Critical Role of Inducible Costimulator Signaling in the Development of Arteriosclerosis

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**Objective**—Proliferation and migration of smooth muscle cells (SMCs) and migration and accumulation of monocytes and T cells are landmark events in the development of arteriosclerosis. SMC proliferation in the intima induces interruption of blood flow and results in ischemia and graft rejection. Inducible costimulator (ICOS) is a major costimulator of T cell activation. However, the effect of costimulatory molecules on the formation of neointimal hyperplasia has not been fully elucidated. We examined the role of the ICOS pathway in SMC proliferation.

**Methods and Results**—ICOS ligand (ICOSL) was detected in SMCs stimulated by interleukin (IL)-1β, and coculture of stimulated SMCs and activated T cells induced SMC proliferation. Inhibition of the ICOS pathway resulted in inhibition of SMC proliferation. In models of transplantation and vascular injury, ICOSL was induced in SMCs in the neointima. Expression of IL-1β, a key inducer of ICOSL expression, was significantly reduced in mice treated with anti-ICOS antibody or soluble form of ICOS (ICOSIg) and in ICOS-deficient mice. Inhibition of the ICOS pathway significantly suppressed neointimal thickening.

**Conclusions**—These results indicate that ICOS on activated T cells contributes to neointimal formation through the regulation of SMC proliferation. These findings provide insights into new therapeutic strategies for arteriosclerosis. (Arterioscler Thromb Vasc Biol. 2006;26:2660-2665.)

**Key Words:** arteriosclerosis ■ costimulation ■ neointima formation ■ smooth muscle cells

Cardiovascular disease continues to be the major cause of death in developed countries.1,2 Neointimal hyperplasia, characterized by graft vasculopathy after transplantation and restenosis after vascular injury, involves infiltration by mononuclear cells including T cells and macrophages. In addition, proliferation of smooth muscle cells (SMCs) plays important roles in neointimal formation, processes that are influenced by multiple cytokines and can be predicted by endothelial damage.3,4 SMC proliferation in the intima induces interruption of blood flow and results in ischemia and graft rejection.

T cell activation requires 2 distinct signals through T cell receptors and costimulatory molecules. Inducible costimulator (ICOS) is a member of the CD28/CTLA4 family and is expressed on T cells after activation. Stimulation of the ICOS pathway promotes secretion of IFN-γ, IL-4, and IL-10.4 Inhibition of the ICOS pathway with anti-ICOS antibody or the soluble form of ICOS (ICOSIg) prolonged cardiac allograft survival in a murine model, and combined treatment with anti-ICOS antibody and cyclosporin A6 or ICOSIg and CTLA4Ig7 prolonged allograft survival indefinitely and prevented the development of graft vasculopathy. Treatment with anti-ICOS antibody in addition to blockade of the CD40 ligand (CD40L)/CD40 pathway also attenuated the development of graft vasculopathy.6,7

ICOS ligand (ICOSL), also known as B7-related protein 1 (B7RP-1), is expressed constitutively on B cells and in peripheral lymphoid tissues.8,9 In vitro studies have shown that ICOSL expression is induced on fibroblasts treated with tumor necrosis factor (TNF)-α and that it is expressed constitutively on endothelial cells and is upregulated by treatment with IL-1β or TNF-α.10 However, it is not known whether ICOSL is expressed on SMCs. Numerous studies have indicated that inhibition of the costimulatory pathway not only prolongs allograft survival11 but also attenuates atherogenesis and neointimal hyperplasia after transplantation or vascular injury.12-15 We recently reported that SMCs stimulated by interferon (IFN)-γ express herpes virus entry mediator (HVEM), which binds to LIGHT, and proliferate by interaction with T cells through the LIGHT pathway.16 Programmed death-ligand 1 (PD-L1) is expressed on SMCs stimulated by IFN-γ and is associated with SMC proliferation.17 Although it has not been shown that other costimulatory molecules are expressed on SMCs, data suggest that the costimulatory pathway plays an important role in...
regulating not only immune responses but also SMC proliferation and subsequent arteriopathy.

Here, we show that ICOSL is expressed on SMCs in neointima formed by vascular injury. The ICOS pathway promotes SMC proliferation in these animal models. Furthermore, we show that inhibition of the ICOS pathway prevents the development of neointimal hyperplasia after transplantation or vascular injury.

Materials and Methods
For complete Materials and Methods, please see http://atvb.ahajournals.org.

