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보건복지부 지정
혁신형 세포치료 연구중심병원 사업단
IRICT Innovative Research Institute for Cell Therapy

Inflammation in experimental and clinical study

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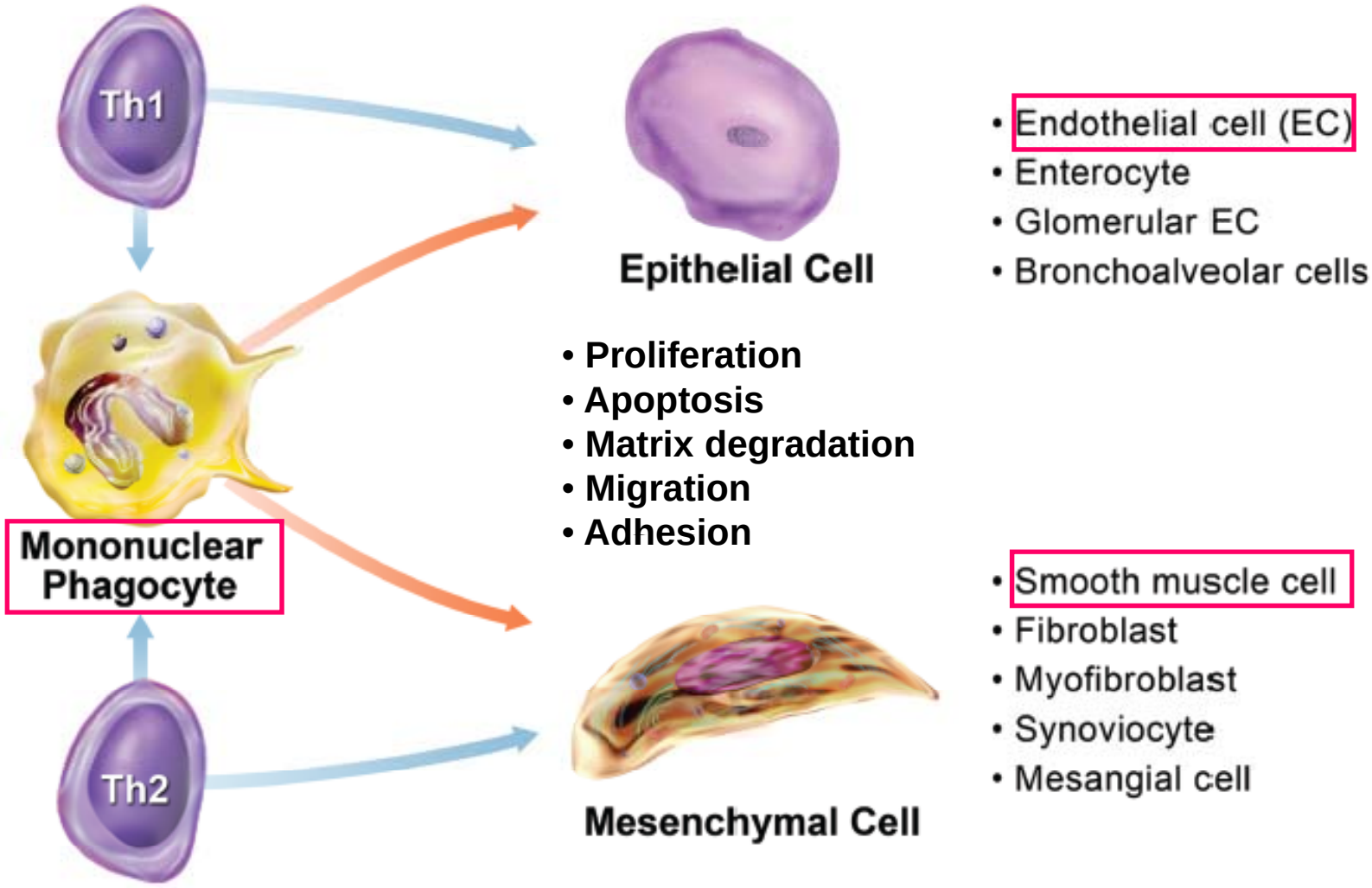
Inflammation in experimental and clinical study: Contents

- Inflammatory processes on cardiovascular cells
  - Inflammatory signaling pathways
  - Endothelial cell dysfunction / Matrix degradation
  - Cellular survival/proliferation with inflammation
- Inflammatory markers in clinical studies
  - Cell adhesion molecules
  - Inflammatory cytokines
  - Clinical implication

Common inflammatory and immune processes act on different cell types

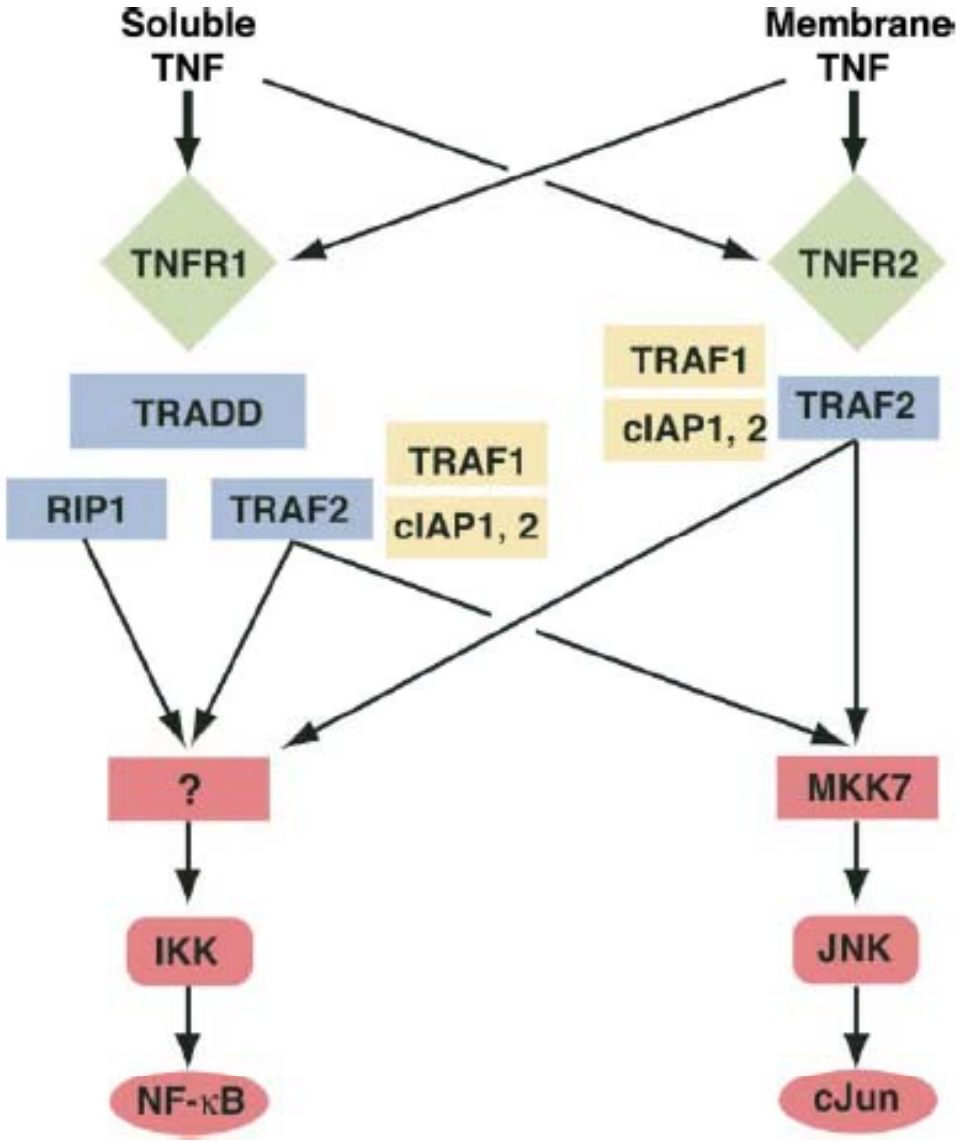
Inflammatory stimuli
- **Angiotensin II**
- **Glucose**
- **oxLDL**
- **CRP, LPS**
- **ROS**
- **AGE**
- **…**



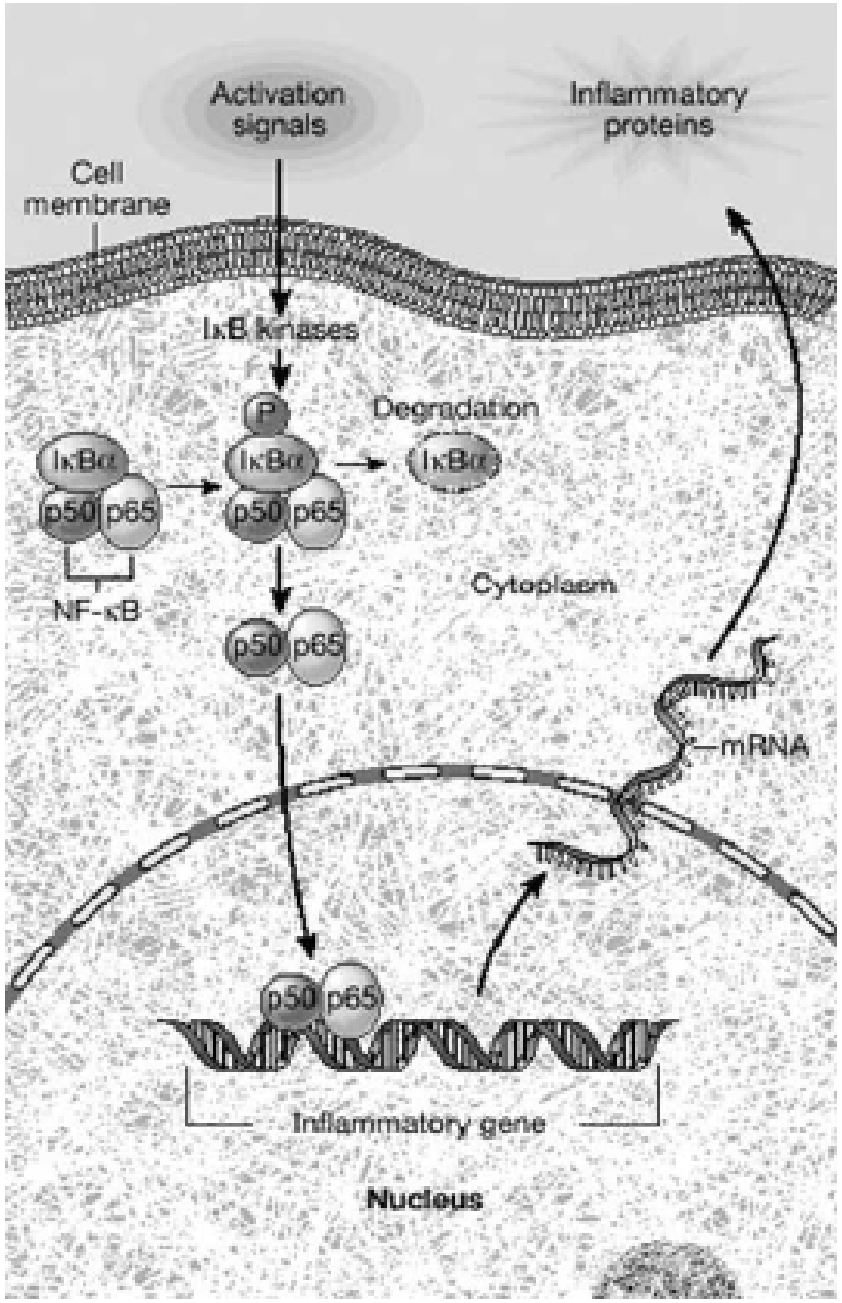
Libby P. Nutrition Review 2007

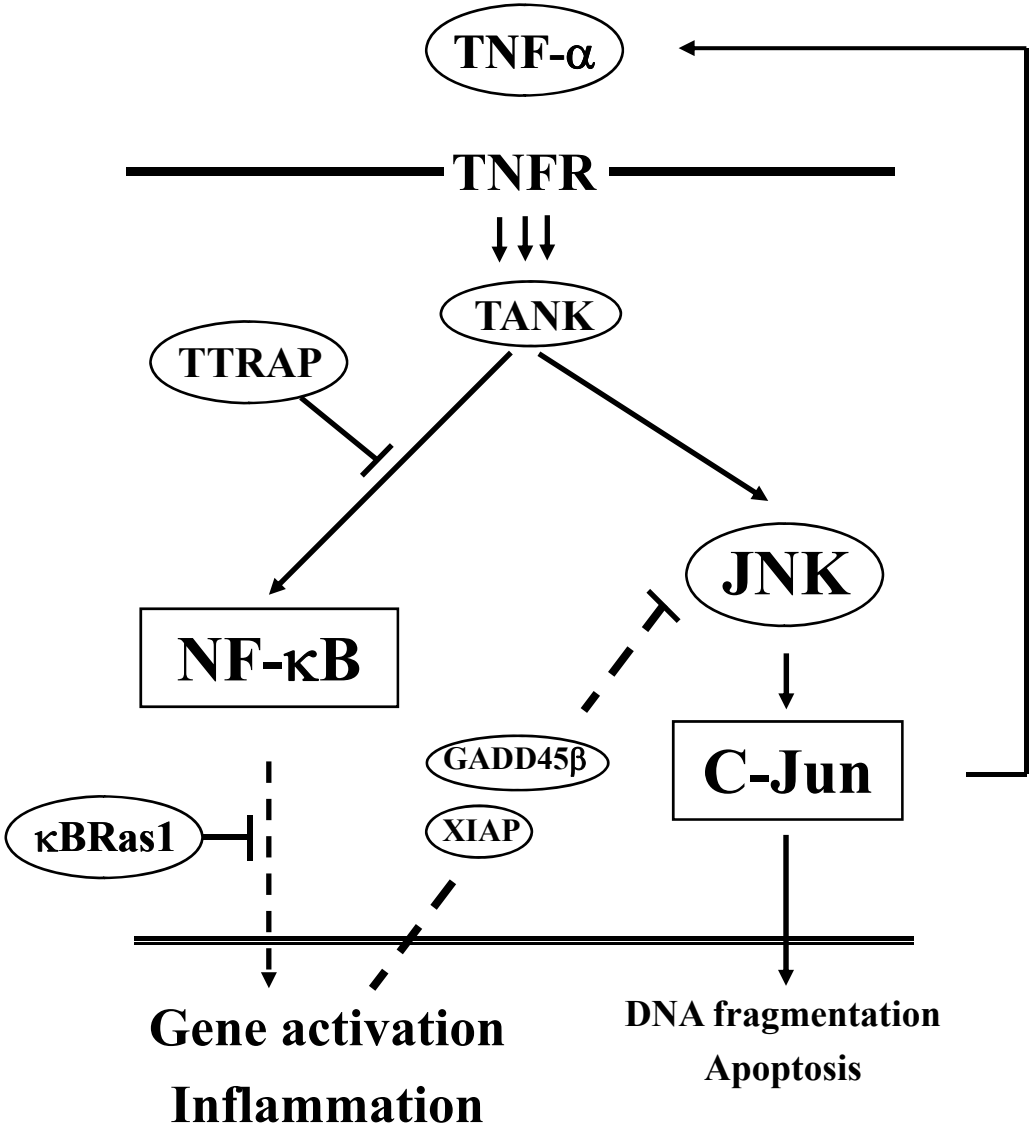
TNF receptor pathways signaling both death and inflammation

