Role of chondrocyte antigen presentation in osteoarthritis

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โรคข้อเข่าเสื่อมเป็นโรคที่เกิดจากการเสื่อมสลายของส่วนประกอบต่างๆบริเวณข้อ โดยเฉพาะอย่างยิ่งการแตกสลายตรงบริเวณกระดูกอ่อนผิวข้อ ส่งผลให้เซลล์กระดูกอ่อนซึ่งเป็นเซลล์สำคัญในบริเวณนี้สูญเสียคุณสมบัติในการควบคุมสมดุลในการสร้างและสลายกระดูกอ่อน ทำให้เกิดอาการเสื่อมของข้อ นอกจากนี้เซลล์กระดูกอ่อนยังสามารถกระตุ้นเซลล์ในระบบภูมิคุ้มกัน อาทิเช่น T cell ในผู้ป่วยข้อเข่าเสื่อมได้ ซึ่งอาจจะเป็นตัวแปรสำคัญที่ทำให้เกิดความรุนแรงของโรคในผู้ป่วยได้ แต่อย่างไรก็ตามยังไม่เป็นที่ทราบแน่ชัดว่าเซลล์กระดูกอ่อนมีหน้าที่หรือบทบาทในการกระตุ้นระบบภูมิคุ้มกันในโรคข้อเสื่อมได้อย่างไร ในงานวิจัยนี้จึงสนใจที่จะศึกษาการตอบสนองของเซลล์กระดูกอ่อนในผู้ป่วยข้อเข่าเสื่อมเมื่อถูกกระตุ้นด้วย IFN-γ และ proteoglycan aggrecan ซึ่งเป็นสารที่เกิดจากการสลายของกระดูกอ่อนที่สามารถพบได้ในน้ำไขข้อของผู้ป่วย รวมทั้งศึกษาความสามารถของเซลล์กระดูกอ่อนในการนำเสนอ proteoglycan aggrecan ให้แก่ T cell ในข้อด้วย โดยจากการศึกษาพบว่า เซลล์กระดูกอ่อนของผู้ป่วยข้อเข่าเสื่อมที่กระตุ้นด้วย IFN-γ จะมีการแสดงออกของ MHC class I และ II บนผิวเซลล์มากขึ้น เมื่อกระตุ้นเซลล์กระดูกอ่อนด้วย IFN-γ ร่วมกับ proteoglycan aggrecan peptide ตำแหน่งที่ P16-31 และ P263-280 สามารถเหนี่ยวนำให้เซลล์กระดูกอ่อน มีการแสดงออกของ CD80 และ CD86 บนผิวเซลล์มากขึ้น และมีการหลั่ง IL-6, IL-8 และ TNFα ในปริมาณที่มากด้วย นอกจากนี้ยังพบว่าเซลล์กระดูกอ่อน สามารถนำเสนอ P263-280 และ P16-31 ให้กับ T cell ที่สกัดมาจาก infrapatellar fat pads ได้ และกระตุ้นให้ T cell นั้นมีการเพิ่มจำนวนได้ (T cell proliferation) จากการทดลองนี้ชี้ให้เห็นว่า IFN-γ ร่วมกับ proteoglycan aggrecan ตำแหน่งที่ P16-31 และ P263-280 อาจเป็นปัจจัยหนึ่งที่ช่วยเพิ่มความสามารถในการเป็น antigen presenting cell ของเซลล์กระดูกอ่อนได้ ผลการศึกษาที่ได้จะเป็นความรู้ที่สำคัญที่จะนำไปพัฒนาการรักษาโรคข้อเข่าเสื่อมต่อไป

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Osteoarthritis (OA) is an inflammatory disorder characterized by degeneration of joint components, especially the cartilage. Chondrocytes are found in articular cartilage and play a role in cartilage production and degradation. Several studies found that chondrocytes not only control the cartilage metabolism, but also play a role in immune responses. However, the role of chondrocytes in the pathogenesis of OA is still unclear. In this study, we investigated the responses of OA chondrocytes after stimulating with IFN-γ and proteoglycan aggrecan and also determined antigen presentation function of chondrocytes to present proteoglycan aggrecan peptide to T cells. We found that OA chondrocyte upregulate MHC class I and II on their cell surface after IFN-γ stimulation. Proteoglycan aggrecan peptides, especially P16-31 and P263-280, can stimulate chondrocyte to express CD80 and 86, and secrete high levels of IL-6, IL-8 and TNFα. Moreover, chondrocytes were able to present the P263-280 and P16-31 peptides to autologous T cells isolated from infrapatellar fat pads and stimulated T cell proliferation. These results indicate proteoglycan aggrecan peptides in the presence of IFN-γ induces antigen presentation function of chondrocytes. The knowledge from this study might be used as a foundation for further therapeutic development.