Reagents
Anti-mouse ICOS monoclonal antibody (JMAb51) was obtained from JT Pharmaceutical Frontier Research Laboratory (Kamagawa, Japan). ICOSIg was prepared as described previously.6

Animals
Six- to 8-week-old male C57BL/6 (B/6, H-2²) mice were obtained from Japan Clea. Male B6.C-H-2bm12Km2Eg (Bm12, H-2bm12) mice were obtained from The Jackson Laboratory (Bar Harbor, Me). Combinations of B/6 and Bm12 mice are class II mismatch. Male ICOS−/− mice on a C57BL/6 background were provided by JT Pharmaceutical Frontier Research Laboratory.6 Animals were maintained in our animal facility and weighed 20 to 25 grams. The present study conformed to the Guide for the Care and Use of Laboratory Animals of Tokyo Medical and Dental University.

Cardiac Transplantation
Donor hearts were transplanted heterotopically into recipient mice as described previously.7 Recipient mice were injected intraperitoneally with anti-ICOS antibody (100 μg per treatment, n=10), ICOSIg (50 μg per treatment, n=10), or isotype-matched control IgG (n=10) every 7 d for 8 weeks after transplantation. ICOS−/− mice were used as recipients (n=10). Allografts were harvested 8 weeks after transplantation.

Mouse Femoral Arterial Injury Model
Transluminal arterial injury of the femoral arteries of B/6 or ICOS−/− mice (n=8) was induced by inserting a straight spring wire as described previously.20 B/6 mice were injected intraperitoneally with anti-ICOS antibody (100 μg per treatment, n=8), ICOSIg (50 μg per treatment, n=8), or isotype-matched control IgG (n=8) on days 0, 2, 4, 6, 8, and 10 after injury. At 4 weeks after injury, the femoral arteries were excised.

Immunohistochemistry
Immunohistochemistry and immunofluorescence double staining were performed as described previously.16

Histological Evaluation
Grafts and arteries were analyzed by Elastica van Gieson staining. The areas within the internal elastic lamina (IEL) and the external elastic lamina (EEL) and the lumen were carefully traced, and the planimetric areas were calculated with an image analysis system (Scion Image beta 4.0.2; Scion).

Coculture of SMCs and T cells
Primary SMCs were obtained from the thoracic aortas of B/6, Bm12, or ICOS−/− mice by an explant technique as described previously.16 Splenocyte suspensions were obtained by disrupting spleens between sterile glass slides. Red blood cells were lysed by ammonium chloride lysis. Cells were washed, and T cells were obtained by depletion of non-T cells (B cells, NK cells, dendritic cells, macrophages, granulocytes, and erythroid cells) by magnetic cell sorting using anti-ICOS antibody (100 μg per treatment, n=10), ICOSIg (50 μg per treatment, n=8), or PD98059 (10 μg/mL) were added to each well for 24 hours. After washing in phosphate-buffered saline, SMCS were incubated with BrdU (Roche) according to the manufacturer’s instructions. Incorporated BrdU was measured by a microplate-imaging system (BioRad). SMC proliferation is expressed as the optical density.

Reverse-Transcription Polymerase Chain Reaction Analysis
Total RNA was extracted from SMCs after 24 hours of cytokine stimulation. cDNA was prepared from 5 μg of RNA by reverse transcription. cDNA (10 μL) was amplified. Polymerase chain reaction products were analyzed by ethidium bromide staining of 1.5% agarose gels.

Western Blot Analysis
Western blot was performed as described previously.17

Fluorescence-Activated Cell Sorter Analysis
Arrested SMCs were stimulated with IL-1β (10 ng/mL) for 24 or 48 hours. SMCs with and without IL-1β stimulation were incubated with anti-ICOS antibody and stained with biotinylated isotype-matched control IgG or goat IgG. Cells were then stained with fluoresein isothiocyanate (FITC)-conjugated anti-α-smooth muscle actin (SMA) antibody and streptavidin-phycocerythrin. Cells were then analyzed by flow cytometry on a fluorescence-activated cell sorter Calibur (Becton Dickinson).

Statistical Analysis
All data are expressed as mean±SEM. Differences between 2 groups were analyzed by Student t test. Differences in data between multiple groups were subjected to 1-way ANOVA followed by Scheffe test. P<0.05 was considered statistically significant.