- TNF as a key inducer of proinflammatory genes
  - Triggering local expression of cytokines
  - Promoting adhesion, extravasation, attraction, leukocyte activation
- Two distinct TNF receptors
  - TNFR1: primary R for sTNF constitutively expressed in most cell types
  - TNFR2: main R for MTNF expression highly regulated



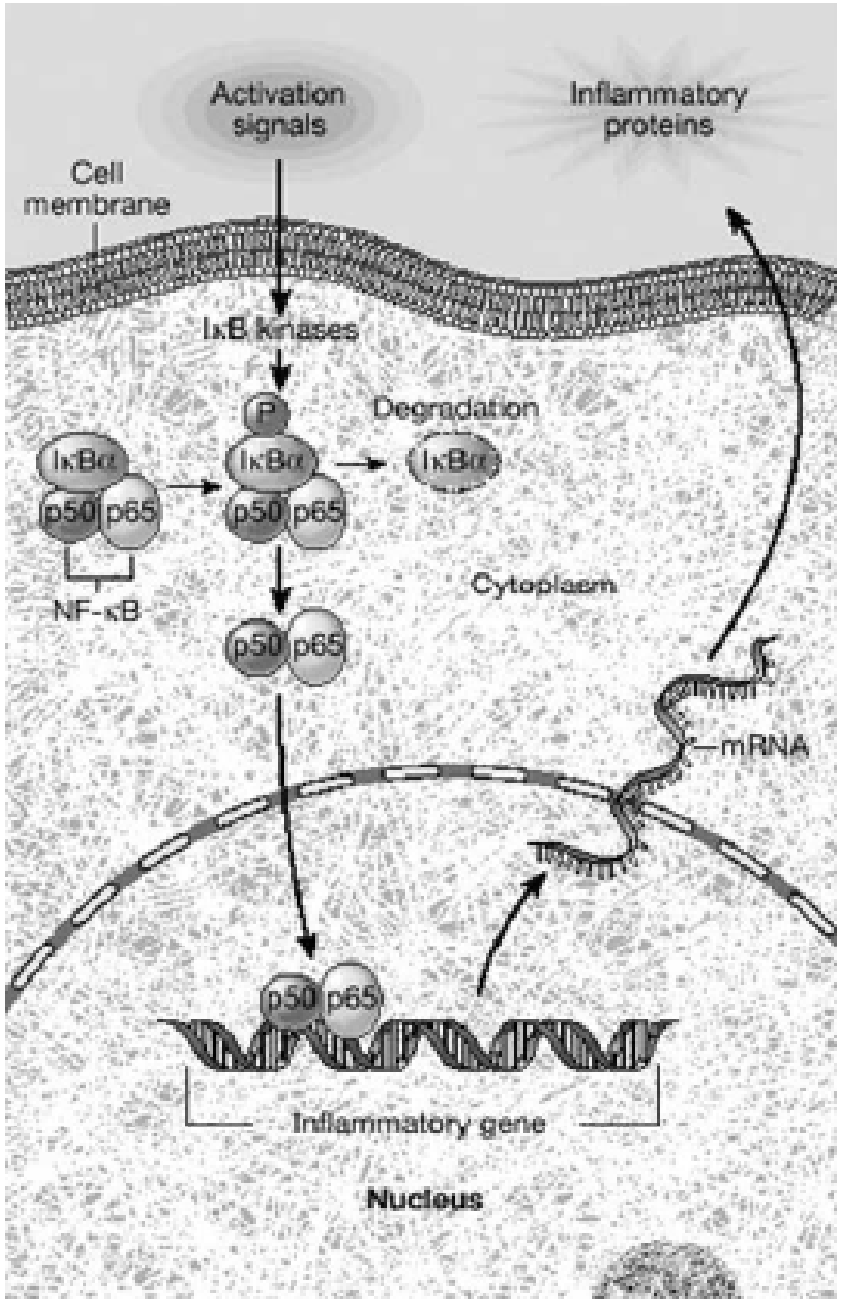
TNF receptor pathways signaling both death and inflammation





Lee HY, Oh BH, Kim HS, et al. ATBV 2008

TNF receptor pathways signaling both death and inflammation



Pro-inflammatory signal

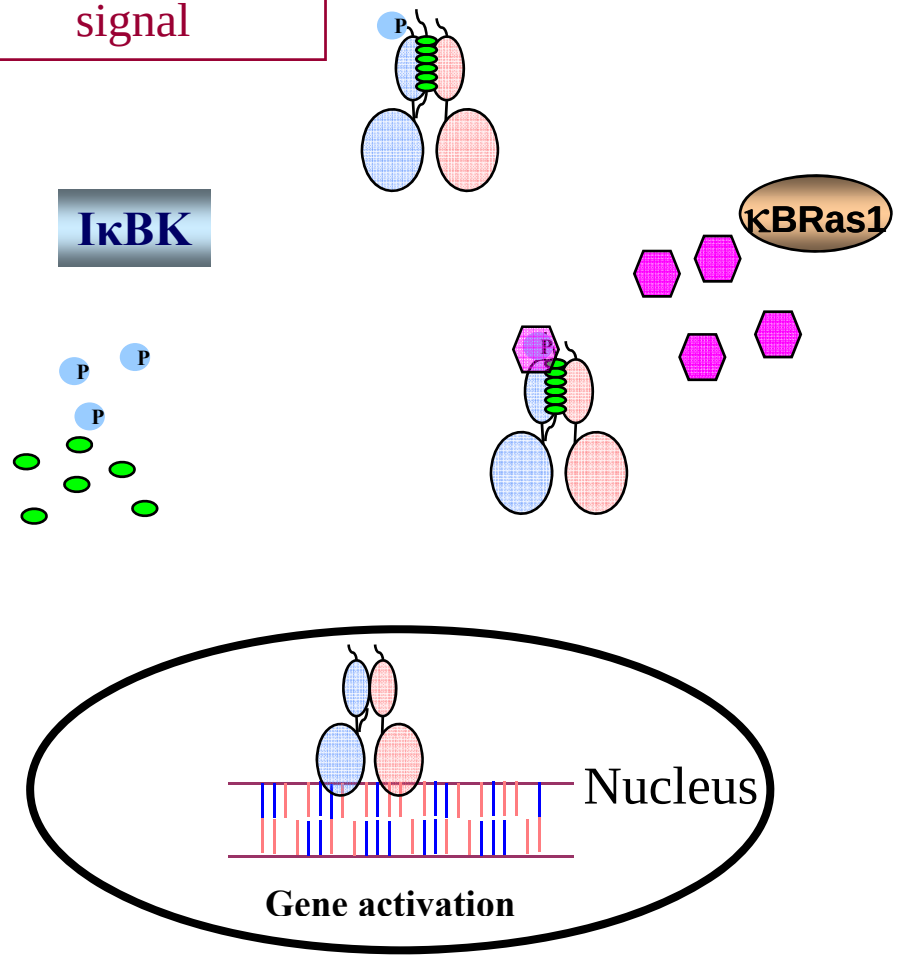
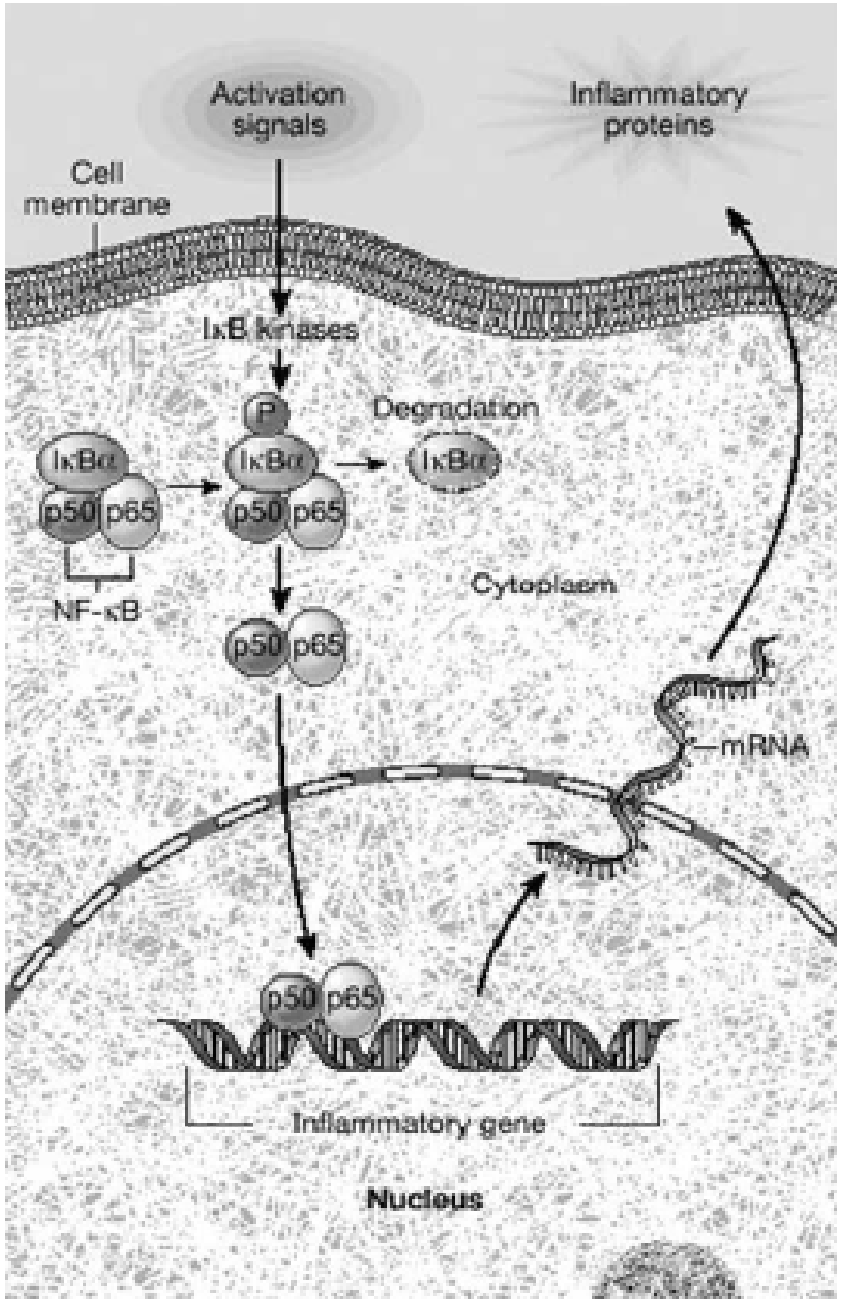


Figure from NEJM 1997;336:1066-1071

TNF receptor pathways signaling both death and inflammation



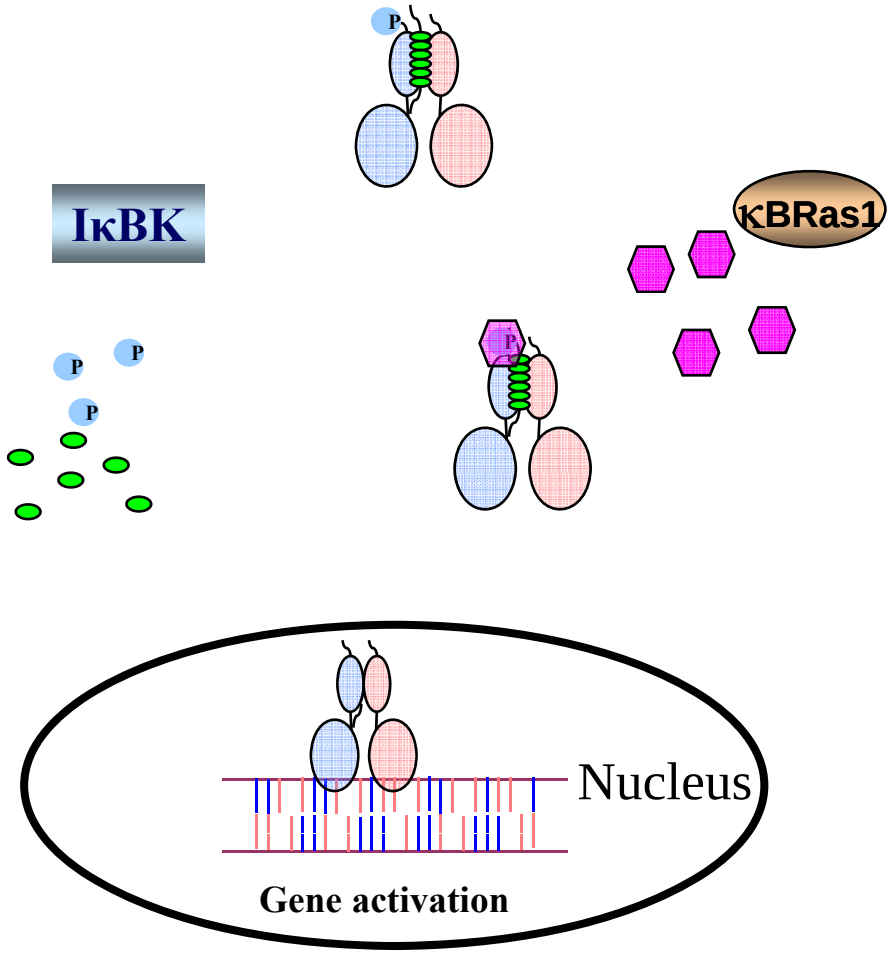
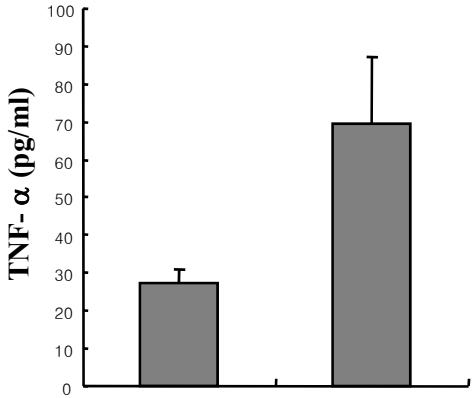


