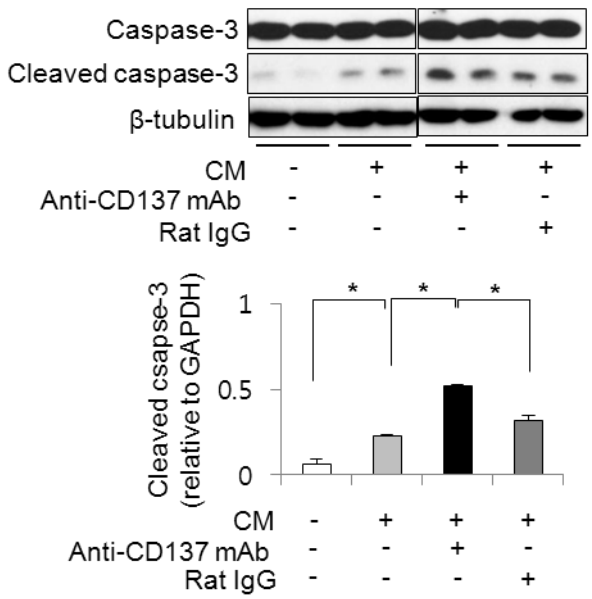

Figure III-8. Continued. B: MOVAS-1 cells were treated with mix of IFN-γ, TNF-α and IL-1β (each 25 ng/ml; referred to as cytokine mix, CM) for 72 h in the presence of 5 μg/ml agonistic anti-CD137 mAb or 5 μg/ml rat IgG. Immnuo-blot analysis of caspase-3, cleaved caspase-3 in cell lysate of MOVAS-1 cells. Band intensity of cleaved caspase-3 relative to β-tubulin was analyzed. Data are means ± SEM from three independent experiments. **P* < 0.05.

C

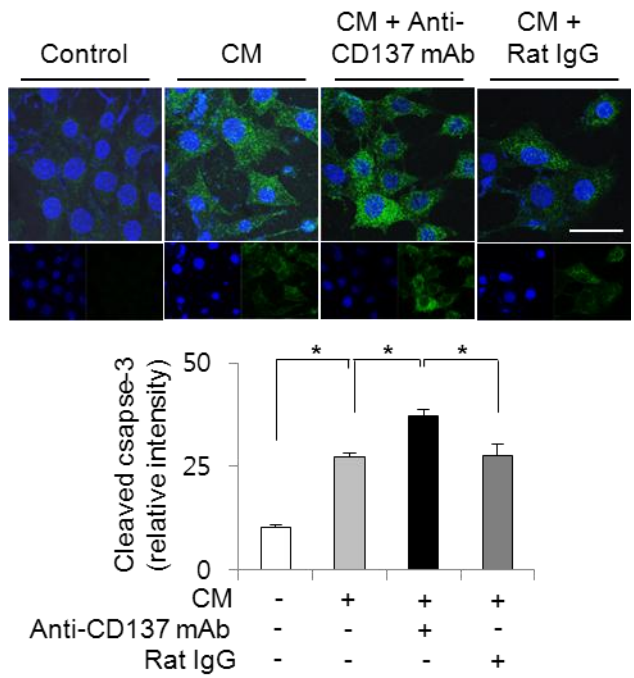

Figure III-8. Continued. C: MOVAS-1 cells were treated with mix of IFN-γ, TNF-α and IL-1β (each 25 ng/ml; referred to as cytokine mix, CM) for 72 h in the presence of 5 μg/ml agonistic anti-CD137 mAb or 5 μg/ml rat IgG. Intracellular staining of cleaved caspase-3 in MOVAS-1 cells. Fluorogenic intensity of cleaved caspase-3 was analyzed. Scale bar, 50 μm. *$P < 0.05$.