Results
Expression of ICOSL on SMCs
We analyzed the expression of ICOSL on SMCs by reverse-transcription polymerase chain reaction and flow cytometry. Treatment with IL-1β induced ICOSL expression on SMCs from B/6 or Bm12 mice (Figure 1 and supplemental Figure I, available at http://atvb.ahajournals.org). ICOSL was not detected on untreated SMCs (Figure 1 and supplemental Figure I).
I) or on SMCs treated with TNF-α, IFN-γ, or IL-4 (data not shown).

**SMC Proliferation Induced by Interaction With T Cells**

Naïve T cells and T cells activated after transplantation were isolated from splenocytes of B/6 or ICOS−/− mice. Naïve T cells or activated T cells from B/6 mice did not induce proliferation of unstimulated SMCs from Bm12 mice (Figure 2A and 2B). Naïve T cells from B/6 mice also did not stimulate proliferation of SMCs from Bm12 mice treated with IL-1β (Figure 2A). However, activated T cells from B/6 mice induced significant proliferation of SMCs from Bm12 mice treated with IL-1β (Figure 2B). This proliferation was inhibited by addition of anti-ICOS antibody or ICOSIg (Figure 2B). Interestingly, activated T cells from B/6 mice induced significant proliferation of IL-1β-treated SMCs from B/6 mice, and addition of anti-ICOS antibody or ICOSIg suppressed SMC proliferation (supplemental Figure IIA and IIB).

We tested whether this effect on SMC proliferation involved activation of mitogen-activated protein kinase (MAPK) signaling. Increased extracellular signal regulated kinase (ERK) 1 and 2 (ERK 1/2) phosphorylation in SMCs was detected in cocultures of IL-1β-stimulated SMCs and activated T cells (Figure 2A and 2B; supplemental Figure IIA and IIB). To clarify the involvement of ERK1/2 in SMC proliferation, we used a MEK inhibitor PD98059. PD98059 significantly suppressed SMC proliferation induced by interaction with activated T cells (Figure 2C and 2D; supplemental Figure IIC and IID). However, Western blot analysis showed no effect on the phosphorylation of c-Jun-N-terminal kinase (JNK) or p38 MAPK (data not shown). Neither naive nor activated T cells from ICOS−/− mice induced proliferation of unstimulated or IL-1β-stimulated SMCs from Bm12 mice (Figure 2E and 2F). Western blot analysis showed no effect on the phosphorylation of ERK 1/2, JNK or p38 MAPK in SMCs from Bm12 mice (Figure 2E and 2F, data not shown).

**Prevention of Graft Vasculopathy by Inhibition of the ICOS Pathway**

We used B/6 or ICOS−/− mice as recipients and Bm12 mice as donors. ICOS staining was detected in graft infiltrating cells (supplemental Figure IIIA) and on cells in thickened neointima (supplemental Figure IIIIB). Although ICOS expression was not detected on naive CD4+ and CD8+ cells, it was induced on activated CD4+ and CD8+ cells (supplemental Figure IIC). In comparison to those in control IgG-treated allografts, the numbers of SMCs in neointima and the numbers of CD4+, CD8+, and CD11b+ cells infiltration was significantly reduced in mice treated with anti-ICOS antibody or ICOSIg and in ICOS−/− mice was significantly decreased (supplemental Figure IIID and IIIIE).

Allografts in control IgG-treated mice at 8 weeks after transplantation showed severe neointimal hyperplasia (luminal occlusion: 70.1±5.5%; Figure 3A and 3F). Interestingly, ICOSL staining was detected on SMCs in grafts with vasculopathic change, but no ICOSL staining was detected on SMCs in native hearts (supplemental Figure IIIIF). In comparison to control IgG-treated allografts, allografts treated with anti-ICOS antibody or ICOSIg at 8 weeks after transplantation showed significantly less severe neointimal hyperplasia (8.3±4.8%, 10.1±5.6%, respectively; Figure 3B, 3C, and 3F). Furthermore, sections from allografts of ICOS−/− recipient mice and isografts showed no intimal thickening by 8 weeks after transplantation (11.8±4.5%, 0.0%; Figure 3D, 3E, and 3F).

To examine cytokine expression in cardiac allografts, we performed Western blot analysis on native hearts and cardiac allografts at 8 weeks after transplantation. Expression of IL-1β, IL-6, and IFN-γ was detected in allografts from control IgG-treated mice (supplemental Figure IIG). However, expression of these cytokines was significantly suppressed in allografts from anti-ICOS antibody-treated mice, from ICOSIg-treated mice, and from ICOS−/− mice (supplemental Figure IIG). Expression of IL-4 and IL-10 was not altered (supplemental Figure IIG).