Field of Study:	Medical Microbiology	Student's Signature .....
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Panjana Sengprasert

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ADAMTS	:	Disintegrin and metalloproteinase with thrombospondin motifs
B2M	:	Beta-2-microglobulin
BMPs	:	bone morphogenetic protein
CD	:	Cluster of differentiation
CEF	:	Cytomegalovirus, Epstein-Barr virus and Influenza virus
CFSE	:	Carboxyfluorescein succinimidyl ester
Col II	:	type II collagen
COL2A1	:	type II collagen alpha chain
COMP	:	Cartilage oligomeric matrix protein
CPPD	:	calcium pyrophosphate dehydrate
CS	:	Chondrocitin sulfate
CTX-II	:	Collagen type II-degrading molecule
DEGs	:	Differential expressed genes
ECM	:	Extracellular matrix
FGFs	:	fibroblast growth factors
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	:	Granulocyte-macrophage colony-stimulaing factor
HA	:	Hyaluronic acid
HC gp-39	:	Human cartilage glycoprotein-39
HLA	:	Human leukocyte antigen
ICAM-1	:	intracellular adhesion molecule-1
IFN-γ	:	Interferon gamma
IGD	:	Interglobular domain
IGF-I	:	insulin-like growth factor I
IL	:	Interleukin
IPFP	:	infrapatellar fat pads
JAK/STAT	:	Janus kinase/signal transducer and activator of transcription
KEGG	:	Kyoto Encyclopedia of Genes and Genomes pathway
KS	:	Keratan sulfate
LAMP2	:	Lysosome-associated membrane protein 2
LFA-1	:	ligand for leukocyte associated antigen-1
LIF	:	Leukemia inhibitor factor
MAPK	:	Mitogen-activated protein kinase
MHC	:	Major histocompatibility complex

MMPS	:	Matrix metalloproteases
MoDC	:	Human monocyte derived dendritic cell
MYD88	:	Myeloid differentiation primary response 88
NF-kB	:	Nuclear factor kappa B
NK	:	Natural killer cells
NOS	:	nitric oxide synthase
OA	:	Osteoarthritis
OVA	:	ovalbumin
PG	:	Proteoglycan
PGE2	:	Prostaglandin E2
PTGS2	:	Prostaglandin-endoperoxide synthase 2
ROS	:	Reactive oxygen species
TAP	:	transporter for antigen processing protein
TGF-β	:	transforming growth factor
TIMPs	:	tissue inhibitors of MMPs
TLR	:	Toll like receptor
TNF-α	:	tumor necrosis factor α
TRAF	:	TNF receptor associated factor
VCAM1	:	Vascular cell adhesion molecule 1

CHAPTER I

INTRODUCTION

Osteoarthritis (OA) is a common degenerative joint disease induced by multi-factorial factors including genetic, biomechanical, biochemical and metabolic factors. This degeneration causes abnormal remodeling of the joint leading to low-grade inflammation, pain and disability in humans, particularly in older adults. Generally, OA can occur in several joints in the body such as the hands, hip or knees; however, knee OA is the most prevalent (over 50%). Pathogenesis of the disease is characterized by joint tissue damage, cartilage degradation, bone remodeling, bone sclerosis, synovial hypertrophy and osteophyte formation. Symptoms of OA compose of joint stiffness, muscle weakness, joint swelling and deformation. In some cases, cracking and creaking sounds, and loss of joint motion are also observed in patients that leads to a reduction in quality of life. Nowadays, treatment of OA is based on exercise therapy and administration of analgesics and non-steroidal anti-inflammatory drugs (NSAIDs). However, these treatment methods cannot completely cure the disease, but only reduce the symptoms of the patients. Besides, cell-based therapy for cartilage repair is alternative treatment. Chondrocyte is one of the optimal cell sources for this therapeutic. However, chondrocytes cannot only control cartilage repair, but they also response to immune system in OA pathogenesis and role of chondrocyte in immune response is still unclear. Therefore, studies on OA, especially on chondrocyte function in immune response and cause of inflammation in OA patients, will be beneficial in further modifying treatment of the disease. Moreover, knowledge of biochemical biomarkers expressed during early cartilage degradation may be useful in developing treatments before cartilage degradation becomes severe.

LITERATURE REVIEW

The human knee joint

Anatomy of human knee

The human knee is a complex joint at the juncture of the thigh bone (femur) and shin bone (tibia) linked to many ligaments and tendons to increase joint strength and joint stability (1). The surface of these bones at the knee joints are covered with cartilage and surrounded by tissues, such as synovial tissue and infrapatellar fat pads (Figure 1) (2). Both the cartilage and surrounding tissues function to absorb and reduce the friction during joint movement. There are extracellular matrixes (ECM) surrounding the joint such as collagen, proteoglycan, glycosaminoglycans, keratin sulfate and chondroitin sulfate, giving strength and flexibility (3). In a healthy knee joint, the smoothness and thickness of the cartilage layer results in a suitable gap size between the femur and tibia that enables the joint to move easily (2). If there are any abnormalities or destruction of the joint cartilage, the gap space will narrow, increasing difficulty in joint movement (1, 4).