D

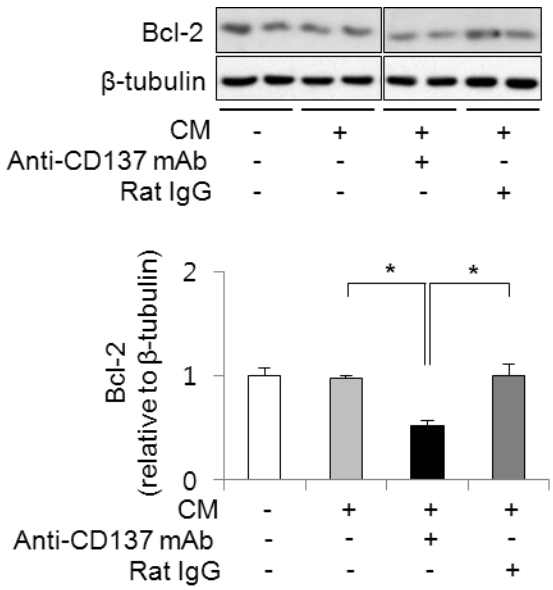

Figure III-8. Continued. D: MOVAS-1 cells were treated with mix of IFN-γ, TNF-α and IL-1β (each 25 ng/ml; referred to as cytokine mix, CM) for 24 h in the presence of 5 μg/ml agonistic anti-CD137 mAb or 5 μg/ml rat IgG. Immuno-blot of Bcl-2 expression in cell lysate of MOVAS-1 cells. Band intensity of cleaved Bcl-2 relative to β-tubulin was analyzed. Data are means ± SEM from three independent experiments. **P* < 0.05.

E

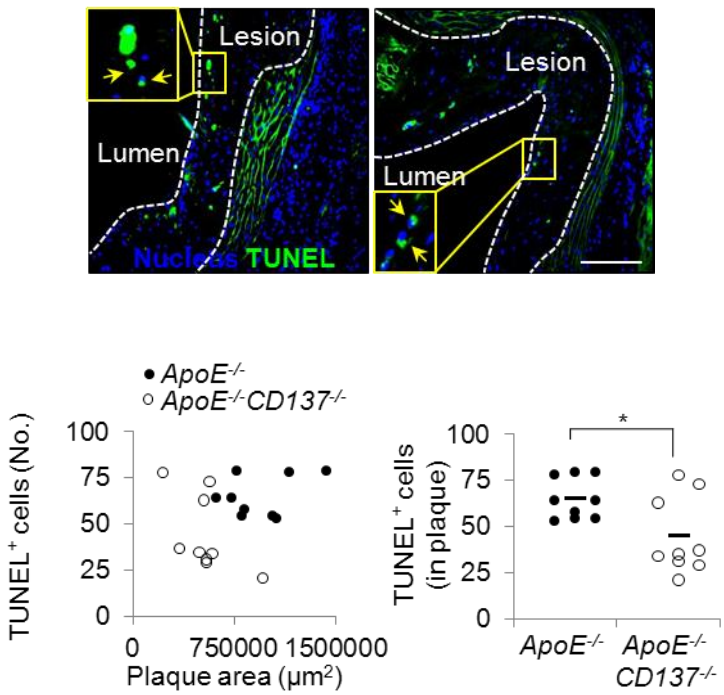

Figure III-8. Continued. E: TUNEL staining for apoptotic cells in plaque (yellow arrows in yellow lined box, n = 9 for *ApoE*$^{-/-}$, n = 9 for *ApoE*$^{-/-}$*CD137*$^{-/-}$). Scale bar, 200 μm. Circles represent individual mice; black lines represent the mean.