**Prevention of Neointimal Hyperplasia After Vascular Injury by Inhibition of the ICOS Pathway**

Femoral arteries of B/6 mice developed severe intimal hyperplasia at 4 weeks after injury (intima-media [I/M] ratio: 2.2±0.3, Figure 4B and 4F). Although ICOS expression was not detected on naive CD4+ and CD8+ cells, it was induced on activated CD4+ and CD8+ cells (supplemental Figure IVA). ICOS-positive cells were detected in the neointima (supplemental Figure IVB). Immunofluorescence showed ICOSL staining on SMCs in neointimal hyperplasia at 4 weeks after injury; no staining was detected on SMCs in uninjured arteries (supplemental Figure IVC). In comparison to that in control IgG-treated mice, neointimal formation was significantly attenuated in mice treated with anti-ICOS antibody or ICOSIg and in ICOS−/− mice (supplemental Figure IVD and IVE) and IL-1β staining was suppressed on SMCs in mice treated with anti-ICOS antibody or ICOSIg and in ICOS−/− mice (supplemental Figure IVF).

**Discussion**

Acute coronary syndromes and chronic rejection of transplanted organs induce disruption of plaque and neointimal hyperplasia, leading to loss of blood flow. These illnesses are associated with immune responses, and SMC proliferation plays a central role in the progression of neointimal hyperplasia. Cytokine stimulation and costimulatory signaling induce SMC proliferation and neointimal hyperplasia, however, the interactions between T cells and SMCs that result in signaling of the costimulatory pathway and in SMC proliferation have not been fully elucidated. To the best of our knowledge, the present study is the first to show that inhibition of the ICOS pathway reduces proliferation of SMCs cultured with activated T cells through abrogation of...
ERK 1/2 phosphorylation and inhibits the development of intimal hyperplasia after transplantation or vascular injury.

ICOSL is expressed on endothelial cells and is upregulated by various inflammatory stimuli. We found that ICOSL expression was induced on SMCs treated with IL-1β. It has been reported that cytokines and growth factors stimulate proliferation of SMCs. Our present data showed that IL-1β stimulated SMC proliferation (Figure 2). A previous study showed that T cells affect SMC proliferation, and we recently reported that costimulatory molecules were expressed on SMCs and that the costimulatory pathway was associated with SMC proliferation. Therefore, we examined whether the ICOS pathway was associated with SMC proliferation. SMCs proliferated in response to IL-1β stimulation; however, synergistic proliferation was induced only when SMCs treated with IL-1β were cocultured with T cells activated after transplantation. This proliferation was decreased by inhibition of the ICOS pathway with anti-ICOS antibody or ICOSIg. These results indicate that SMC proliferation in vitro is induced by signaling through the ICOS pathway, and activated T cells from B/6 mice stimulate proliferation not only of SMCs from Bm12 mice but also SMCs from B/6 mice.

MAPKs are known to play roles in regulating cell proliferation, survival, and differentiation. ERK 1/2 regulates cell proliferation and differentiation, and p38 MAPK and JNK are activated by cellular stresses such as inflammation and apoptosis. In this study, phosphorylation of ERK 1/2 in SMCs stimulated by IL-1β was detected after addition of activated T cells, and inhibition of the ICOS pathway inhibited ERK 1/2 phosphorylation. Phosphorylation of p38 MAPK and JNK in IL-1β-stimulated SMCs was not detected after addition of activated T cells. These data suggest that SMC proliferation is induced by activation of ERK 1/2 signaling in the ICOS pathway.