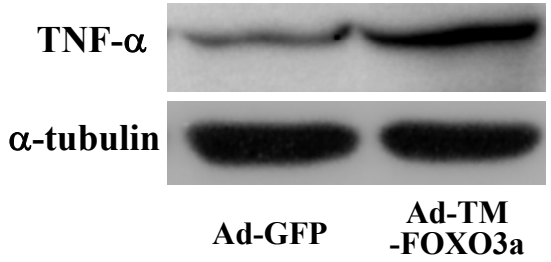
Figure from NEJM 1997;336:1066-1071

Evaluating TNF receptor pathways: TNF and NF-κB

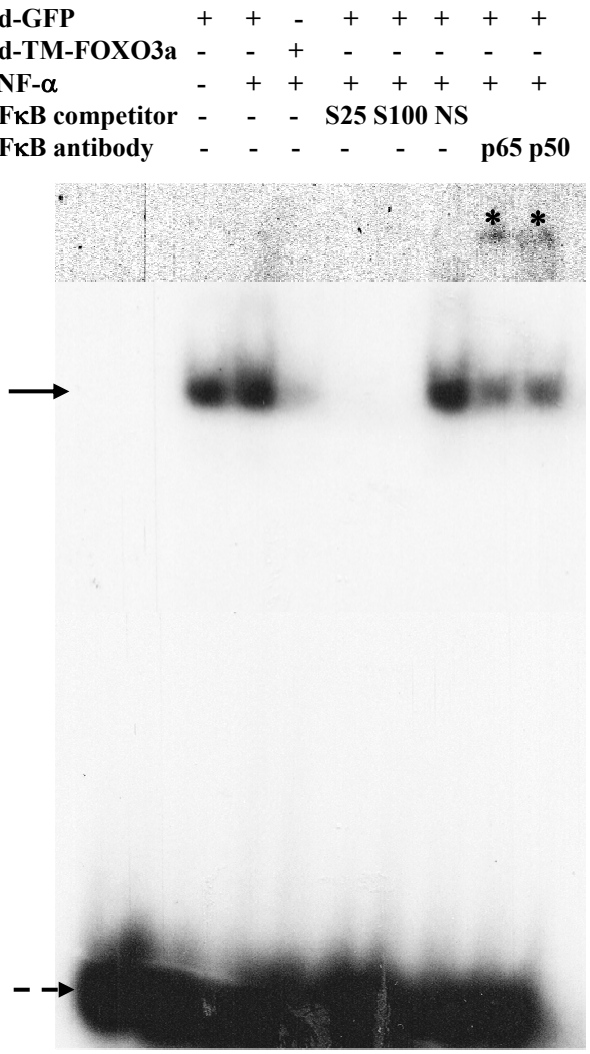
ELISA in supernatant



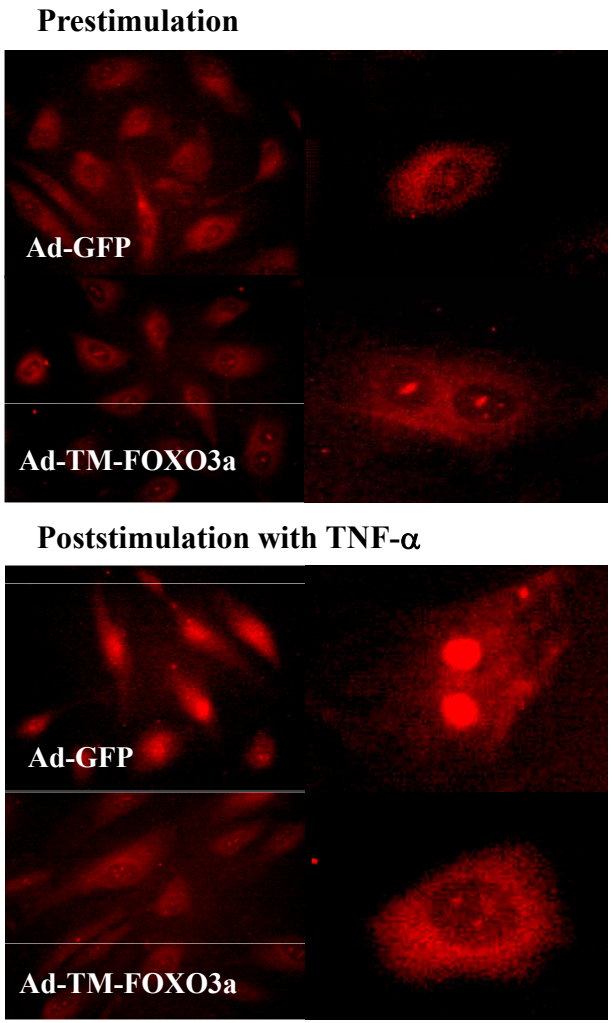
Immunoblot in cell fraction



EMSA of NF-κB

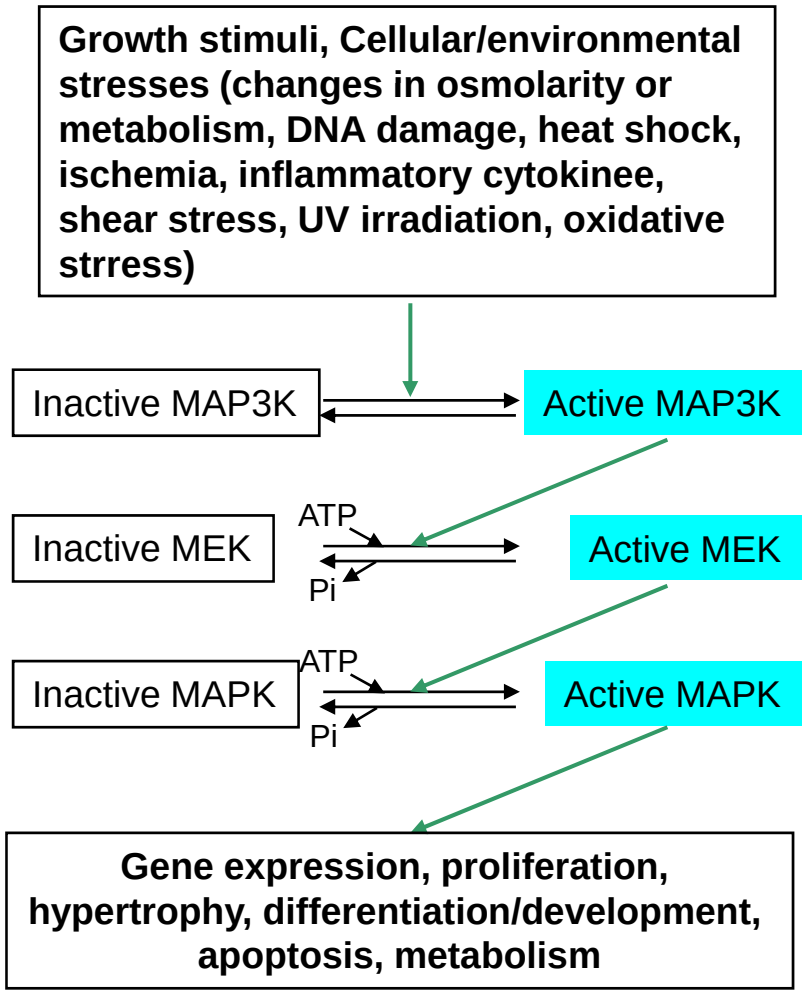


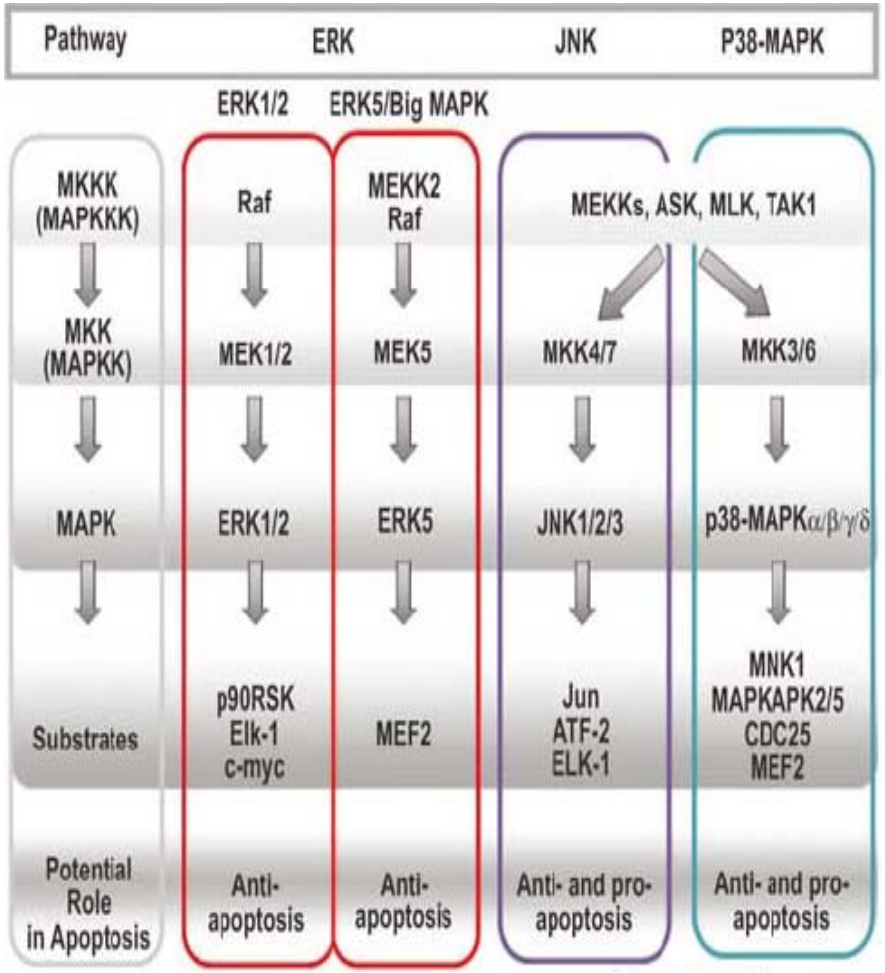
Immunofluorescent microscopy for p65 subunit



Lee HY, Oh BH, Kim HS, et al. ATBV 2008

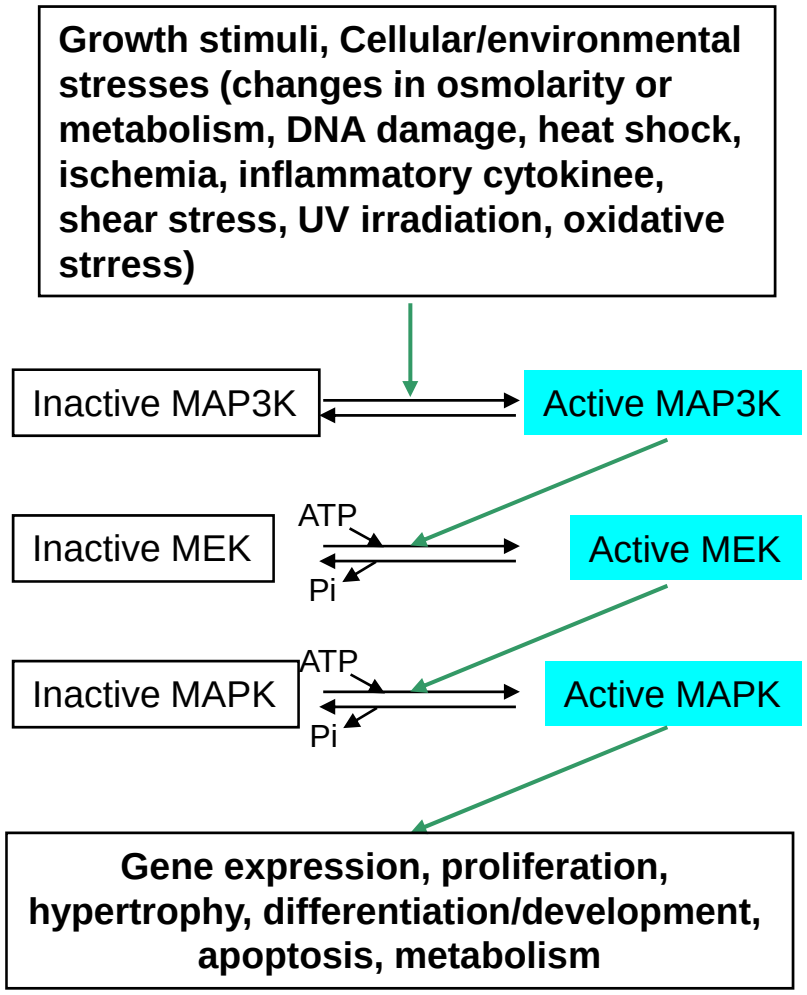
Mitogen-activated protein kinases in inflammation / apoptosis regulation