F

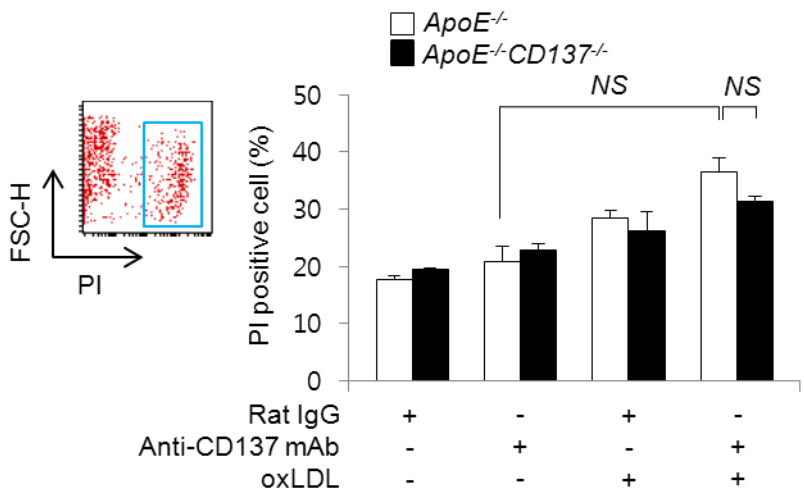

Figure III-8. Continued. F: Peritoneal macrophages were isolated from *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice at 9 weeks of age. Cells were stimulated with 50 μg/ml oxLDL in the presence of 5 μg/ml agonistic anti-CD137 mAb or same dose of rat IgG for 24 h. The survival rate of the macrophages was determined by measuring cell viability using propidium iodide staining by flow cytometry. Data are means ± SEM. NS, not significant.

G

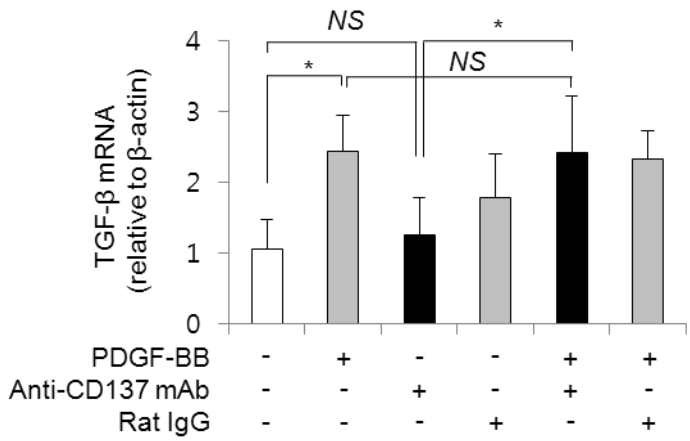

Figure III-8. Continued. G: Activation of CD137 signaling does not induce TGF-β expression in VSMCs. Expressions of TGF-β mRNA was determined by quantitative real time-PCR analysis. MOVAS-1 cells were stimulated with 25 ng/ml PDGF-BB in the presence of 5 μg/ml agonistic anti-CD137 mAb or same dose of rat IgG for 72 hours. The data were normalized to β-actin mRNA expression. Data are means ± SEM from three independent experiments. * $P < 0.05$. NS, not significant.

7. Agonistic anti-CD137 mAb treatment induces vulnerable plaque phenotype.

It is of note that *ApoE*$^{-/-}$ mice were characterized as having a more vulnerable plaque phenotype than *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice. These results support that further activation of CD137 signaling would aggravate atherosclerotic plaque instability. Thus, to examine the *in vivo* effects of CD137 signaling activation on atherosclerotic plaque stability, we used diet induced *Ldlr*$^{-/-}$ mice which were fed a high fat diet for 16 weeks. Mice were injected intraperitoneally with agonistic anti-CD137 mAb or a control (rat IgG) once a week for the entire 16 weeks. We found that atherosclerotic plaque lesions at the beginning of aorta were comparable between two groups of mice (Fig. III-9A). To test the involvement of *in vivo* CD137 activation in atherosclerotic plaque stability, we analyzed phenotypes of atherosclerotic plaque. The acellular, nuclear free area in atherosclerotic plaque was significantly increased in agonistic anti-CD137 mAb injected mice compared with rat IgG injected mice (Fig. III-9B). Distribution and expression of collagen were slightly decreased in anti-CD137 agonistic mAb injected mice but showed no significant difference between mice groups (Fig. III-9C). However, migrated VSMCs into atherosclerotic plaque were decreased (Fig. III-9D), and infiltrated macrophages were increased in agonistic anti-CD137 mAb injected mice compared with rat IgG injected mice (Fig. III-9E). These results indicate that *in vivo* CD137 activation facilitates the progression of atherosclerosis, as characterized by unstable plaque phenotypes.