On the basis of in vitro data, we examined the relation between SMC proliferation and the ICOS pathway in vivo. Although survival early after transplantation has been improved by advances in immunosuppressive therapy, graft vasculopathy remains a major problem limiting long-term survival of cardiac transplant recipients. T cells and macrophages are activated and result in the secretion of proinflammatory cytokines. Although these cytokines affect SMC proliferation, it has been reported that costimulatory pathways such as those involving CD28/B7 and CD40L/CD40 are also associated with the development of neointimal hyperplasia after transplantation or vascular injury remains unknown. Immunofluorescence staining indicated that ICOSL expression was induced on SMCs in thickened neointima after transplantation or vascular injury. Therefore, we examined whether the development of neointimal hyperplasia is
associated with the ICOS pathway. Compared with treatment with control IgG, treatment with anti-ICOS antibody or ICOSIg significantly reduced the development of neointimal hyperplasia after transplantation or vascular injury. Neointimal hyperplasia was also significantly attenuated in allografts and arteries from ICOS−/− mice. Compared with mice treated with control IgG, proinflammatory cytokines, including IL-1β, an important regulator of ICOSL expression, were significantly suppressed in mice after transplantation or vascular injury treated by inhibition of the ICOS pathway. There is the limitation to this study. It is not clear how the blockade of the ICOS pathway inhibits the development of arteriosclerosis in vivo. The infiltration of T cells and the expression of proinflammatory cytokines were significantly suppressed by inhibition of the ICOS pathway. Cytokine stimulation plays critical roles in SMC proliferation and the development of neointimal hyperplasia after transplantation or vascular injury. T cell activation and differentiation are defective in the absence of ICOS.29–31 Therefore, it is probable that the development of arteriosclerosis is inhibited by the attenuation of inflammatory responses. However, interaction between activated T cells and SMCs may be involved with SMC proliferation in vitro and in vivo. Our data also suggest that interaction between homotypic activated T cells and SMCs synergistically induces SMC proliferation and may contribute to restenosis after injury. Further studies are necessary to clarify the mechanism by which the interaction between T cells and SMCs inhibits arteriosclerosis.

Afek et al reported that blockade of the ICOS pathway increased atherosclerotic lesions in apoE knockout mice.32 The data of Afeck et al are based on one side that ICOSIg fusion protein alone was administrated to block the ICOS pathway. However, we used not only ICOSIg fusion protein but also anti-ICOS antibody or ICOS−/− mice. It is possible that how to block the costimulatory pathways induces different actions. Also, the model of vasculopathy was used in our study, although the model of atherosclerosis was used in Afeck et al study. The ICOS pathway may play different roles in vasculopathy and atherosclerosis. Although acquired im-

Figure 3. Photomicrographs of paraffin sections of cardiac allografts at 8 weeks after transplantation stained with Elastica van Gieson stain. Representative vessels from allografts treated with control IgG (A), anti-ICOS antibody (B), or ICOSIg (C), an allograft from an ICOS−/− recipient mouse (D), and an isograft (E) are shown (original magnification, 400×). F, The degree of graft vasculopathy in each group was quantified. Data are expressed as mean±SEM of 10 mice in each group. *P<0.05.

Figure 4. Photomicrographs of paraffin sections of femoral arteries at 4 weeks after vascular injury stained with Elastica van Gieson stain. Representative vessels from arteries treated with control IgG (A), anti-ICOS antibody (B), or ICOSIg (C), and arteries from ICOS−/− mice (D) are shown (original magnification, 400×). E, Quantitative analysis of I/M ratio in each group is shown. Data are expressed as mean±SEM of 8 mice in each group. *P<0.05.
munity is related to the development of vasculopathy, the role of acquired immunity in atherosclerosis is unclear. These points could explain the discrepancy between our data and Afek et al study.

In conclusion, the present study shows that ICOSL is expressed on SMCs stimulated by IL-1β and that the ICOS pathway plays a pivotal role in regulating SMC proliferation in vitro and in vivo. Reduction of neointimal hyperplasia occurs not only by modulation of immune responses but also by suppression of SMC proliferation through the ICOS pathway. Therefore, the ICOS pathway may comprise an important target for the prevention of neointimal formation.

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Disclosures
None.

References

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Data supplement

Materials and Methods

Reagents

Anti-mouse ICOS monoclonal antibody (JMAb51) was obtained from JT Pharmaceutical Frontier Research Laboratory. ICOSIg was prepared as described previously. Human IgG was obtained from Jackson ImmunoResearch Laboratories. Nonneutralizing anti-mouse CD18 monoclonal antibody (M18/2, rat IgG2a) was purchased from American Type Culture Collection. M18/2 or human IgG was used as an isotype-matched control IgG. PD98059 was obtained from Sigma-Aldrich. Recombinant mouse IL-1β, TNF-α, IFN-γ and IL-4, anti-mouse CD4, CD8, IL-6, TNF-α, IFN-γ, IL-4 and IL-10 monoclonal antibodies, FITC-conjugated anti-α-smooth muscle actin (SMA) antibody and avidin-Texas Red were obtained from Pharmingen. Anti-goat IL-1β antibody and anti-goat ICOSL antibody were obtained from Santa Cruz Biotechnology. Anti-mouse actin monoclonal antibody was obtained from Chemicon International. Phospho-ERK 1/2, p44/42 MAPK, phospho-p38 MAPK, p38 MAPK, phospho-JNK and JNK antibodies were obtained from Cell Signaling.