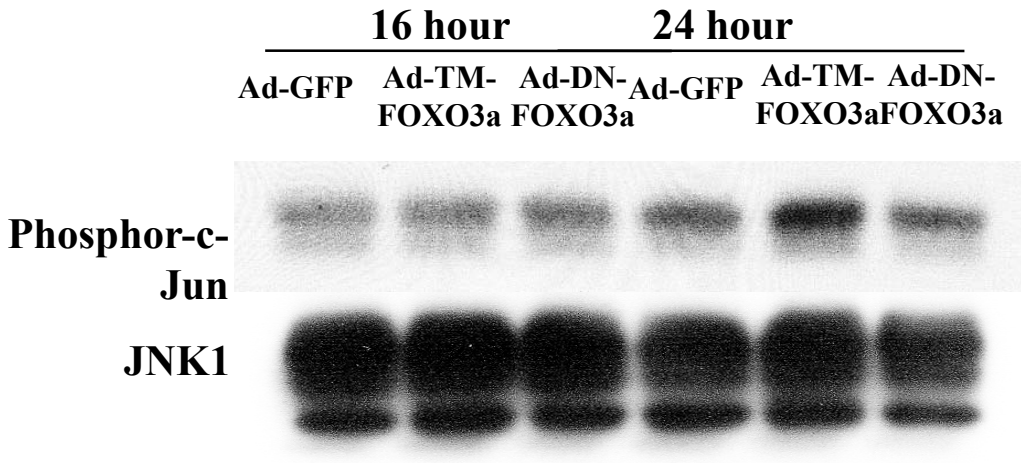




Deng et al. Cell. 2003; 115: 61-70

Mitogen-activated protein kinases in inflammation / apoptosis regulation

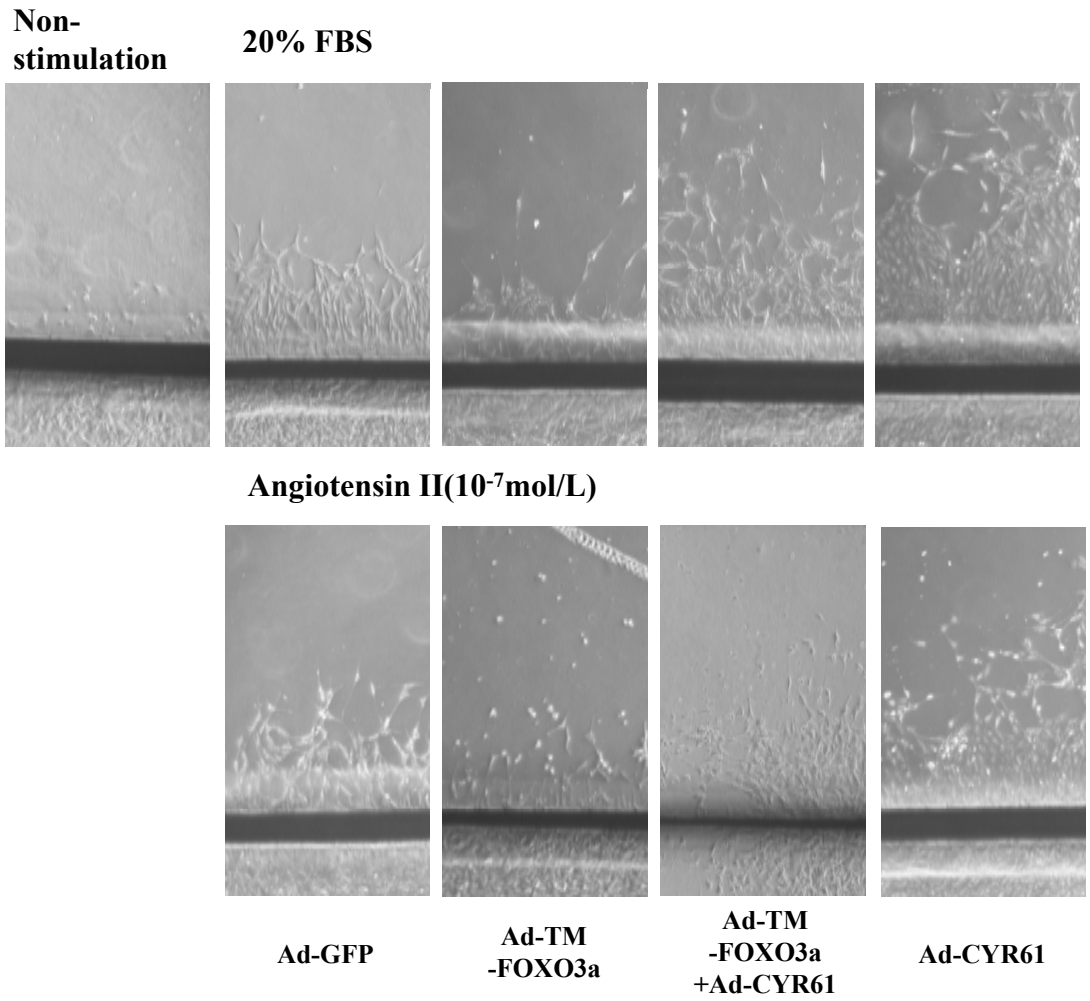


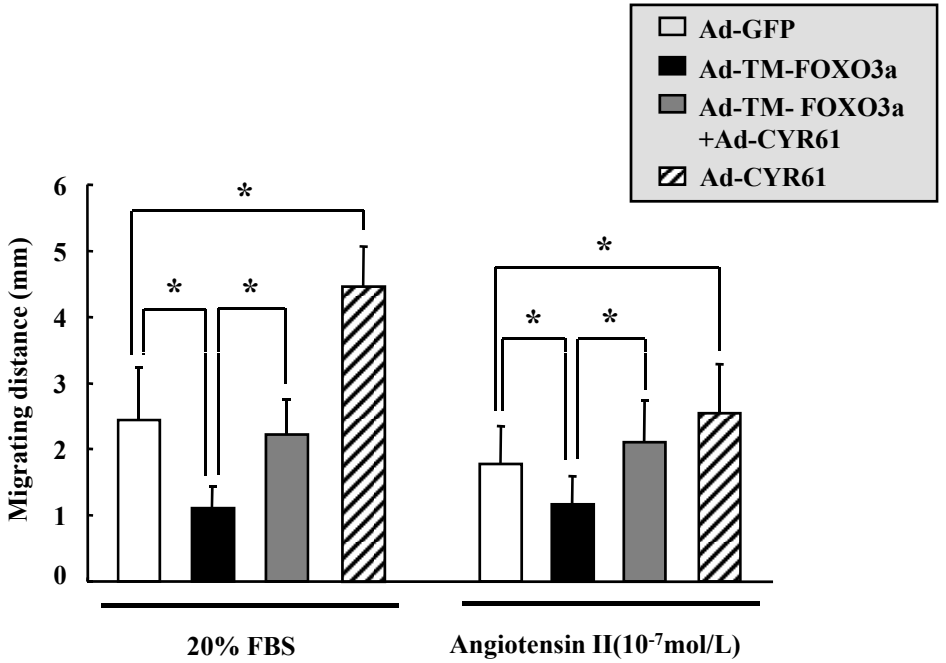


Lee HY, Oh BH, Kim HS, et al. ATBV 2008

VSMC migration following inflammatory chemotactic cytokines

- Scratch wound migration assay
  - Cells on glass coverslips scratched with a cell scraper to make the wound area
  - Indicated cytokines applied to VSMCs on the coverslips
  - Incubated for 96 hours and the migration distance quantified using Image-Pro 4.5



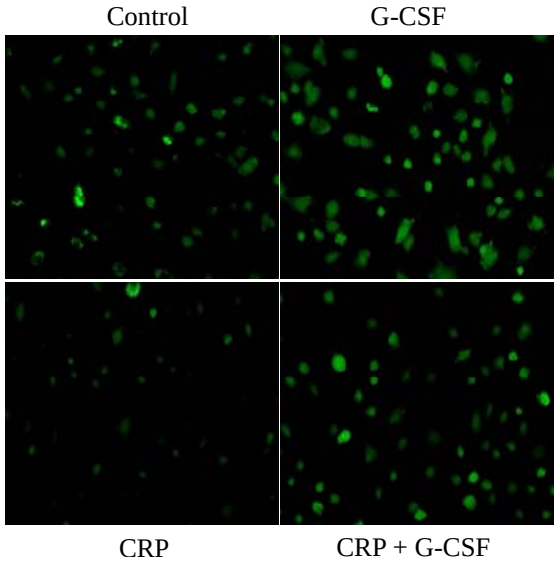


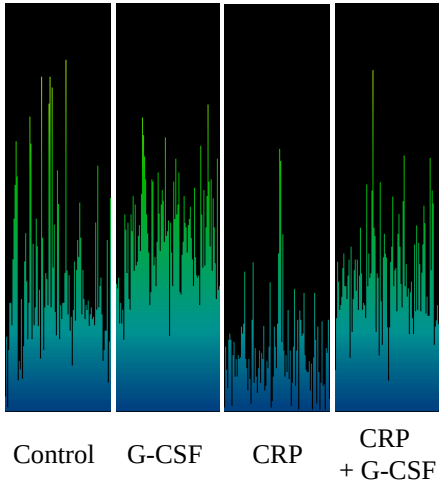
Lee HY, Oh BH, Kim HS, et al. Circulation Research 2007

Nitric oxide production form endothelial cells

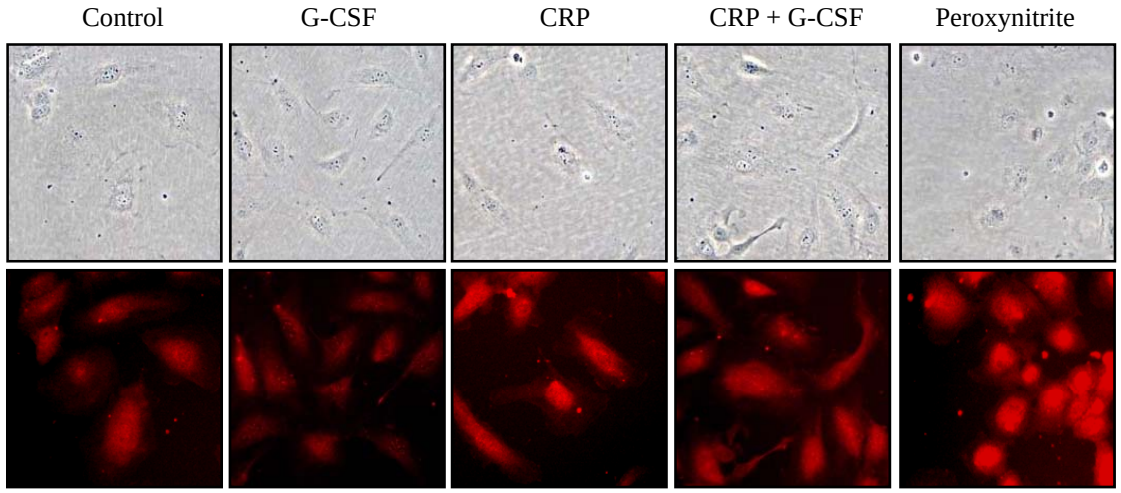
- Diamino-fluorescein-2 diacetate (DAF-2 DA) staining
  - DAF-2 DA: a membrane NO-specific fluorescence indicator
  - ECs bathed in buffer containing L-arginine (1 mM) and DAF-2 DA (10 uM) for 15 minutes.
  - Intensity profile of fluorescence of each cell in captured image during a fixed exposure time measured semiquantitatively with Image-Pro Plus® (MediaCybernetics)
- Immunofluorescent microscopy for peroxynitrite formation

Nitric oxide production





Peroxynitrite formation

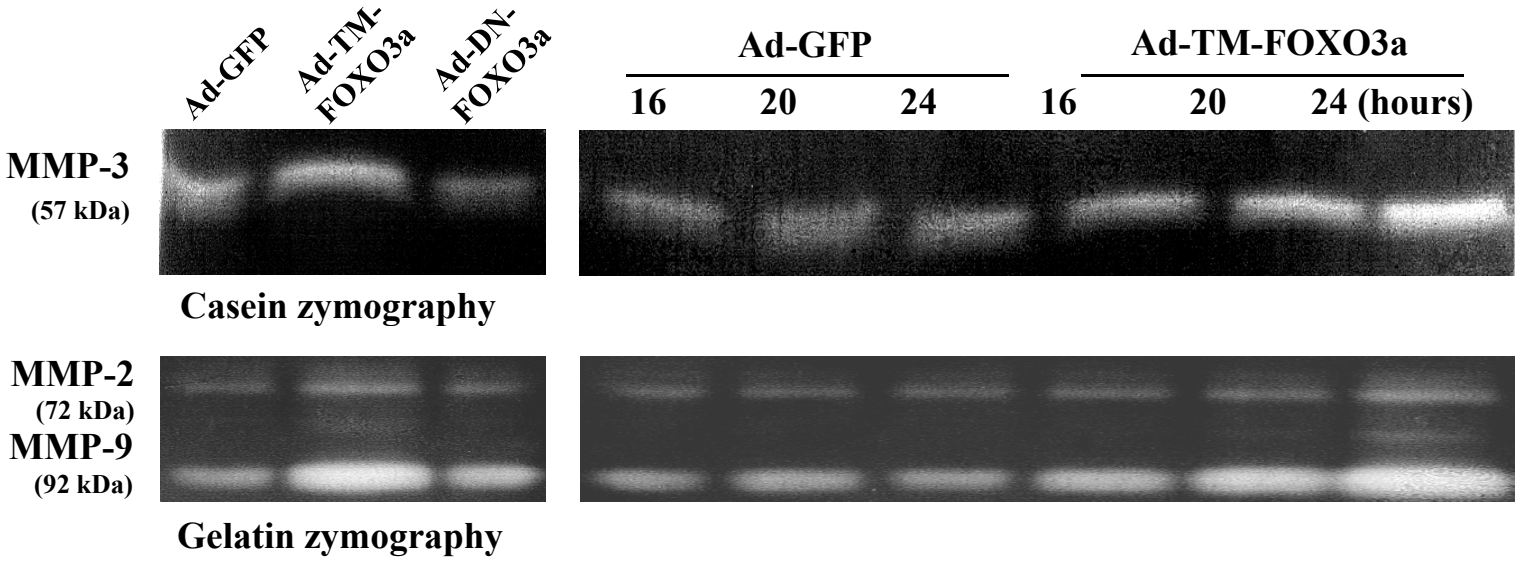


Shin JI, Kim YJ, Park KW, Lee HY, Kim HS, et al. In submission

Extracellular degradation by metalloproteinase

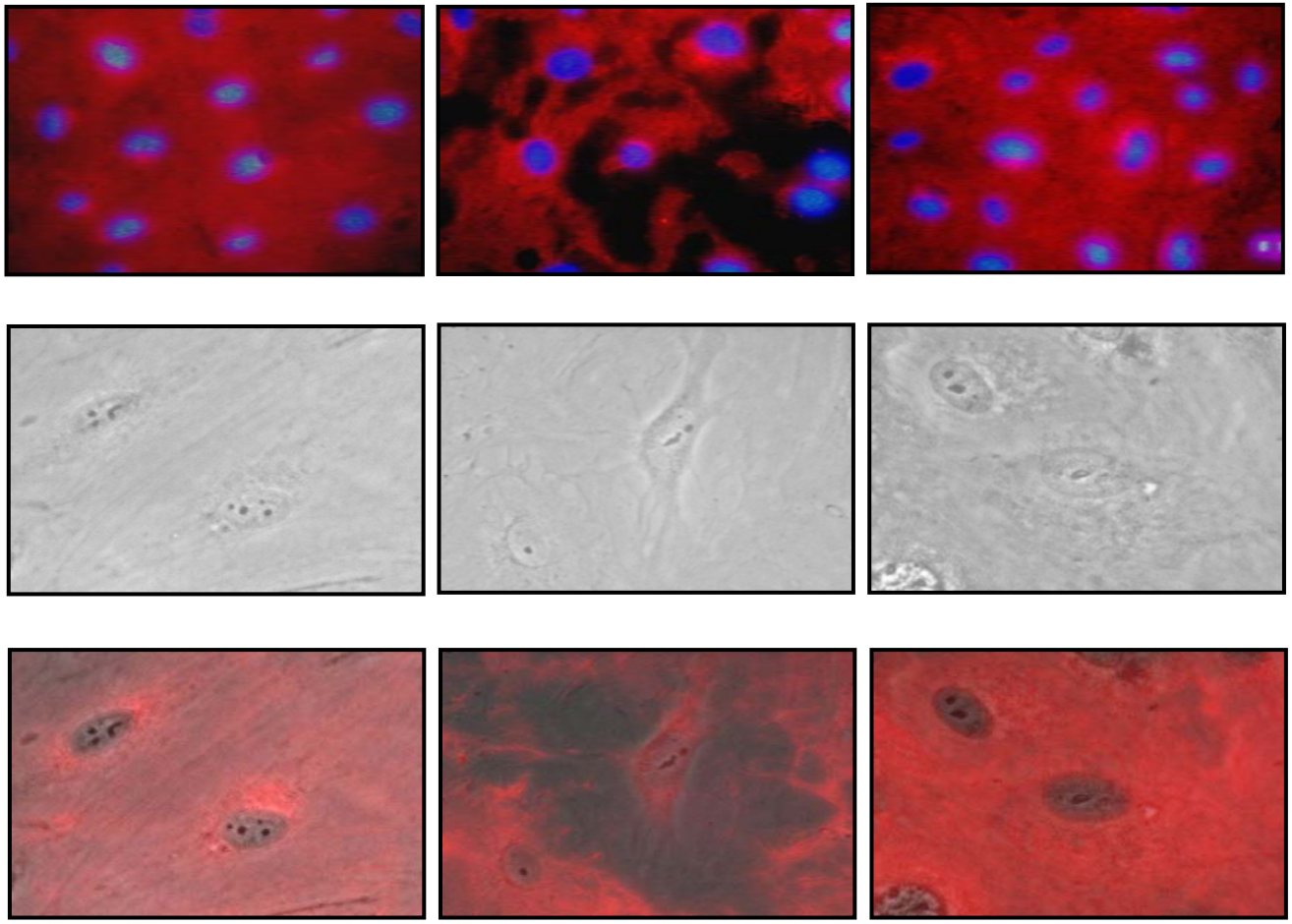
- Casein and gelatin zymography
  - Media conditioned by cell culture under different experimental conditions separated on an 8% polyacrylamide gel containing 0.1% casein or gelatin
  - After electrophoresis, gels stained with 0.05% Coomassie Brilliant Blue R250 (Sigma Co.) and location of enzymolytic activity detected as clear bands
- Immunofluorescent staining for extracellular matrices
  - Monoclonal antibody against fibronectin, beta-catenin, or VE-cadherin
  - Counter stained with 4,6-diamidino-2-phenylindole(DAPI) to visualize nuclei