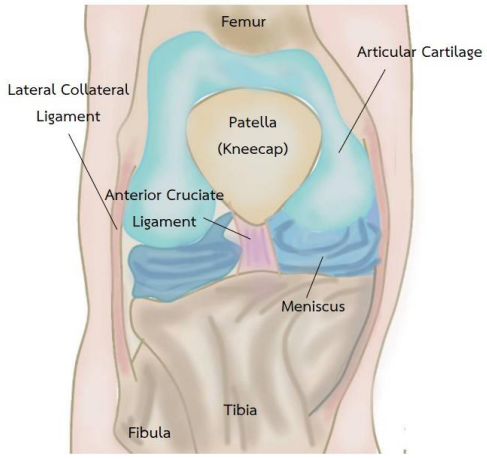


Figure 1 Knee anatomy (adapted from Wilson C., 2016)

The human articular cartilage

The human articular cartilage

The human articular cartilage exists at the end of the tibia and femur and function to distribute loads across the joints. It is composed of two important parts; (i) a dense ECM compartment and (ii) a cellular compartment, in which the majority of cells are the articular chondrocytes (5). The articular cartilage function to reduce friction surface during joint motion, and to resist tensile, shear and compressive forces. The cartilage contains 4 tissue layers: a superficial, middle, deep, and cartilage calcification layer (Figure 2). The superficial layer is the outer most layer and function to protect the deeper layers from shear stress (3, 5). Each layer contains a large number of ECM components such as glycoproteins, proteoglycans, hyaluronan and collagen (6, 7). The cartilage is formed predominantly from type II collagen (8). Proteoglycans and other non-collagenous proteins bind and stabilize fibrillar collagen network (12). Intrafibrillar space within the collagen also contains water (over 60%) and nutrients, including sodium, calcium, chloride, potassium and oxygen dissolving in this fluid (5). The flow of water from synovial fluid into the intrafibrillar space help transport and distribute nutrients to chondrocytes (5).

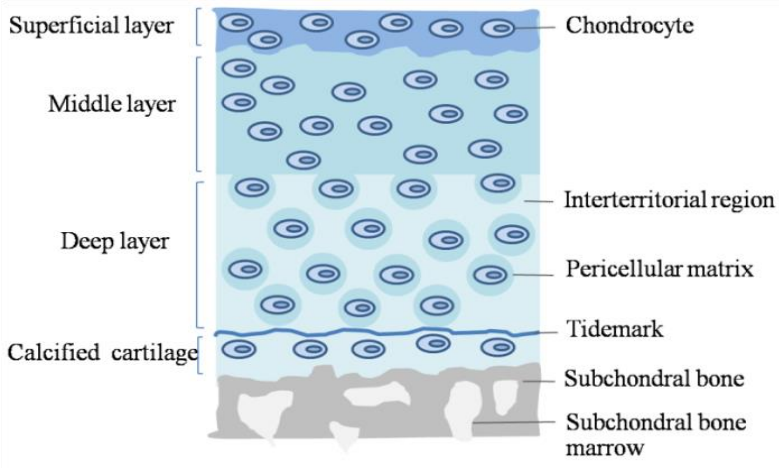


Figure 2 Schematic of articular cartilage matrix region (adapted from Martel-Pelletier I., et al., 2016)

Articular chondrocytes and their biological function

Articular chondrocytes are resident cells located in the matrix cavity of cartilage and occupy about 2% of the cartilage volume in adults (5). Chondrocytes originate from condensation of mesenchymal progenitors and differentiate to chondroprogenitors, which predominantly produce collagen types II, IX and XI (8). The morphology of chondrocytes on cartilage surface is flattened and becomes rounder and larger into the deeper zones (9). Although chondrocytes function to synthesize, maintain and repair ECM; however, they produce different types of ECM components when located in different cartilage layers. Chondrocytes in the superficial layer are multipotent and produce hyaluronic acid and proteoglycan 4 (lubricin) to allow for better movement of the joint (3). Chondrocytes in the middle and deep layers function to produce collagen and proteoglycan aggrecan to maintain cartilage formation and turnover of these ECM is very low (3).

Collagen

Collagen is the major protein found in cartilage and make up about 60% of cartilage matrix (10). Collagen fibril acts as a structural backbone and is mainly composed of type II collagen. During type II collagen synthesis, N-terminal and C-terminal of pro-collagen are removed by proteolytic enzymes and fibrils assembly occurs (10). Also, type II collagen fibrils associate with other components, including type IX, XI, and VI collagen, fibromodulin, decorin, biglycan and cartilage oligomeric matrix protein (COMP) to form collagen network and endoskeleton (11). These collagen scaffold maintain and provide tensile strength of cartilage (12). Under normal conditions, the turnover rate of collagen network is very low and has a long half-life of 117 years or until they are damaged (8).