Animals

Six- to 8-week-old male C57BL/6 (B/6, H-2b) mice were obtained from Japan Clea. Male B6.C-H-2bml12-KhEg (Bm12, H-2bml12) mice were obtained from The Jackson Laboratory. Combinations of B/6 and Bm12 mice are class II mismatch. Male ICOS-/- mice on a C57BL/6 background were provided by JT Pharmaceutical Frontier Research Laboratory. Animals were maintained in our animal facility and weighed 20-25 g. The present study conformed to the Guide for the Care and Use of Laboratory Animals of Tokyo Medical and Dental University.

Cardiac transplantation

Donor hearts were transplanted heterotopically into recipient mice as described previously. Recipient
mice were injected intraperitoneally with anti-ICOS antibody (100 µg per treatment, \( n = 10 \)), ICOSIg (50 µg per treatment, \( n = 10 \)) or isotype-matched control IgG (\( n = 10 \)) every 7 d for 8 weeks after transplantation. ICOS\(^{-/-}\) mice were used as recipients (\( n = 10 \)). Allografts were harvested 8 weeks after transplantation. After harvesting, allografts were sectioned transversely into three parts; the basal section was fixed in 10% formalin and embedded in paraffin for morphologic examination, the midsection was embedded immediately in OCT compound and flash frozen in liquid nitrogen for immunohistochemistry and the apical section was used to extract proteins for Western blot analysis.

**Mouse femoral arterial injury model**

Transluminal arterial injury of the femoral arteries of B/6 or ICOS\(^{-/-}\) mice (\( n = 8 \)) was induced by inserting a straight spring wire as described previously\(^4\). B/6 mice were injected intraperitoneally with anti-ICOS antibody (100 µg per treatment, \( n = 8 \)), ICOSIg (50 µg per treatment, \( n = 8 \)) or isotype-matched control IgG (\( n = 8 \)) on days 0, 2, 4, 6, 8 and 10 after injury. At 4 weeks after injury, the femoral arteries were excised, fixed in 10% formalin and embedded in paraffin for morphologic examination. Arteries were also embedded immediately in OCT compound and flash frozen in liquid nitrogen for immunohistochemistry.

**Immunohistochemistry**

Immunohistochemistry was performed as described previously\(^5\). Frozen sections (5 µm) were fixed in acetone for 10 min at 4°C. After a wash in PBS, sections were incubated with primary antibodies overnight at 4°C. Sections were then incubated with biotinylated secondary antibodies at room temperature for 30 min. Antigen-antibody conjugates were detected with avidin-biotin-horseradish peroxidase complex (Nichirei) according to the manufacturer’s instructions. The chromogen was 3-amino-9-ethylcarbazole. Sections were counterstained with hematoxylin. Quantitative analysis of lesional SMC content was expressed as the percentage of the immunostained area in relation to the
total intimal area⁶. The numbers of SMCs was quantified by counting stained cells in neointima in mice. The numbers of CD4+, CD8+, and CD11b+ cells was quantified by counting stained cells in 20 nonoverlapping high-power fields per graft.

Immunofluorescence double staining was performed to examine ICOSL staining on SMCs. After incubation with anti-ICOSL antibody and biotinylated secondary antibody, sections were stained with FITC-conjugated anti-α-SMA antibody and avidin-Texas Red. Sections were observed by confocal microscopy.

**Histologic evaluation**

Grafts and arteries were analyzed by Elastica van Gieson staining. The areas within the internal elastic lamina (IEL) and the external elastic lamina (EEL) and the lumen were carefully traced, and the planimetric areas were calculated with an image analysis system (Scion Image beta 4.0.2; Scion). Cross-sectional area luminal stenosis was calculated as follows: luminal occlusion = (IEL area – luminal area)/IEL area × 100 (%). The intima-to-media (I/M) ratio was calculated as follows⁷: I/M = (IEL area – lumen area)/(EEL area – IEL area).

**Coculture of SMCs and T cells**

Primary SMCs were obtained from the thoracic aortas of B/6, Bm12 or ICOS⁻⁻⁻ mice by an explant technique as described previously⁵. Cultured SMCs were identified by typical hill-and-valley morphology and immunostaining with monoclonal antibody to α-SMA. All experiments were performed with cells between passages three and eight.