Zymography

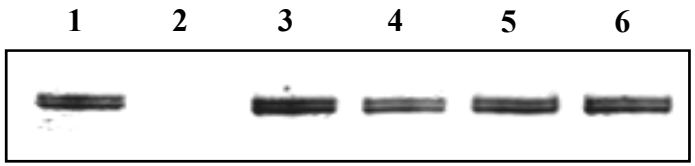


Lee HY, Oh BH, Kim HS, et al. ATBV 2008

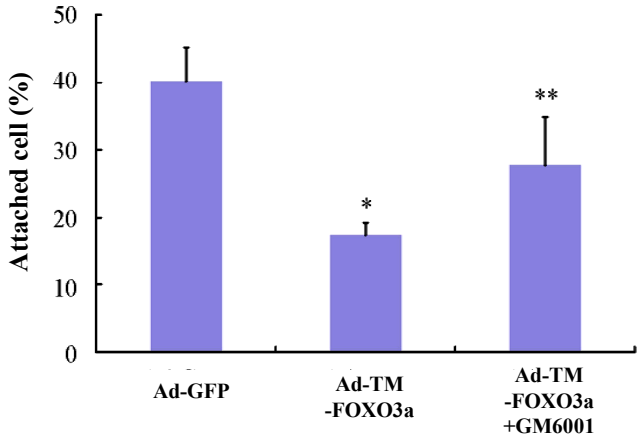
Fibronectin immunofluorescent stain



Ad-GFP | Ad-TM-FOXO3a | Ad-TM-FOXO3a + GM6001



1. Only Fibronectin,
2. Only supernatant
3. Ad-GFP, 4. Ad-TM-FOXO3a
5. Ad-TM-FOXO3a + GM6001 (5μM)
6. Ad-TM-FOXO3a + GM6001 (10μM)



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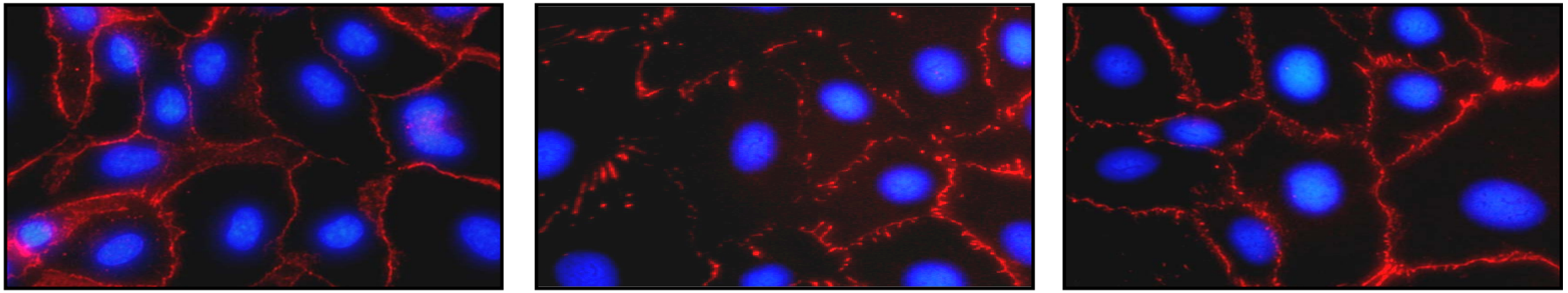
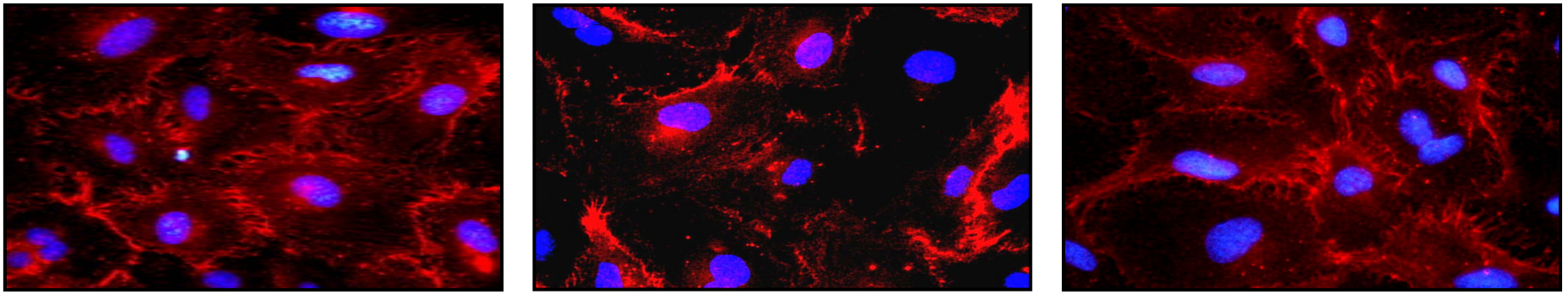
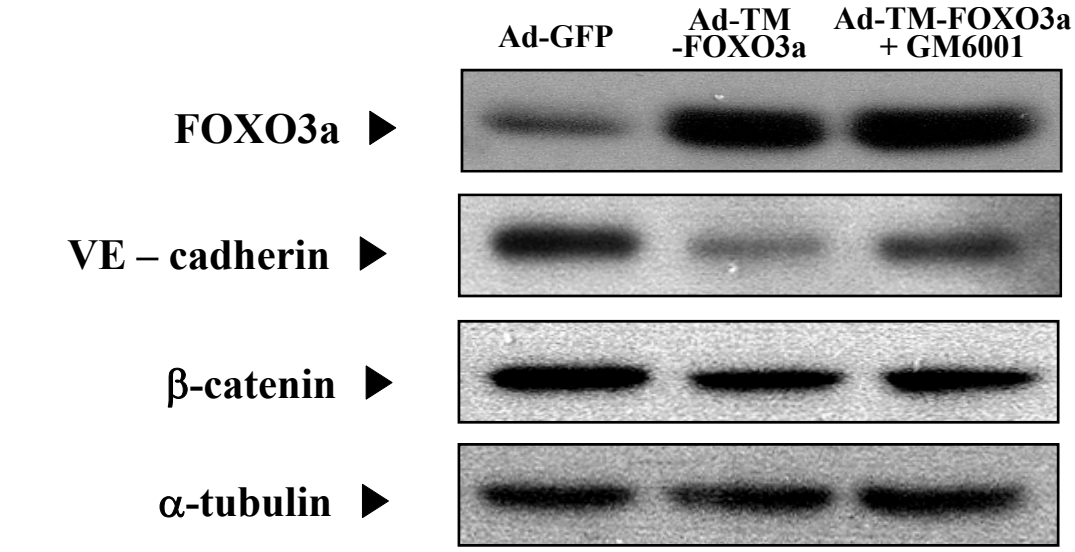
VE-cadherin

Ad-GFP | Ad-TM-FOXO3a | Ad-TM-FOXO3a + GM6001



β-catenin

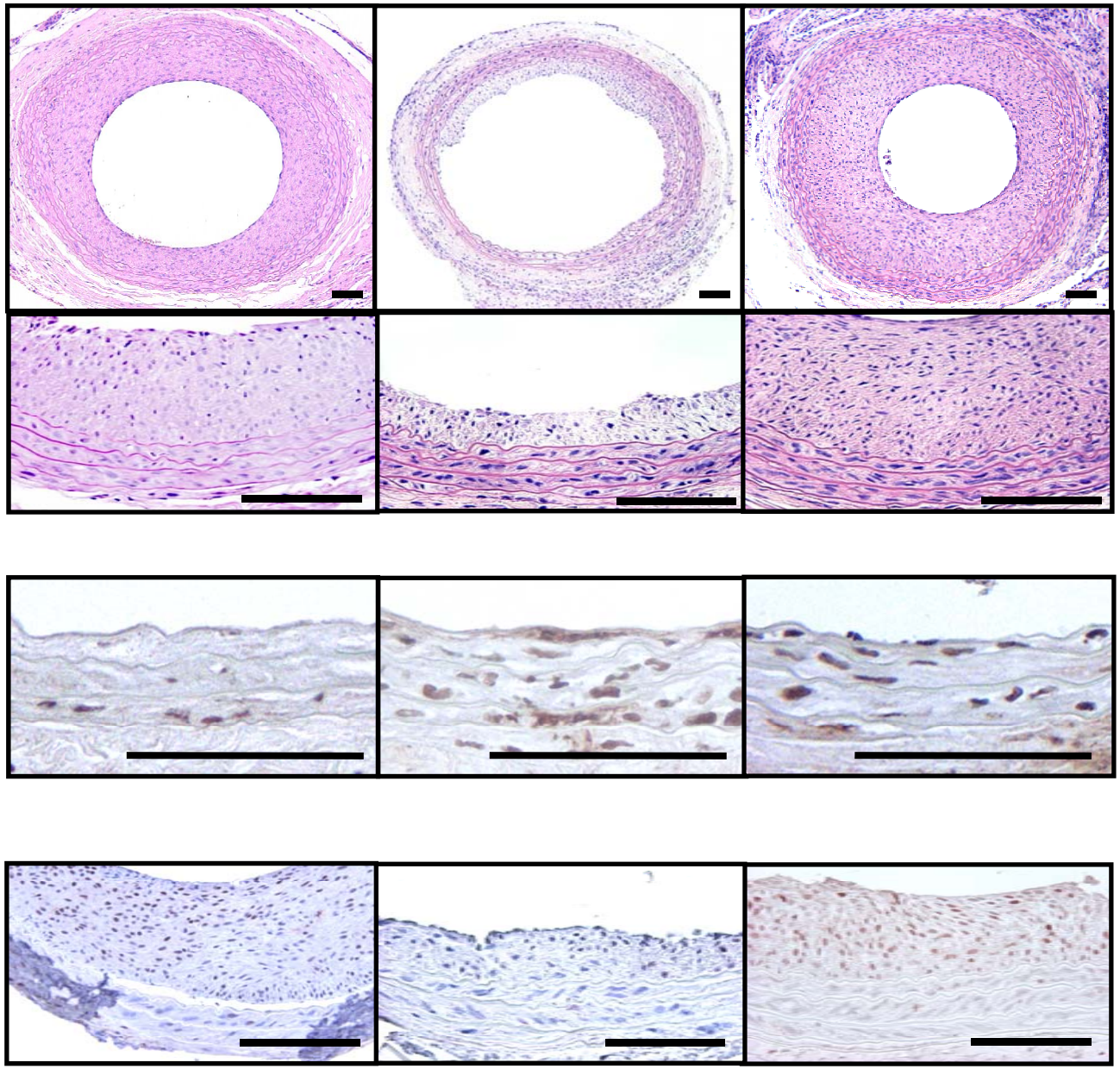


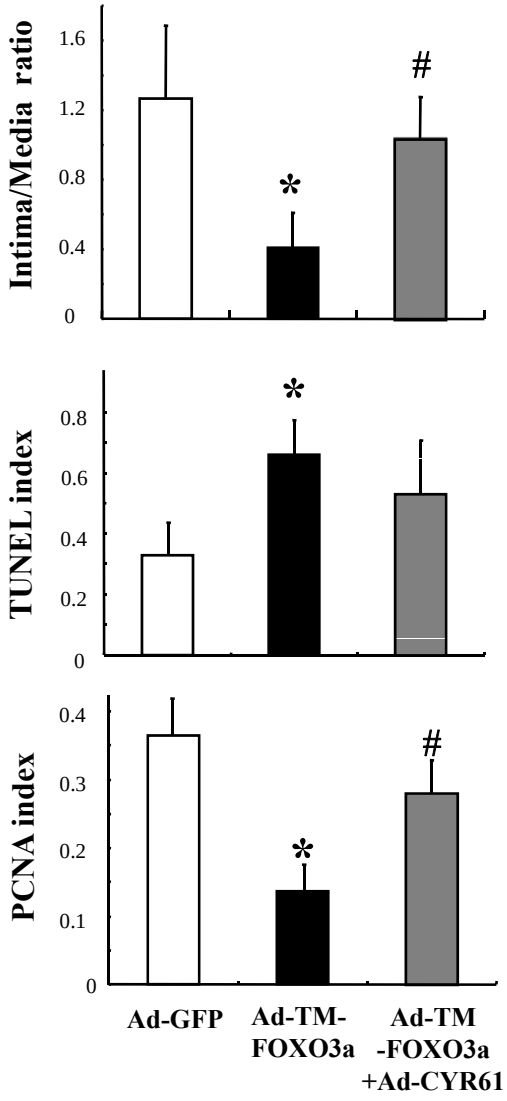


Lee HY, Oh BH, Kim HS, et al. ATBV 2008

Histologic analysis of VSMC proliferation and apoptosis

- Morphometric analysis
  - Extent of neointima quantified by computed planimetry of stained sections
  - Cross-sectional areas of the blood vessel layers quantified by using Image Pro Plus Analyzer Version 4.5 (Media Cybernetics)
  - Intima-to-media ratios were calculated from the mean of these determinations.
- Detection of proliferating cells or fraction
  - FACS analyzing cells with S phase using Modfit LT software (Verity Software)
  - Immunohistochemical stain for proliferating cell nuclear antigen (PCNA)
- Detection of apoptotic fraction
  - FACS counting cells with hypodiploid DNA contents
  - Terminal deoxynucleotidyl transferase-mediated dUTP nick endlabeling (TUNEL) stain