Proteoglycan aggrecan

Aggrecan is a large aggregate of chondroitin sulfate proteoglycans, the second most abundant component found in cartilage (8). Monomers of aggrecan consist of more than 2,400 amino acids of core protein containing three globular domains: G1, G2 and G3; linked to glycosaminoglycan attachment regions, including keratin sulfate domain (KS) and two chondroitin sulfate domains (CS1 and CS2) on this core protein

(Figure 3) (13). The G1 domain is located on the amino terminus of the core protein. This domain interacts with hyaluronic acid (HA) and this interaction is stabilized by a link protein. Moreover, there is immunoglobulin folding motif in this domain. The protein containing immunoglobulin structures are mostly related to cell recognition or immune reaction (13). The G2 domain contains homologous region with the G1 domain; however, it does not bind to HA (13). The G1 and G2 domains are separated by interglobular domain (IGD) which is a peptide containing proteolytic cleavage sites of many proteinases (13). The G3 domain is located on the carboxy terminus of the core protein and has a predominant function for aggrecan trafficking and secretion from within chondrocytes into ECM (13). Between G2 and G3 domains contains a KS-domain and two CS-domains. The function of these two types of glycosaminoglycan domains is not known, however, they are important in enhancing the load-bearing capability of aggrecans (14). Binding of these glycosaminoglycans provide an increase of negative charges on aggrecan (13, 15). The negatively charged group on glycosaminoglycan carry a lot of mobile counter positively charged ions inducing difference in ion concentration between inside and outside of cartilage (14). This osmotic imbalance causes water to be drawn into the cartilage and induce aggrecan to become swollen and expanded, resulting in increased elasticity of cartilage to resist compressive loads (13, 15). Nonetheless, glycosaminoglycans can mask T cell epitopes of core proteins and prevent T cell stimulation in the immune system (16).

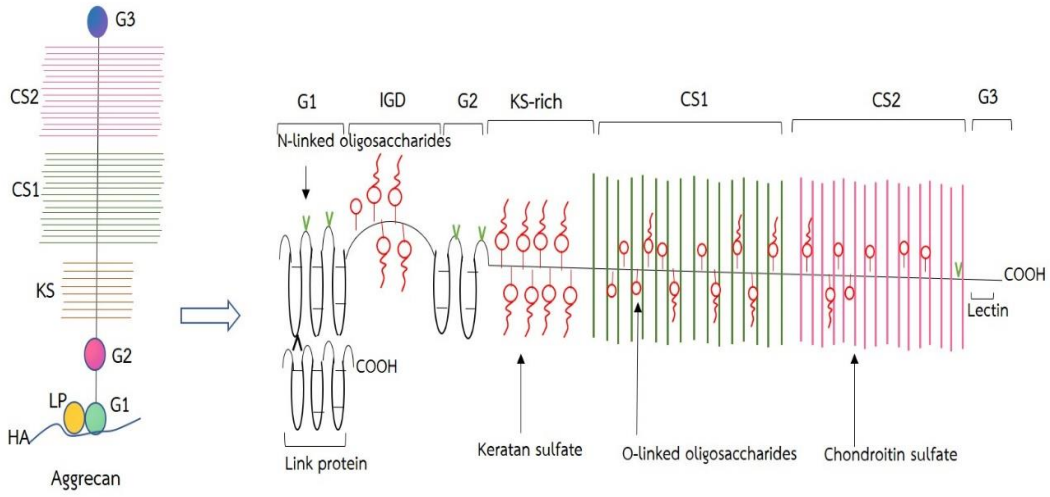


Figure 3 Structure of proteoglycan aggrecan (adapted from Roughley, P.J. and Mort, J.S., 2014)

Turnover of aggrecan (with age)

Unlike type II collagen, the structure of proteoglycan aggrecan structure is not stable throughout life. Proteoglycan aggrecan has a half-life of 25 years (13). The core protein of proteoglycan aggrecan is susceptible to digestion by protease enzymes, including matrix metalloproteases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) (13). These enzymes are zinc-dependent endopeptidase that are produced by chondrocytes and synovial cells (13). In normal cartilage, both MMP and ADAMTS digestion of aggrecan in normal cartilage is controlled by physiological activators including cathepsin B and tissue inhibitors of MMPs (TIMPs) (17). MMPs, especially MMP3, can digest aggrecan in the IGD at various cleavage sites including Asn^{360}-Phe^{361}, Pro^{403}-Ile^{404} and Asp^{460}-Leu^{461}, resulting in two fragments generated (Figure 4) (18-22). One fragment is released into the synovial fluid, while the other one that contains the G1 domain remain bound to HA and could be used for binding of newly synthesized aggrecan (13). In addition to MMPs, aggrecan contains several cleavage sites for ADAMTS, one site located in the IGD (Glu^{373}-Ala^{374}) and four additional sites (Glu^{1564}-Gly^{1565}, Glu^{1733}-Gly^{1734}, Glu^{1838}-Ala^{1839} and Glu^{1938}-Leu^{1939}) located in the CS2 region (Figure 4) (18, 23-25).