Splenocyte suspensions were obtained by disrupting spleens between sterile glass slides. Red blood cells were lysed by ammonium chloride lysis. Cells were washed, and T cells were obtained by depletion of non-T cells (B cells, NK cells, dendritic cells, macrophages, granulocytes, and erythroid cells) by magnetic cell sorting (Miltenyi Biotec). The purity of separated T cells was consistently over
95% as assessed by flow cytometry.

SMCs were trypsinized and seeded onto 96-well plates. At confluence, SMCs were arrested in medium with 0.4% FBS for 5 days. SMCs were then stimulated with recombinant mouse IL-1β (10 ng/ml) for 48 hours. After a wash in PBS, mitomycin-C-inactivated T cells (total 5 × 10⁵) and anti-ICOS antibody, ICOSIg (10 µg/ml), or PD98059 (10 µg/ml) were added to each well for 24 hours. After washing in PBS, SMCs were incubated with BrdU (Roche) according to the manufacturer’s instructions. Incorporated BrdU was measured by a microplate-imaging system (BioRad). SMC proliferation is expressed as the optical density.

RT-PCR analysis

Total RNA was extracted from SMCs after 24 hours of cytokine stimulation. cDNA was prepared from 5 µg of RNA by reverse transcription. cDNA (10 µl) was amplified according to the following parameters: 94°C, 1 min; 58°C, 1 min; 72°C, 1 min for 30 cycles. Primers for ICOSL and β-actin were as follows: for ICOSL, 5’-GTCCCCACCGAAGCTATACA-3’ and 5’-GAGGCTCCATATTCCGATGA-3’; for β-actin, 5’-AACTGGGACGACATGGAGAA-3’ and 5’-CATGAGGTAGTCTGTCAGGT-3’. PCR products were analyzed by ethidium bromide staining of 1.5% agarose gels.

Western blot analysis

Western blot was performed as described previously⁸. Heart sections were homogenized in extraction buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM EGTA, 10 mM EDTA, 100 mM NaF, 1 mM Na₃P₂O₇, 2 mM Na₃VO₄, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF) and cocktail tablets (Roche)⁸. After coculture of SMCs and T cells for 15 min, SMCs were washed in PBS. SMCs were incubated in medium with 10% FBS for 24 hours. SMCs were lysed with buffer containing 25 mM HEPES (pH 7.4), 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate,
5 mM EDTA, 50 mM NaF, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM sodium orthovanadate (Na3VO4). After centrifugation, supernatants were stored. The protein concentration of each sample was measured with a protein assay kit (Bio-Rad). Protein concentrations were similar between samples in all experiments. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and incubated with primary antibodies at 4°C overnight. Membranes were incubated with secondary antibody for 2 hours and developed with enhanced chemiluminescence reagent (Amersham Biosciences). Enhanced chemiluminescence was detected with an LAS-1000 imaging system (Fujifilm). Blot density was analyzed by with an image analysis system (Scion Image beta 4.0.2; Scion).

FACS analysis
Arrested SMCs were stimulated with IL-1β (10 ng/ml) for 24 or 48 hours. SMCs with and without IL-1β stimulation were incubated with anti-ICOSL antibody and stained with biotinylated isotype-matched control IgG or goat IgG at 4°C for 20 min. Cells were then stained with FITC-conjugated anti-α-SMA antibody and streptavidin-phycoerythrin (PE) at 4°C for 20 min. Splenocytes were isolated from mice after cardiac transplantation or vascular injury. Cells were stained with biotinylated anti-ICOS antibody or isotype-matched control IgG at 4°C for 20 min. Cells were then stained with FITC-conjugated anti-CD4 or anti-CD8 antibody and streptavidin-PE 4°C for 20 min. Cells were then analyzed by flow cytometry on a FACS Calibur (Becton Dickinson).

Statistical analysis
All data are expressed as mean ± SEM. Differences between two groups were analyzed by Student’s t test. Differences in data between multiple groups were subjected to one-way ANOVA followed by Scheffe’s test. P < 0.05 was considered statistically significant.
References


Figure legends

I Expression of ICOSL in SMCs by flow cytometry. SMCs were stained with control IgG (black) and antibody against ICOSL (solid line).

II (A and B) Coculture of T cells from B/6 mice and SMCs from B/6 mice and Western blotting. Naïve T cells (A) did not induce proliferation of IL-1β-untreated or IL-1β-treated SMCs. Activated T cells (B) induced proliferation of IL-1β-untreated SMCs and significantly stimulated proliferation of IL-1β-treated SMCs. Addition of anti-ICOS antibody or ICOSIg suppressed SMC proliferation. Phosphorylation of ERK 1/2 in SMCs stimulated by IL-1β was detected after addition of activated T cells, and inhibition of the ICOS pathway inhibited ERK 1/2 phosphorylation. Results are representative of three independent experiments. Blot density was defined as ratio to p-ERK of untreated SMCs. Data are expressed as mean ± SEM of three independent experiments. *P < 0.05.