A

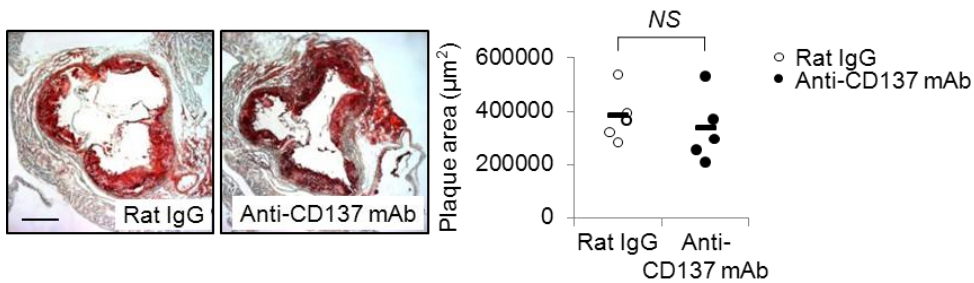

Figure III-9. *In vivo* activation of CD137 signaling induces vulnerable plaque phenotypes. *Ldlr*$^{-/-}$ male mice were fed a high-fat diet for 16 weeks. During this period, mice were injected i.p. with 50 μg rat IgG or 50 μg agonistic anti-CD137 mAb once a week. **A:** Oil red O stained atherosclerotic plaque in the aortic root. *n* = 5 for rat IgG group, *n* = 5 for agonistic anti-CD137 mAb group. Scale bar for **A**, 200 μm. NS, not significant.