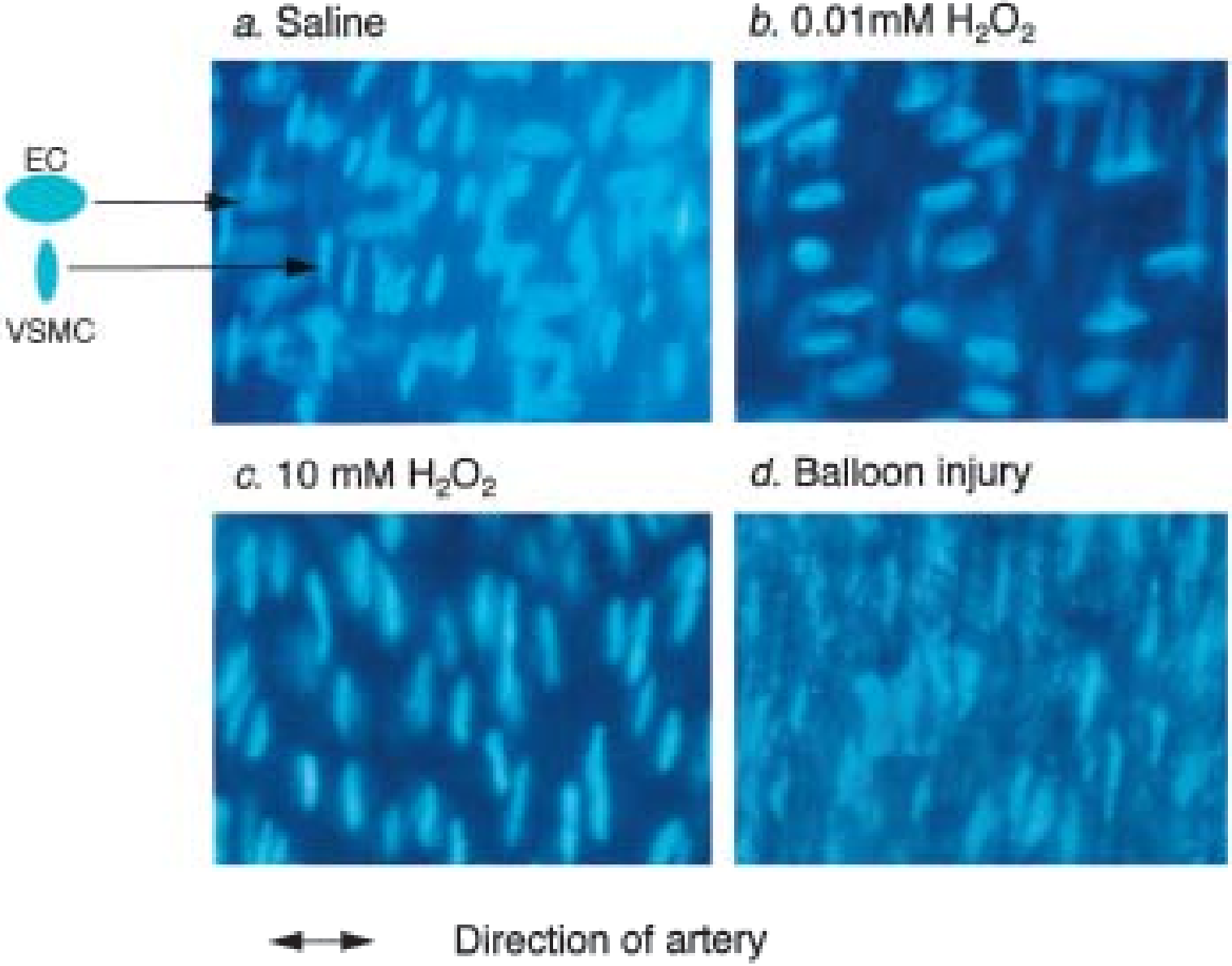


Lee HY, Oh BH, Kim HS, et al. Circulation Research 2007

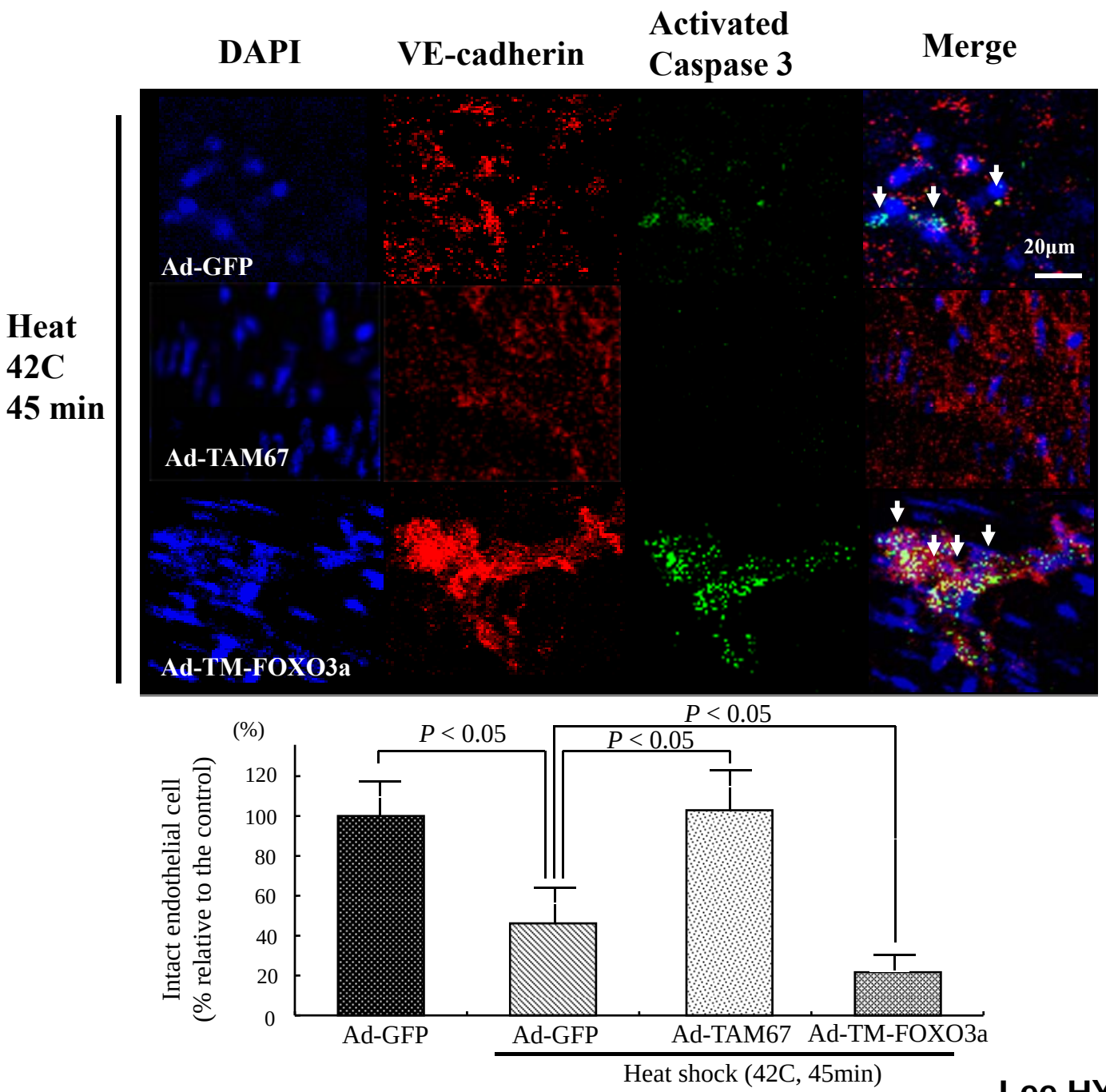
Ex vivo visualization of endothelial denudation in vessels

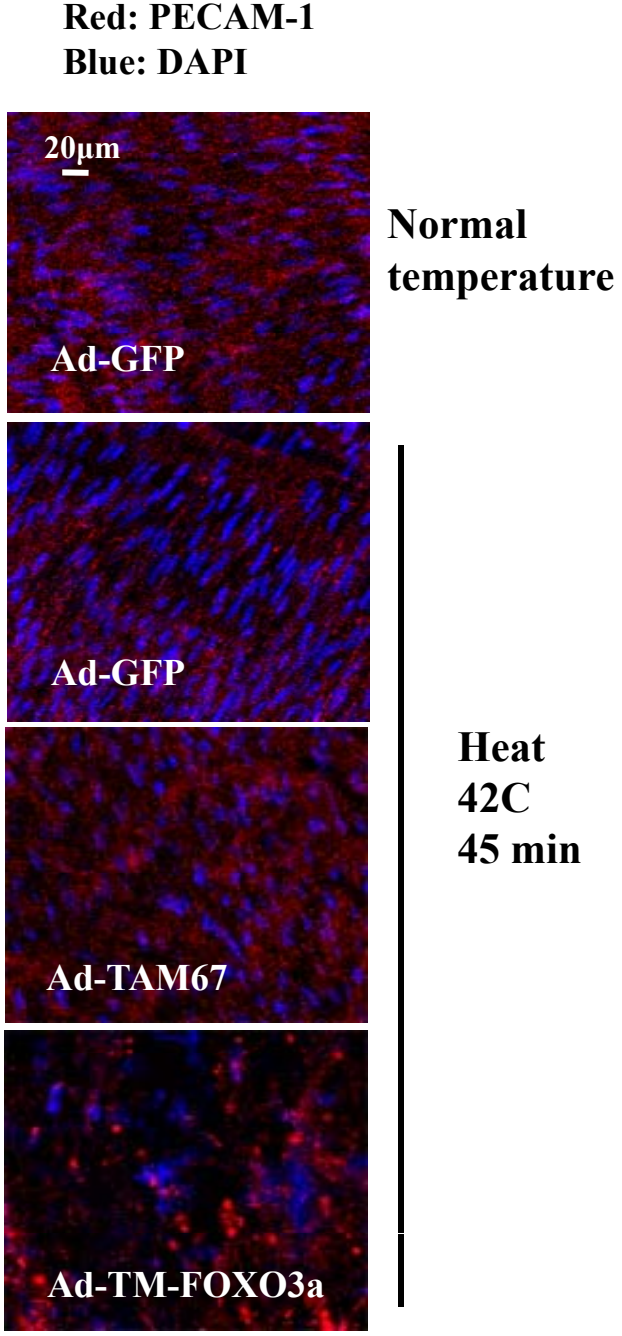
- The indicated artery was dissected, incised longitudinally, and pinned to plastic board
- The specimens were immunostained with antibodies against
  - **activated caspase-3 and VE-cadherin to visualize apoptotic Ecs**
  - **PECAM-1 to visualize ECs lining**
  - **4,6-diamidino-2-phenylindole(DAPI) to visualize nuclei**
- After IF staining, the luminal sides of the harvested vessels were observed en-face with the confocal microscope.
  In immuno-staining of PECAM-1 with DAPI staining,
  - **Viable ECs were quantitated by counting the round nuclei with PECAM-1 positivity**
  - **Apoptotic ECs were identified by double positive for the activated caspase-3 and VE-cadherin**

Ex vivo visualization of endothelial denudation induced by ROS



Estrogen prevents oxidative stress–induced endothelial cell apoptosis in rats. Ouchi Y, et al. Circulation 2001

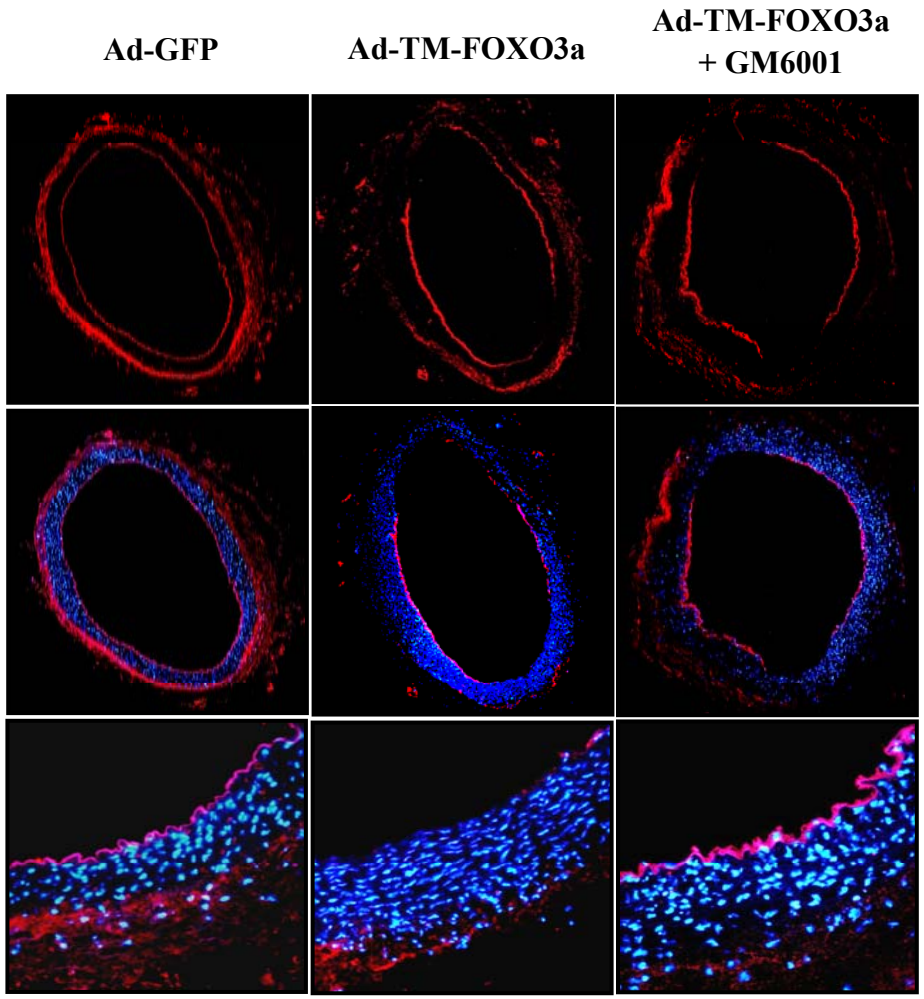


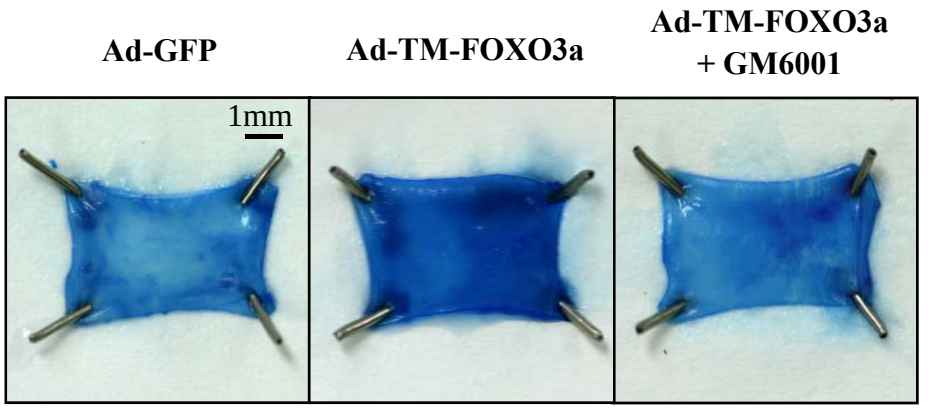


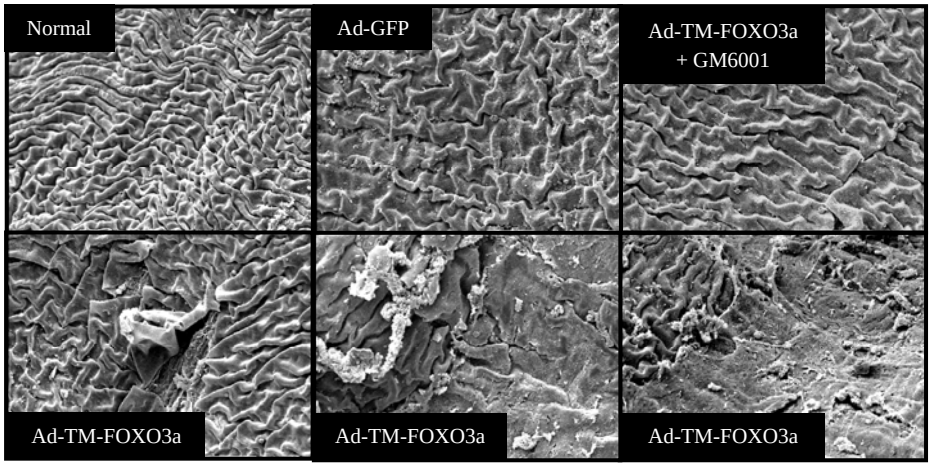
Lee HY, Oh BH, Kim HS, et al. ATBV 2008

Histologic analysis of endothelial denudation

- Evans blue stained on the denudated area
  - Immediately before sacrifice, animals were perfused with Evans blue dye14 to identify the remaining denuded area
  - endothelium-retained area was defined macroscopically as the area that was not stained with the Evans blue dye
- Cross sectional immunohistochemical stain
  - **EC visualized with PECAM-1 monoclonal antibody**
  - **4,6-diamidino-2-phenylindole(DAPI) used for nuclei visualization**
  - **Endothelial coverage assessed by the percentage of PECAM-1 positive circumference versus total in three different sections PECAM-1 to visualize ECs lining**
- Scanning electron microscope