(C and D) Inhibition of SMC proliferation by a MEK inhibitor PD98059. (C) Addition of PD98059 failed to inhibit SMC proliferation induced by interaction with naïve T cells. (D) PD98059 significantly inhibited SMC proliferation induced by interaction with activated T cell. Data are expressed as mean ± SEM of three independent experiments. *P < 0.05.

III (A) Immunohistochemical staining of ICOS in cardiac allografts treated with control IgG for 8 weeks. Representative frozen sections stained with antibody against ICOS (left) and isotype-matched control IgG (right) are shown. ICOS staining was identified in cells infiltrating the allografts (original magnification, 400×). (B) Representative frozen sections stained for ICOS. ICOS-positive cells were detected in the neointima of mice treated with control IgG for 8 weeks (original magnification, 400×). (C) Naïve (black) and activated (solid) T cells were stained with antibody against ICOS. (D) Representative frozen sections stained for SMCs, CD4+, CD8+, and CD11b+ cells (original magnification, 400×). (E) Quantitative analysis of SMCs, CD4+, CD8+, and CD11b+ positive cells.
Data are expressed as mean ± SEM of 10 mice in each group. *P < 0.05. (F) Representative vessels from native hearts (upper) or allografts from mice treated with control IgG for 8 weeks (lower) were double stained with anti-α-SMA antibody and anti-ICOSL antibody (original magnification, 400×).

(G) Expression of cytokines in allografts by Western blotting. Representative data of three independent experiments shows cytokine expression in native hearts and allografts at 8 weeks after transplantation.

IV (A) Naïve (black) and activated (solid) T cells were stained with antibody against ICOS. (B) Representative frozen sections showing ICOS immunostaining in the neointima. ICOS-positive cells were detected in the neointima of mice treated with control IgG for 4 weeks (original magnification, 400×). (C) Representative vessels from uninjured artery (upper) or injured artery treated with control IgG for 4 weeks (lower) were stained with anti-α-SMA antibody and anti-ICOSL antibody (original magnification, 400×). (D) Representative frozen sections stained for SMCs, CD4⁺, CD8⁺, and CD11b⁺ cells (original magnification, 400×). (E) Quantitative analysis of SMCs, CD4⁺, CD8⁺, and CD11b⁺ positive cells. Data are expressed as mean ± SEM of eight mice in each group. *P < 0.05. (F) Representative frozen sections stained for IL-1β in the neointima (original magnification, 400×).
**B/6**

- **0 hour**: 1.4%
- **24 hours**: 3.6%
- **48 hours**: 53.5%

**ICOSL**

**Bm12**

- **0 hour**: 0.9%
- **24 hours**: 8.7%
- **48 hours**: 71.6%

**ICOSL**

*Data supplement I*
A

IL-1β

Naïve T cell (B/6)

ICOSAb

ICOSIg

SMC(B/6)

Optical density

P-ERK

ERK

B

IL-1β

Activated T cell (B/6)

ICOSAb

ICOSIg

SMC(B/6)

Optical density

P-ERK

ERK

Data supplement II
**C**

- IL-1β: +
- Naïve T cell (B/6): +
- PD98059: +
- SMC(B/6): +

**D**

- IL-1β: +
- Activated T cell (B/6): +
- PD98059: +
- SMC(B/6): +

**Optical density**

- **C**: 0.08, 0.12, 0.16
- **D**: 0.1, 0.15, 0.2

*Data supplement II*
A

ICOS

Control

B

ICOS

Control

Data supplement III
Data supplement III C

**CD4⁺ cells**

**CD8⁺ cells**

17.0%

1.1%
Data supplement III D

CD4+ T cell  CD8+ T cell  CD11b+ cell  Control

Control IgG

Anti-ICOS antibody

ICOSIg

ICOS-/-

Isograft
Data supplement III F

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Data supplement III G

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Data supplement IV A

CD4⁺ cells

CD8⁺ cells
Data supplement IV B

ICOS          Control
Data supplement IV C

Uninjured artery

Artery after injury
Data supplement IV D

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Data supplement IV F

Control IgG

Anti-ICOS antibody

ICOSIg

ICOS−/−