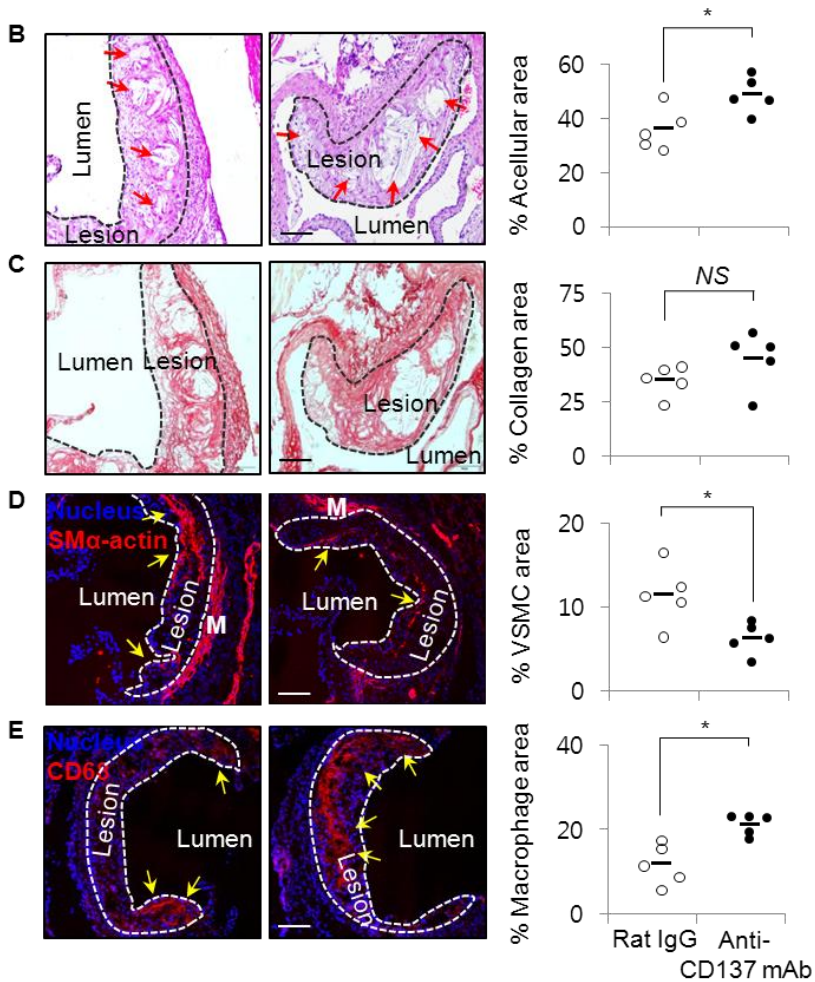

Figure III-9. Continued. B: Hematoxylin and Eosin staining for acellular, nuclear free area (red arrows). **C:** Picrosirius red staining for collagen. **D:** Smooth muscle α-actin (SMα-actin) immunostaining for detecting migrated VSMCs into intima (yellow arrows). **E:** CD68 immunostaining for detecting infiltrated macrophages (yellow arrows). **B-E**, n = 5 for rat IgG group, n = 5 for agonistic anti-CD137 mAb group. Scale bars for **B-E**, 100 μm. Circles represent individual mice; black lines represent the mean. *P < 0.05. NS, not significant.

Discussion

Although CD137 is known to be a powerful T cell co-stimulatory molecule, it has recently been revealed that CD137 is expressed and has diverse functions on a variety of immune and vascular cells. Thus, we investigated the effect of CD137 on plaque stability in advanced atheroma, and mainly focused on T cells, macrophages, and VSMCs. Our first aim was to determine whether the effect of CD137 was maintained in advanced atheroma as well as during the pathogenesis of atherosclerosis. To characterize which cells in atherosclerotic plaque are controlled under CD137 signaling, we examined the CD137 expression of T cells, macrophages, and VSMCs. Initially, we found that CD137 expresses and contributes to IFN-γ production under atherosclerotic environment in T cells from *ApoE*$^{-/-}$ mice. Also, both infiltrated macrophages and migrated VSMCs expressed CD137 in atherosclerotic plaque of *ApoE*$^{-/-}$ mice. Accordingly, inflammatory and atherosclerotic condition dependent CD137 mRNA, protein expressions on these cells were confirmed using an *in vitro* cell culture. A previous study had shown that CD137L was expressed in macrophages, and that activation of macrophages was induced by reverse signaling through CD137 in activated T cells (Jeon *et al*., 2010). However, in this study, we found that CD137 was also expressed in macrophages, and focused our research on CD137 signaling in macrophages.

Vulnerable plaque is associated with an excessive production of MMPs,

which are assumed to orchestrate widespread matrix destruction. We found that high levels of MMP-9 were expressed in macrophages in atherosclerotic plaque. To investigate whether MMP-9 expression is regulated by activation of CD137 signaling, we analyzed the mechanisms of MMP-9 expression. Previous studies suggested that treatment of macrophages with oxLDL up-regulates MMP-9 (Chen *et al*., 2011; Li and Renier, 2009), and CD137 activates intracellular MAPK signaling pathways, such as p38 and SAPK/JNK (Arch, 1998; Kwon, 2002). As shown in Fig. III, oxLDL is a strong inducer of CD137 expression. Upon treatment of oxLDL, significant increases in MMP-9 expression were detected, and CD137 was highly expressed in macrophages. Furthermore, co-treatment of oxLDL and agonistic anti-CD137 mAb enhanced the production of MMP-9 through the phosphorylation of p38 MAPK and ERK1/2 pathways. In advanced atherosclerotic plaque, active MMPs were significantly higher in *ApoE*$^{-/-}$ than in the same age *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice. Vascular cells, such as endothelial cells and VSMCs are known to express MMPs. Our results showed that the expression of active MMPs in atherosclerotic plaque was mostly due to that of macrophages. Among MMPs, macrophage MMP-9 expression was significantly increased in *ApoE*$^{-/-}$ mice compared to *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice. However, we found no differences in MMP-2 expression in aortic root between both mice groups. There was little MMP-2 expression in atherosclerotic plaque in our study. So, it is possible that elevated levels of pro and active MMP-2 in whole aorta are involved in abdominal aortic aneurysm (AAA). Actually, we could observe some formation of AAA in 66 week old *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ CD137$^{-/-}$ mice. Some studies have suggested that AAA