Although aggrecans can be newly synthesized by chondrocytes to substitute the digested aggrecan fragments; however, the complete structure of proteoglycan aggrecan is synthesized lower in aging (13). With increasing age, the length of glycosaminoglycan side chains, which render core proteins from proteolytic enzymes become shorter and have changes in sulfonation patterns of CS and KS (13, 26). While the ability of chondrocytes in proteoglycan aggrecan synthesis decreases, the production of proteolytic enzymes by chondrocytes increase with age. The imbalance between aggrecan production and degradation might be induce progressive destruction of cartilage in osteoarthritis (27).

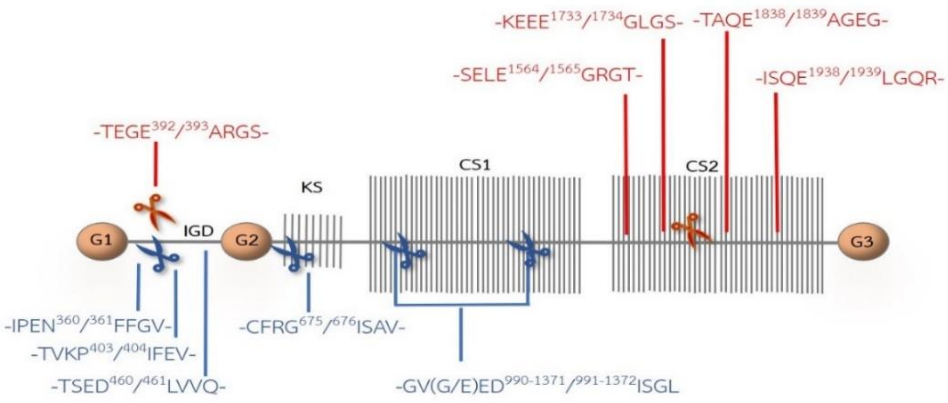


Figure 4 Illustration of digestion point of MMPs (below, blue) and ADAMTS (above, red) on human proteoglycan aggrecan. G1, G2 and G3 are globular domains 1-3, IGD is interglobular domain, KS is keratin sulfate binding region, CS is chondroitin sulfate binding region and arrows represents the cleavage sites of the enzymes (adapted from Struglics, A., et al., 2006)

Osteoarthritis

Osteoarthritis (OA) is the most common type of arthritis found in arthritic patients. It is a multi-factorial disease which is characterized by synovial inflammation and cartilage loss, resulting in joint stiffness, swelling and mobility loss (28). OA can be classified into 2 different forms, including primary OA, which is a genetic-dependent disease, and secondary OA, which is a post-traumatic disease (29). Although primary and secondary OA occur form different factors; however, their pathology and complication of inflammation reactions are similar (29).

Risk factors of OA

Risk factors of the disease can be divided into 2 main groups: predisposition factors and susceptibility factors, as shown in Figure 5 (28). Predisposing factors include age, gender, genetics, obesity and bone metabolism (28, 30). Aging causes muscle mass loss, leading to changes in joint loading and progression to OA. Over 70% of OA patients was aged over 65 years (31). Indeed, chondrocytes cannot replicate throughout their lives because of cell senescence, which increases with age. Senescent chondrocytes lose their ability to repair and maintain ECM (32). Moreover, chondrocytes in the aging population undergo apoptotic death which results in a

decrease in chondrocyte number in the cartilage (33). Women aged over 60 years have symptomatic knee OA more than men (34). Obesity can initiate pathogenesis of OA due to overload of the joint. Overload on weight-bearing joints can activate chondrocytes to degrade cartilage. A reduction of 5 kg of body weight can reduce the risk of OA by 50% (35).

Susceptible factors include daily habitual activities such as exercise and diet (30). Physical activity and movement are predominant factors that induce cartilage destruction in healthy joints. Repetitive movements or wrong movements from occupation or exercise can cause joint injury and lead to secondary OA (36, 37). Not only habitual activities, but diet and nutrition also influence the quality of life. Lack of vitamin C and vitamin K can increase the risk of OA (38-40) . Low levels of vitamin C, which has antioxidant properties, can cause the inability to inhibit reactive oxygen species (ROS) in chondrocytes, leading to collagen destruction in cartilage and viscosity reduction (38, 39). Low levels of vitamin K in plasma increases osteophyte formation and joint space narrowing in the knee (40). All of these different risk factors can cause the development of OA by alteration of biomechanical loading and biochemical factors in the joint (31, 41).