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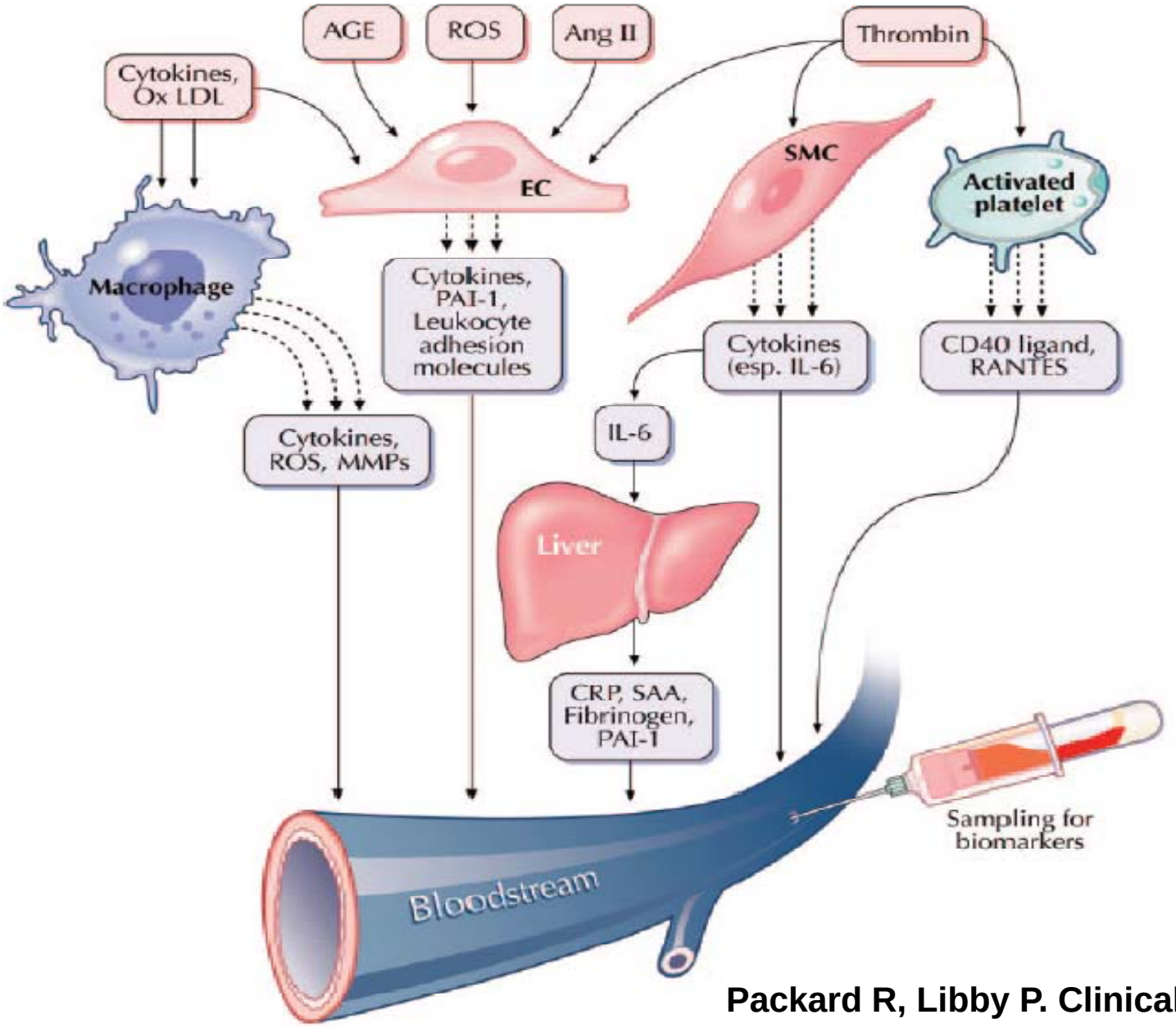
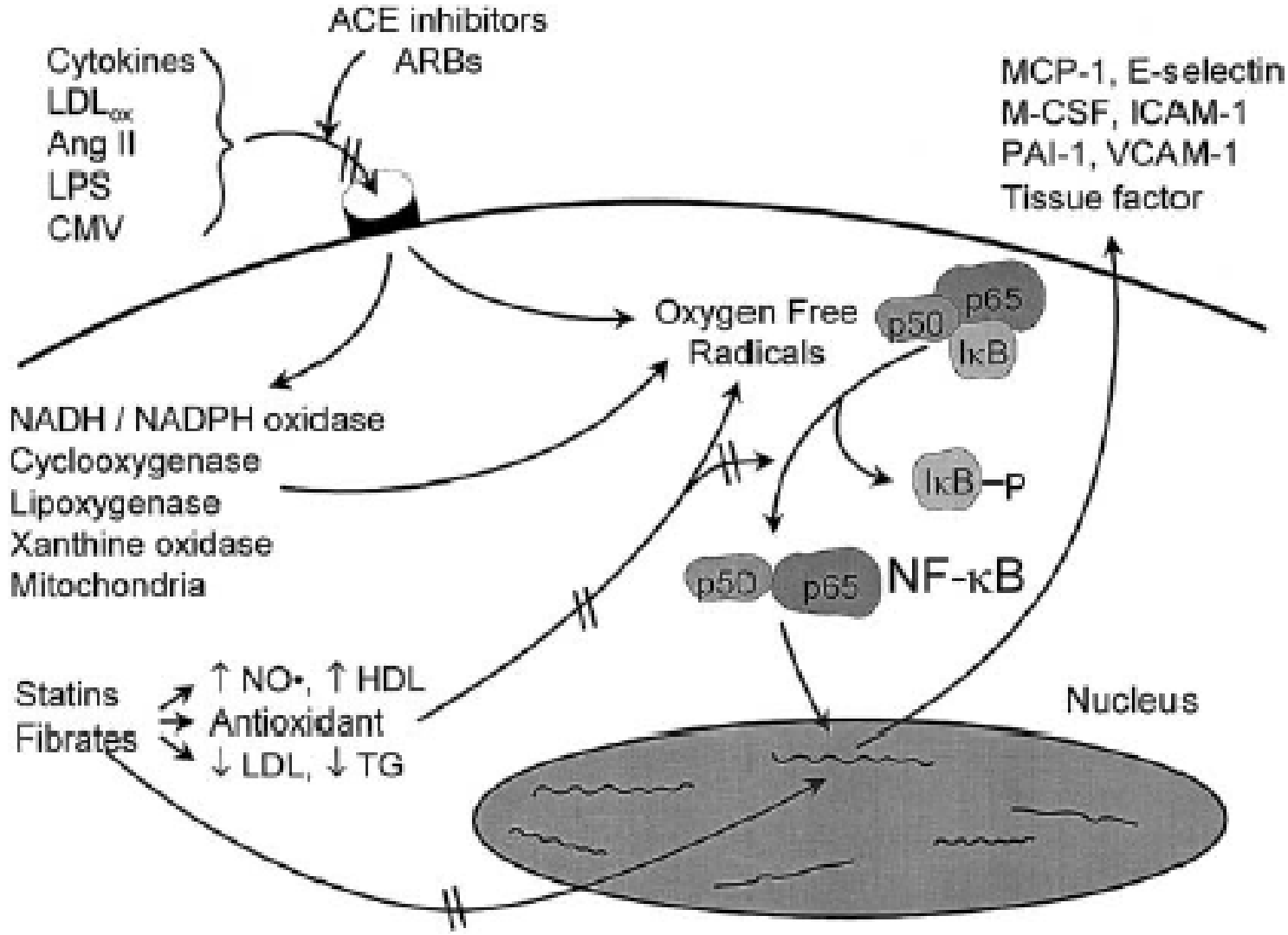
Inflammatory mediators released at different stages of atherosclerosis



Packard R, Libby P. Clinical chemistry 2008

Many proinflammatory stimuli initiated gene transcription through NF-κB



Koh KK, Han SH, Quon MJ. JACC 2007

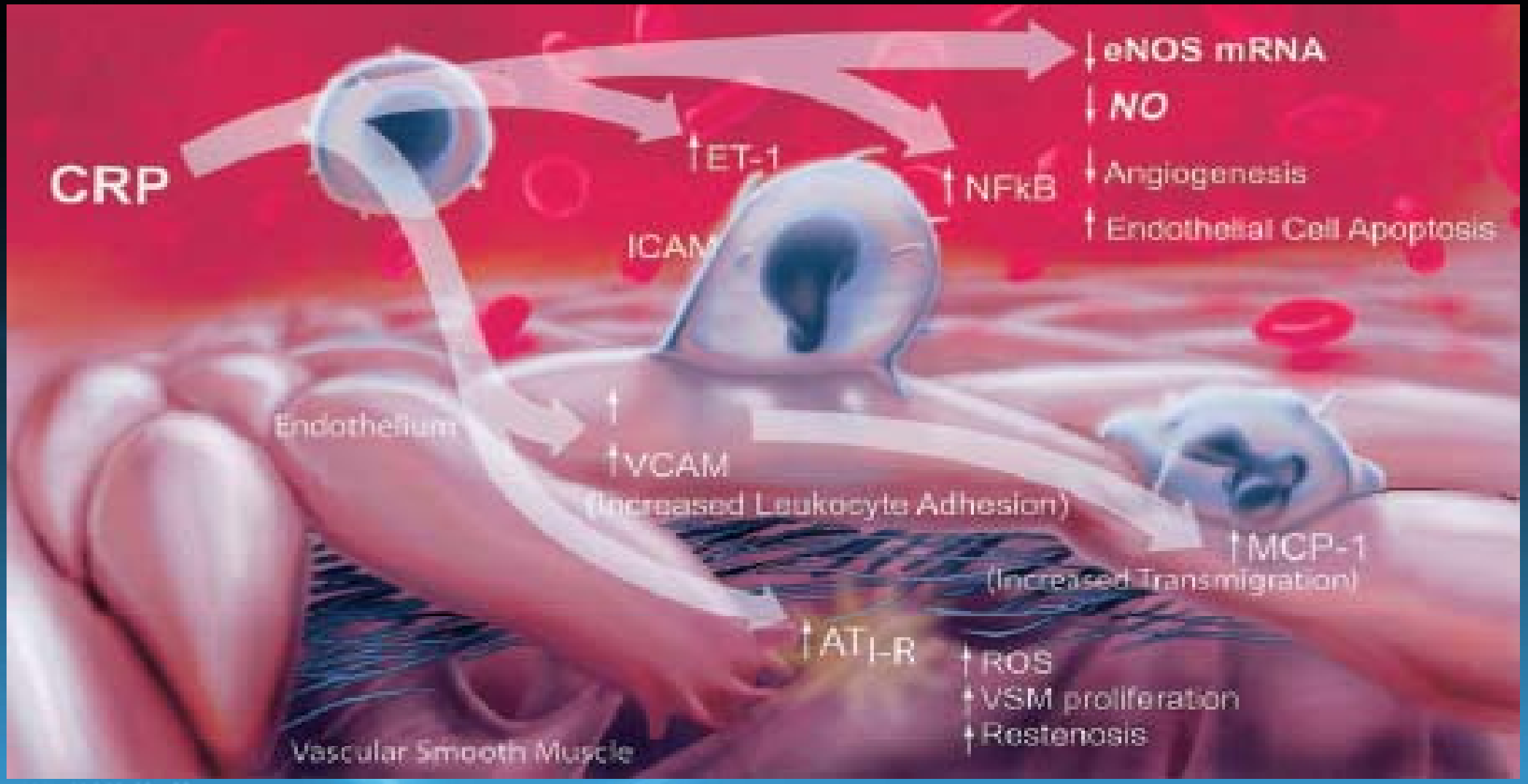
Mediators affecting various stage of inflammatory response

- **Cell adhesion: LFA-1, ICAM-1, VCAM-1, E-selectin, L-selectin**
- **Cell migration: MCP-1, IL-8, RANTES**
- **Matrix degradation:**
  - **Interstitial collagenases: MMP-1/-13**
  - **Gelatinases: MMP-2/-9**
  - **Stromelysin MMP-3**
  - **Negative regulator of MMPs: TIMP-1**
- **Inflammatory mediator: CD40/CD40L, sCD40L, IL-1, IL-6, TNF, C-reactive protein, serum amyloid A**
- **Thrombosis: Tissue factor, Factor VIIa, t-PA, Fibrinogen, PAI-1, PGI2, TxA2**
- **Apoptosis: Caspase-3, -9, Bcl-2**

C-reactive protein

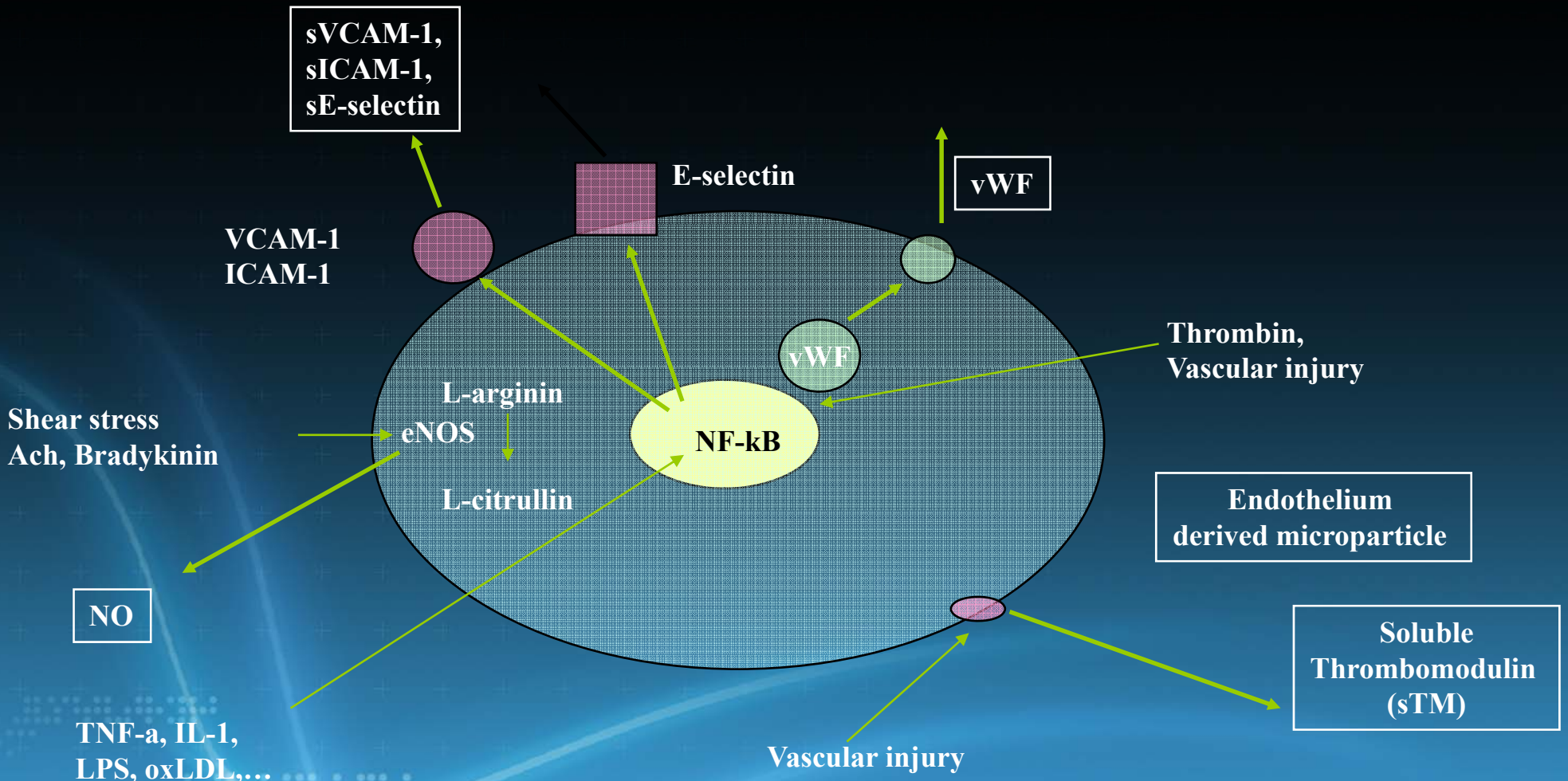
- Not only an inflammatory marker but also a inflammatory mediator
- Inducing synthesis of cytokines, adhesion molecules, tissue factor in monocytes and endothelial cells
- Contributing atherosclerosis progress in
  - atheroma formation / plaque rupture / coronary thrombosis
- Reported as an independent predictor of
  - myocardial infarction, stroke, PAD, and sudden cardiac death, even in apparently healthy individuals
- CRP can be reduced by medication such as
  - statin, fibrate, ARB, ACEI, Glitazones

CRP as a direct effector more than a marker of inflammation



Verma S, et al. Circulation, 2003

Subcellular origin of markers of endothelial activation / dysfunction



Constans J, et al. Clin Chim Acta, 2006.