development depends on macrophage-derived MMP-9 and VSMC-derived MMP-2 (Longo *et al*., 2002; Saraff, 2003). In AAA, we found enhanced proliferation of VSMC and expression of MMP-2 (data not shown). In this study, we focused only on atherosclerotic plaque stability in the aortic root, and not in AAA. Therefore, we can summarize that enhanced macrophage MMP-9, via activation of CD137 signaling mainly affected atherosclerotic plaque stability in the aortic root. It is of note that MMP inhibition is regulated not only by tissue inhibitors of metalloproteinases (TIMPs), but also by anti-inflammatory cytokines. Previous studies have suggested that IL-10 inhibits monocyte/endothelial cell adhesion by inhibiting MMP-9, and enhances the TIMP-1 production by endothelial cells (Mtairag, 2001) and mononuclear phagocytes (Lacraz, 1995). TGF-β, other anti-inflammatory cytokine, inhibits MMP-2 production in VSMCs in response to platelet derived growth factor (PDGF) (Risinger, 2009). Although we could not find a direct link between MMP and the expression of anti-inflammatory cytokines in atherosclerotic plaque stability, we detected that both IL-10 and TGF-β mRNA expression were significantly elevated in aorta, skin draining lymph nodes, and spleen of *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice compared with *ApoE*$^{-/-}$ mice (data not shown). These data raise the interesting possibility that T_{reg} cells, major cells expressing IL-10 and TGF-β (Ait-Oufella *et al*., 2006; Sakaguchi *et al*., 2010), are involved in atherosclerotic plaque stability. In our study, CD137 was not involved in the development and suppressive function of T_{reg} cells.

Our data showed that CD137 signaling induced apoptosis of VSMCs via

up-regulation of cleaved caspase-3, which is consistent with the results of a previous human study. We confirmed that CD137 induced an up-regulation of cleaved caspase-3 which was controlled by inhibition of Bcl-2. Oxidized lipids or lipoproteins induced macrophage apoptosis and endoplasmic reticulum stress activated the apoptotic pathway in advanced lesional macrophages (Seimon and Tabas, 2008; Timmins *et al*., 2009). In our system, we found that resident apoptotic cells were increased in atherosclerotic plaque of *ApoE*$^{-/-}$ mice compared to that of *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice. However, we could not determine any difference of apoptotic macrophages in the plaque of *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice (data not shown). Consistent with *in vivo* results, *in vitro* culture of peritoneal macrophages of both mice treated with oxLDL in the presence of agonistic anti-CD137 mAb revealed that apoptosis of macrophages was not increased by activation of CD137 signaling. It is well known that, TGF-β production has a beneficial role in the vascular stability of VSMCs (Amento *et al.*, 1991; Bobik, 2006). However, no significant difference in TGF-β production via CD137 activation was observed in our experiment. An increased production of TGF-β was only observed upon treatment with PDGF-BB.