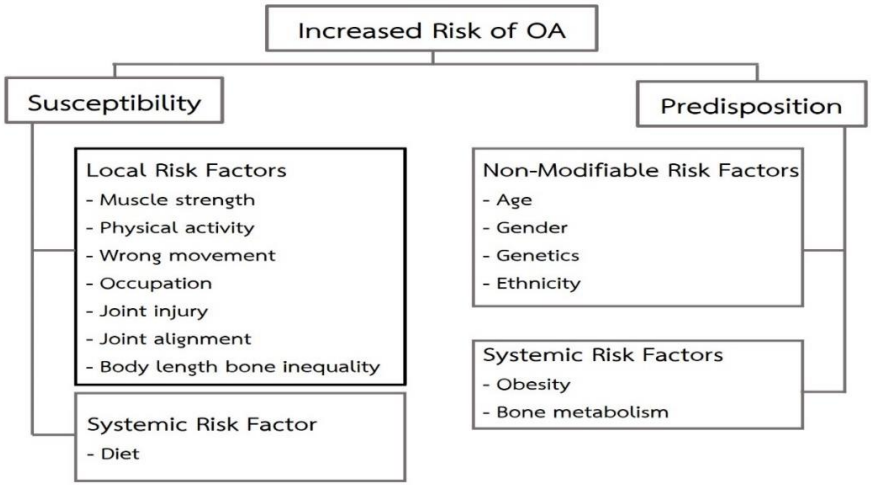


Figure 5 Schematic represents factors on OA pathogenesis (adapted from Musumeci, G., et al., 2015)

Mechanical changes in OA

Joint overload is a major factor that can cause unfavorable biomechanical conditions, including malalignment, loss of meniscal tissue, cartilage lesions, ligament laxity, joint instability and trauma (42). Malalignment causes alteration in load distribution across the tibiofemoral compartment, increasing stress on cartilage and subchondral bone (42). Loss of meniscal tissue, joint instability and ligament laxity shifts the central loading area to a more peripheral location, leading to abnormal overload of the cartilage and additional trauma to the joint (43). This trauma can increase metabolic and oxidative stress of chondrocytes and promote chondrocyte senescence and cartilage damage (42, 43). Lesions on the cartilage are exposed to the subchondral bone, resulting in subchondral bone endplate stiffening and microcracks (43). These biomechanical changes exceed the ability of the joint to maintain and repair itself, resulting in joint destruction and a decrease in bone remodeling (Figure 6) (28, 43, 44).

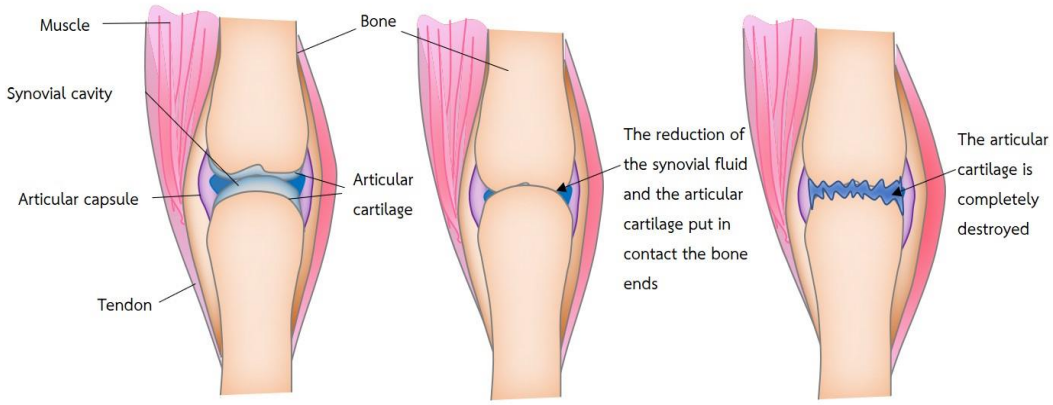


Figure 6 Schematic shows joint alteration during the onset of OA (adapted from Musumeci, G., et al., 2015)

Biochemical changes in OA

In normal cartilage, ECM biosynthesis and degradation are in balance; however, in knee OA, this balance is disturbed and biochemical substances related

to the progression of OA are also altered (45). Cartilage mineralization is one of the causes of OA. Accumulation of mineral in the form of crystal structures, such as calcium pyrophosphate dehydrate (CPPD) and hydroxy apatite, which are caused by an imbalance of phosphate and pyrophosphate level controls was observed in hip and knee OA (46). These calcium crystal accumulation affect cartilage calcification and its prevalence increases with age (46).