Adhesion molecules

E-selectin

- **Specifically expressed on** surface of stimulated EC
- **Markers of** EC damage or activation
  - **Measured by ELISA**
- Large inter-individual variation
  - **Children (9-16) > adult**
  - **Male > Female**
  - **Menopause > Premenopause**
- Clinical implication
  - **Increased in CAD and DM**
  - **Prediction of DM**

ICAM-1 / VCAM-1

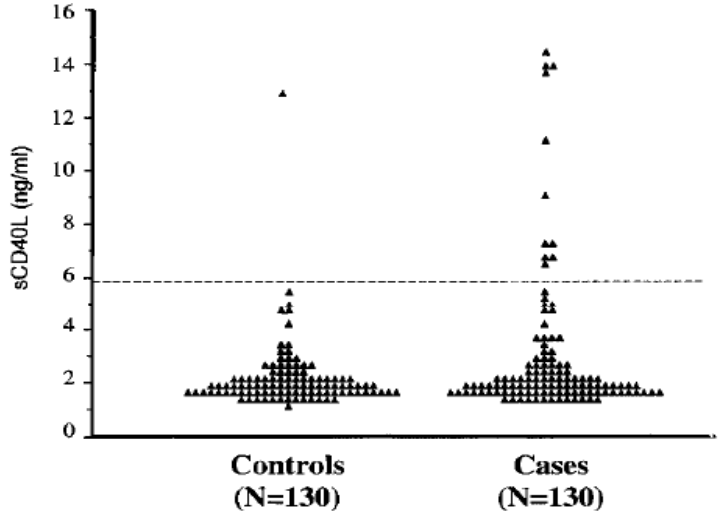
- Expressed on a variety of cell types including VSMC, Monocytes, EC
  - Not endothelial-specific
- **Measured by ELISA**
- Inter-individual variation
  - **Race**
  - **No age or menstruation variation**
- Clinical implication
  - **sICAM-1 predicting CHD and DM in healthy individuals**
  - **sVCAM-1 predicting CHD recurrence**

Other markers of inflammation

- CD40/CD40L
  - Expressed on CD4+ T cells and activated platelets
  - Both membrane-bound and soluble CD40L interacts with CD40 expressed on vascular cells
  - Roles in inflammatory cascade and prothrombotic functions
- Interleukin-18 (IL-18)
  - IL18 co-localized with mononuclear phagocytes in atheroma
  - Inducing essential effectors in atherosclerosis: VCAM-1, IL-8, IL-1, MMPs
  - Proximal inducer and regulator of IFN-gamma

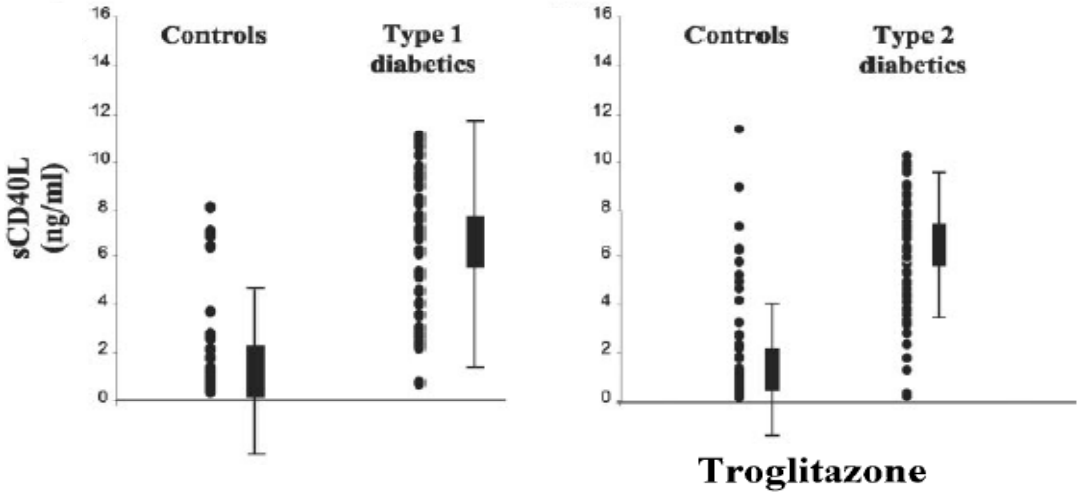
sCD40L as a biochemical marker of risk stratification

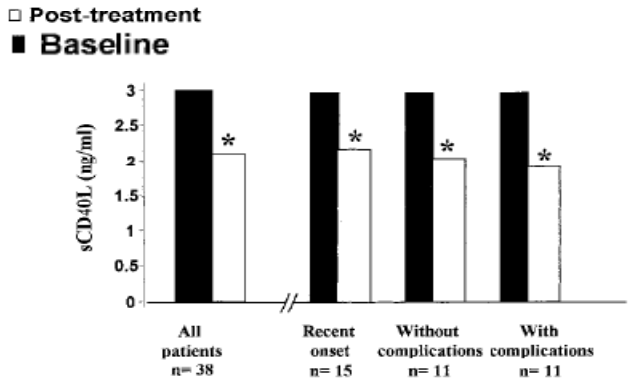
sCD40L among middle-aged healthy women or CAD patients



Cutpoint, percentile	sCD40L, ng/mL	Controls, % (n)	Cases, % (n)	RR	*P*
50th	>1.76	50.4 (65)	50.8 (66)	1.02	0.95
75th	>2.15	24.9 (32)	30.8 (40)	1.35	0.29
90th	>2.92	10.1 (13)	17.7 (23)	1.92	0.08
95th	>3.71	5.0 (6)	13.9 (18)	3.29	0.02
99th	>5.54	0.8 (1)	8.5 (11)	11.83	0.01

sCD40L with or without diabetes





Libby P, et al. Circulation, 2001, 2003.

Matrix metalloproteinases

- Degrades ECM, creating uncoiled, less effective collagen and broken / frayed elastin molecules

- MMP-9 as a most compromising MMP
  - Genetic activity related with presence and severity of CAD
  - Blood MMP-9 levels elevated in

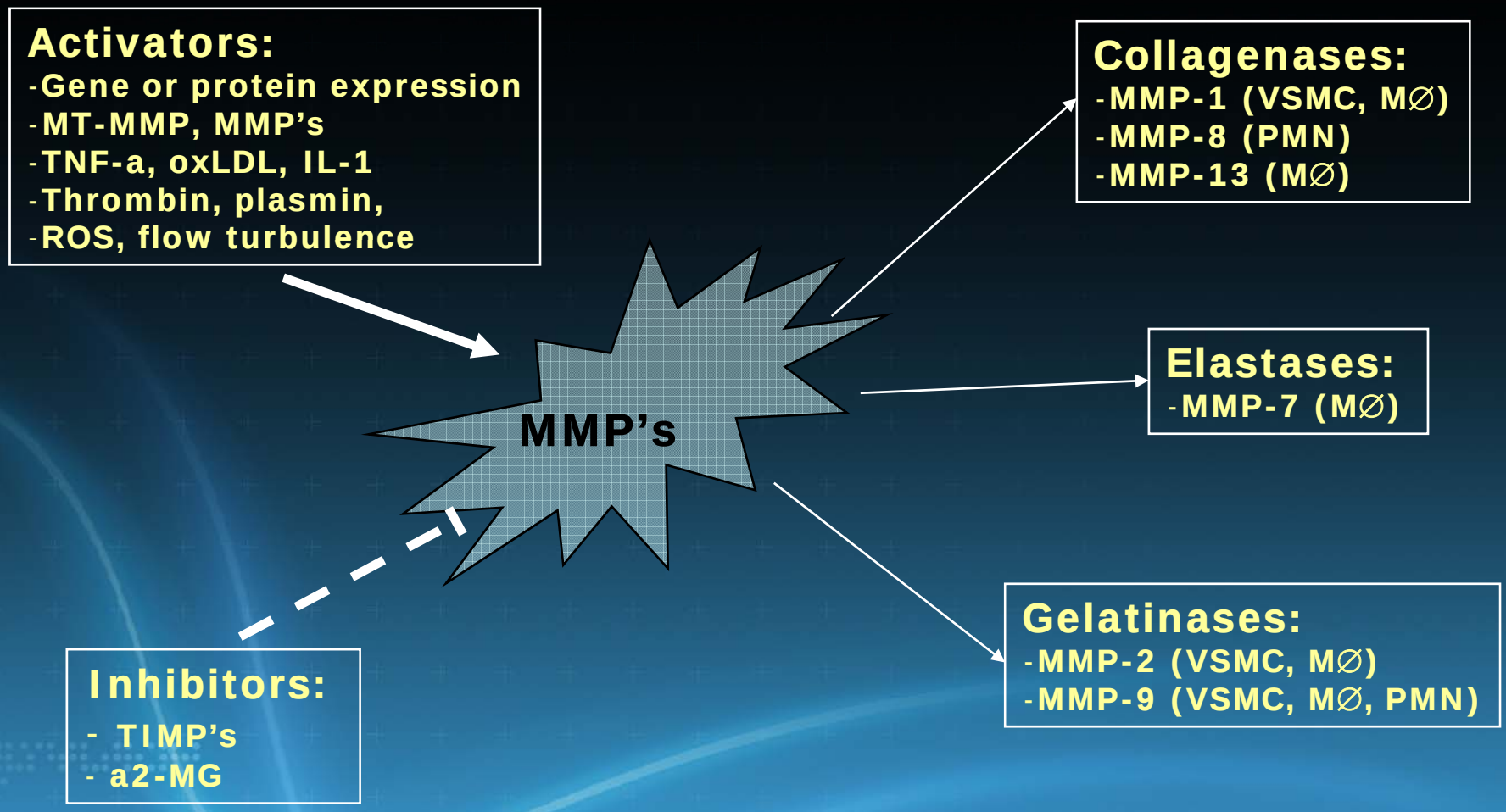
    Type 2 DM with CAD, HT, smoking, Premature CAD

    Abdominal aortic aneurysm expansion

    Worse outcome in stroke, Cardiovascular death

- Tissue inhibitor of metalloproteinase (TIMPs): TIMP-1
  - Related with CV risk factors, LVH, carotid atherosclerosis
- Procollagen markers:
  - N-terminal propeptides of collagen III, C-terminal propeptides of procollagen I

ECM remodeling by matrix metalloproteinases



Response of surrogate markers of inflammation by various intervention

		Valsartan	*Candesartan*	*Irbesartan*	*Losartan*	*Enalapril*	*Ramipril*	*Quinapril*
sICAM-1	Decrease		Rosei *et al.*,[30] Tsutamoto *et al.*[44]		Graninger *et al.*[84]	Rosei *et al.*,[30] Graninger *et al.*[84]		
	No change							
s-VCAM	Decrease		Tsutamoto *et al.*[44]	Navalkar *et al.*[47]	Graninger *et al.*[84]	Graninger *et al.*[84]		
	No change							
CRP	Decrease	Dandona *et al.*,[85] Anand *et al.*,[86] Ridker *et al.*[45]		Schieffer *et al.*[85]				Trikouras *et al.*[88]
	No change		Koh *et al.*[26]	Navalkar *et al.*[47]		Schieffer *et al.*[85]		Dandona *et al.*[25]
Oxidative stress	Decrease	Dandona *et al.*[25]	Koh *et al.*[26]	Sola *et al.*,[28] Navalkar *et al.*,[47] Khan *et al.*[48]				
	No change							Dandona *et al.*[25]
MCP-1	Decrease		Koh *et al.*[26]					
	No change							
TNF-α	Decrease	Neri Serneri *et al.*[41]	Koh *et al.*,[26] Tsutamoto *et al.*[44]				Neri Serneri *et al.*[41]	
	No change							
PAI-1	Decrease		Koh *et al.*[26]	Sola *et al.*[28]			Vaughan *et al.*[51]	
	No change							
MMP-9	Decrease			Schieffer *et al.*[85]		Schieffer *et al.*[85]		
	No change							
IL-6	Decrease	Neri Serneri *et al.*[87]	Tsutamoto *et al.*[44]	Sola *et al.*,[28] Lauten *et al.*,[89] Schieffer *et al.*[85]			Neri Serneri *et al.*[87]	Lauten *et al.*[89]
	No change					Schieffer *et al.*[85]		
NF-κB	Decrease	Dandona *et al.*[25]						
	No change							
IκB	Increase	Dandona *et al.*[25]						
	No change							
IL-10	Increase			Schieffer *et al.*[85]		Schieffer *et al.*[85]		
	No change							
Platelet aggregation	Decrease			Schieffer *et al.*[85]				
	No change					Schieffer *et al.*[85]		

Dandora P, et al. Journal of Human Hypertension. 2007;21:20-27

Conclusions

- Inflammatory processes play a fundamental role in the initiation, propagation, and complications of atherosclerosis.
- Various surrogate markers for inflammation have been studied for reflecting inflammatory condition and evaluating pleiotropic effects of various medication in anti-inflammatory mechanism
- In vitro and in vivo studies of inflammatory response could uncover pathogenic mechanism, thus establishing new therapeutic strategy against atherosclerosis

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