To validate the *in vivo* effect of CD137 signaling in atherosclerotic plaque stability, we used two mice models. First, we found that atherosclerotic plaque of normal chow diet fed 66 week age of *ApoE*$^{-/-}$ mice showed more advanced, unstable plaque phenotypes, which were characterized by enhanced plaque necrosis, decreased collagen, VSMCs content, and enhanced macrophage infiltration

compared to *ApoE*$^{-/-}$*CD137*$^{-/-}$ mice. These results can be explained by the development of advanced plaques as well as increased lesion formation in CD137 intact mice. Second, *in vivo* activation of CD137 signaling using an agonistic anti-CD137 mAb did not affect the lesion formation in high fat diet fed *Ldlr*$^{-/-}$ mice compared to rat IgG injected *Ldlr*$^{-/-}$ mice. Therefore, we analyzed lesion composition in this model. Treatment of an agonistic anti-CD137 mAb showed unstable advanced phenotypes by increased plaque necrosis, decreased VSMCs content, and enhanced macrophage infiltration. Therefore, we could conclude that *in vivo* CD137 activation augments the progression of atherosclerotic plaque lesions to vulneravle plaques.

In summary, we have provided evidence that CD137 signaling destabilizes atherosclerotic plaque. In advanced atheroma, CD137 expresses and contributes to IFN-γ production under atherosclerotic environment in T cells. And oxLDL induced CD137 expression on macrophages and activation of CD137 signaling boosted the production of MMP-9 via the up-regulation of phosphorylated p38 MAPK, ERK1/2 pathways. Then secreted MMP-9 leads to degradation of extracellular matrix components, such as collagens. In VSMCs, inflammatory cytokines induced CD137 expression, and the activation of CD137 signaling inhibited anti-apoptotic factor Bcl-2, followed by the up-regulation of cleaved caspase-3, which leads to apoptosis of VSMCs. Taken together, CD137 signaling exacerbates plaque stability in advanced atherosclerotic plaque. These findings could provide a promising therapeutic strategy in patients with vulnerable

atherosclerotic plaque lesions.

CONCLUSION

The tumor necrosis factor receptor superfamily, which includes CD40, OX40, and LIGHT, plays important roles in atherogenesis. CD137, a member of the tumor necrosis factor receptor superfamily, has been reported to be expressed in human atherosclerotic lesions. In this study, we show that CD137 deficiency leads to attenuation of atherosclerosis in 2 different mouse atherosclerosis models. We propose a model mechanism for the roles of CD137/CD137 ligand signaling in atherosclerosis on the basis of the results of the present study. CD137/CD137 ligand signaling can induce activation of macrophages and lymphocytes through its bidirectional signaling. Endothelial CD137 activation also can induce the production of monocyte chemoattractant protein-1 and cell adhesion molecules, which leads to enhanced monocyte attachment to endothelium. In advanced plaque, we have provided evidence that CD137 signaling destabilizes atherosclerotic plaque. CD137 expresses and contributes to IFN-γ production under atherosclerotic environment in T cells. And oxLDL induced CD137 expression on macrophages and activation of CD137 signaling boosted the production of MMP-9, which leads to degradation of extracellular matrix components, such as collagens. In VSMCs, inflammatory cytokines induced CD137 expression, and the activation of CD137 signaling inhibited anti-apoptotic factor Bcl-2, followed by the up-regulation of cleaved caspase-3, which leads to apoptosis of VSMCs. Taken together, CD137 can be an effective therapeutic target for the development and stabilization of anti-atherogenic drugs.