Indeed, hypercalcification of cartilage is also caused by hypertrophy of chondrocytes after exposure to several cytokines secreted from adipose tissue (46). Increase in inflammatory cytokines, such as IL-1β, IL-6 and tumor necrosis factor α (TNF-α) can effect on cartilage destruction by promoting chondrocytes to produce cartilage catabolic enzymes (MMPs and ADAMTS) and decrease production of catabolic enzyme inhibitors (47). Also, these cytokines can induce damage of subchondral bone and induce chondrocytes to produce anabolic mediators including transforming growth factor (TGF)-β, insulin-like growth factor I (IGF-I), bone morphogenetic protein (BMPs) and fibroblast growth factors (FGFs) at high levels, leading to osteophyte formation in OA patients (8). Alteration of these biochemical substances may further induce chondrocyte senescence and hypertrophy and stimulate OA-associated cascades, resulting in further cartilage catabolism and increased degradation (Figure 7) (48).

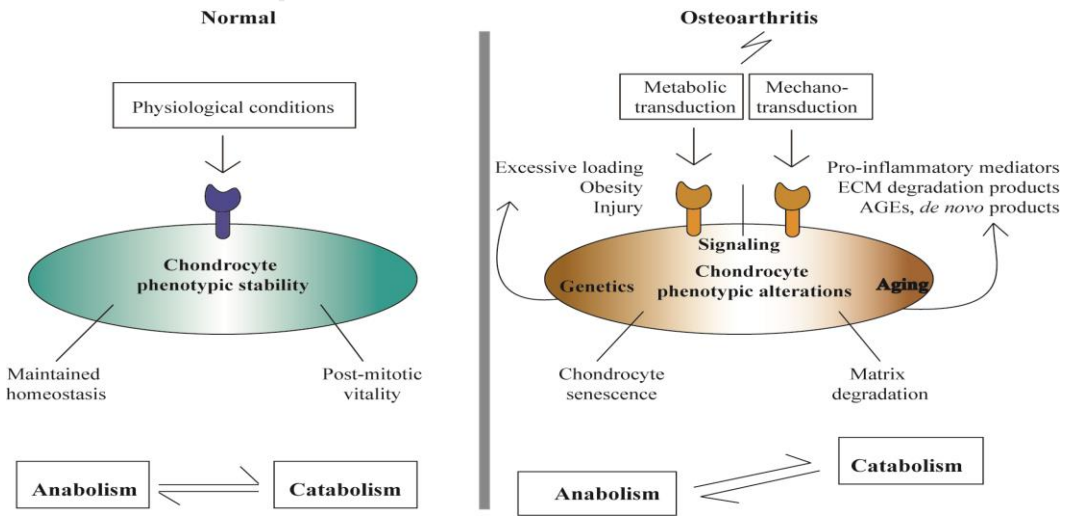


Figure 7 Cartilage homeostasis in normal (left) and pathological OA (right) conditions (adapted from Cucchiarini M., et al., 2016)

Biomarkers in OA

According to the biochemical changes in chondrocytes, several biological processes generate molecules that are secreted into the synovial fluid that can be used as an indicator to estimate disease progression. One biomarker that is widely detected to identify the disease level is protease enzymes in synovial fluid or peripheral blood of patients. Synovial fluid MMP1 and MMP3 levels are elevated after knee injury and are used to predict joint space narrowing in knee OA (17).

Another type of biomarker is cartilage matrix molecules released into the synovial fluid (48). Among the collagen degradation markers, CTX-II (a collagen type II-degrading molecule) and COMP (cartilage oligomeric matrix protein) are present in synovial fluid and serum at high levels in early OA (45). Increasing levels of these molecules correlates with a higher severity of knee OA on radio imaging (45). Moreover, high concentrations of type II procollagen, which indicates collagen synthesis, is found in synovial fluid and correlates with early stage of OA (49).

Because proteoglycan aggrecans have the half-life shorter than collagen, therefore aggrecan fragments generated from aggrecan turnover in OA cartilages are also proposed as one of OA biomarker (26). After proteolysis by proteases, several neoepitopes are generated and secreted into the synovial fluid (Figure 8). In pericellular matrix, MMPs cleave IGD on proteoglycan aggrecan and neo-epitope FFGV-G2-G3 fragments and HA bound G1 fragments are generated. The HA bound G1 fragments on chondrocyte surface are endocytosed into the cells, while the FFGV-G2-G3 fragments are further digested by ADAMTS and their digested fragments secreted into the synovial fluid (18). For interterritorial matrix, turnover of proteoglycan aggrecan starts by ADAMTS cleavage at CS2 region and followed by digestion in IGD domain, resulting in generation of neoepitope G1-TEGE fragments and ARGS-G2 fragments. These fragments are also released into the synovial fluid

(18). In OA, these neoepitopes can be used as biomarkers indicating the early state of the disease (50).