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국문초록

CD40, LIGHT, OX40과 같은 tumor necrosis factor 수용체는 동맥경화증의 발달에 중요한 역할을 하는 것으로 알려져 있다. 또 다른 tumor necrosis factor 수용체의 일종인 CD137은 T 세포 공동자극 분자로 알려진 유전자로, CD137 신호 전달의 활성화는 세포 생존, 세포 분열과 관련된 유전자의 발현을 증가시키고, 염증 사이토카인의 생산을 증가시키는 역할을 한다. 최근 연구에서는 CD137이 사람 동맥경화 환자의 병변에서 발견되었고, 그 중요성이 보고되었는데, 동맥경화 마우스 모델에서 CD137 유전자의 분자 기전과 역할에 대해서 알려진 바가 없는 실정이다. 본 연구에서는 먼저 CD137이 결핍된 ApoE/CD137 이중유전자적중 마우스와 Ldlr/CD137 이중유전자적중 마우스를 이용하여, 동맥경화 병변 내로 유입된 T 세포에서 CD137이 발현되었고, 동맥경화를 촉진하는 기능을 가진 주요 인자라는 것을 확인하였다. 또한 CD137의 결핍이 ApoE/CD137 이중유전자적중 마우스와 Ldlr/CD137 이중유전자적중 마우스의 대동맥에서 동맥경화 병변 형성을 저해시킨다는 사실을 확인하였다. 동맥경화 병변에는 대식세포, T 세포와 같은 염증 세포뿐만 아니라 혈관 세포들도 존재한다. 동맥경화 병변은 크게 증가된 염증, 지질 단백질의 축적, 세포 괴사의 특징을 띄고 있다. 그 중 T 세포가 관여하는 후천성 면역이 주요한 역할을 담당하게 되는데, 활성화된 T 세포가 IFN-r와 같은 염증 사이토카인의 분비를 촉진하여, 동맥경화 병변 형성을 증가시킨다는 사실은 여러 연구에서 보고되었다. 특히 CD137과 같은 T 세포 공동자극 수용체에 의한 T 세포 활성화는 대식세포에서의 염증 사이토카인의 분비를 증가시킴으로써 동맥경화 병변 형성과 염증 상태의 지속을 유발하게 된다. 이번 연구에서는 CD137 신호 전달이 T 세포의 활성화를 촉진하여 IFN-r 염증 사이토카인의 분비를 증

가시켰고, 증가한 IFN-r는 대식세포와 혈관내피세포에서의 염증 사이토카인 및 세포 부착 물질의 분비를 유발하여 동맥경화를 악화시킨다는 분자 메커니즘을 규명하였다.

후기 동맥경화 단계에서의 취약성 동맥경화 병변은 세포괴사반의 존재, 대식세포와 혈관세포의 세포사멸, 염증 상태의 지속과 같은 특징을 가짐으로써, 결국은 동맥경화반 파열을 유도하여, 심근경색을 초래한다. 또한 MMP가 높은 수준으로 존재함으로써, 동맥경화반의 구조적 안정성을 가져다 주는 콜라젠의 분해를 촉진하여, 취약한 동맥경화 병변의 형성을 유도한다고 많은 연구에서 보고되었다. 선행 연구에서 CD137이 동맥경화 발병의 중요 인자라는 사실을 바탕으로 동맥경화 후기 단계, 즉 취약성 동맥경화 병변에서 CD137의 역할을 규명하고자 하였다. 먼저, 동맥경화 병변에 존재하는 T 세포, 대식세포, 평활근세포에서 CD137이 높은 수준으로 발현하였다. T 세포에서는 CD137 신호 전달의 활성화가 IFN-r 염증 사이토카인의 발현을 증가시켰고, 대식세포에서는 MMP-9의 발현이 CD137에 의해 조절됨을 알 수 있었다. 또한 혈관 내로 유입된 평활근 세포에서 CD137 신호 전달의 활성화에 의해 세포내 항세포사멸 인자인 Bcl-2 단백질이 저해됨에 따라 caspase-3 활성화가 촉진되어 세포 사멸이 촉진되는 것을 확인하였다.

결론적으로 CD137은 동맥경화의 발병 기전을 촉진시킬 뿐 아니라, 동맥경화 병변 안정성까지 저해하는 주요 인자라는 것을 본 연구에서 증명함으로써, 항염증 및 항동맥경화 치료제로서 CD137이 주요 목표 물질이 될 수 있음을 제안하는 바이다.