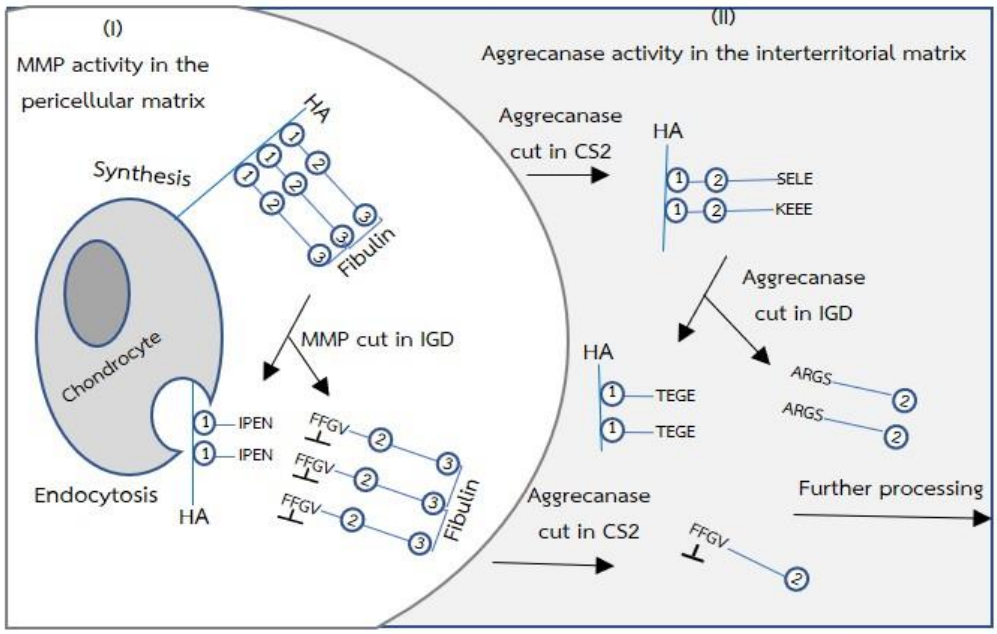


Figure 8 Illustration of proteolysis of proteoglycan aggrecan in pericellular matrix and interterritorial matrix of OA cartilages (adapted from Struglics, A., et al., 2006).

Immunopathogenesis of knee OA

Inflammation

In knee OA, cartilage breakdown is a predominant characteristic that can attribute to inflammation within the knee joint (51). Synovitis, inflammation of the synovium, is caused by contraction of degraded cartilage fragments into the synovium (31). Damaged-associated molecular patterns (DAMPs) can stimulate synovium tissue-resident cells, including synoviocytes, fibroblasts, and synovial macrophages, to produce inflammatory mediators via Toll-like receptor (TLR) engagement (52). Inflammatory mediators found in OA synovium include nitric oxide synthase (NOS), prostaglandin and cytokines (IL-1, IL-6, IL-18 and TNF-α) (52, 53). Synoviocytes and synovial macrophages also secrete several chemokines including

CXCL-1, CXCL-8, CCL3, CCL5 and CCL2 into the joint space (51). These mediators activate articular chondrocytes to synthesize MMPs and ADAMTs, resulting in further cartilage degradation (54).

Innate immunity

The innate immune response is known to be activated in knee OA. Chemokines, particularly CCL2 and CCL5, recruit innate immune cells, such as natural killer (NK) cells, mast cells and macrophages into the synovium (55). Macrophages are the most abundant immune cell infiltrating in inflamed synovium that produce IL1β and TNF-α. These cytokines increase MMP9 and MMP13 production in the cartilage, promoting cartilage breakdown and facilitating progression of OA (56). Mast cells are also present in OA synovial tissue and a high number of these cells correlates with greater degree of synovitis (54). Neutrophils are also found in synovial tissue and produce IL-1 and IL-8, increasing MMP8 production and cartilage breakdown (57). However, activated dendritic cells and natural killer (NK) cells are present in synovial tissue at low levels (51). In addition, studies have shown that synovial membrane and synovial fluid from OA patients have high levels of the complement proteins C3 and C5 convertases, which promote membrane attack complex (MAC) and pore formation on chondrocytes to lyse them (51, 54).

Adaptive immunity

Studies have also shown the presence of B cells and T cells within the synovium, suggesting a role for the adaptive immune response in knee OA. Over 50% of infiltrated cells in the synovial tissue are primarily CD3+ T cells and express early activation marker (CD69), intermediate activation markers (CD25 and CD38) and late activation marker (CD45RO) on their surface, indicating their activation within the synovium (58). In synovial tissue and infrapatellar fat pads of OA patients there were higher percentages of CD4+ T cells than CD8+ T cells (59). However, the percentage of peripheral blood activated CD8+ T cells was higher than CD4+ T cells (51, 60). Effector T cells, Th1 and Th2 T cells could be found in synovial fluid and in the sublining layer of synovial membranes (61). There were five times as higher of Th1 T

cells than the Th2 subset (60). In addition, CD4+CD25+Foxp3+ regulatory T cells (Tregs) are also increased in synovial fluid and peripheral blood of OA patients. These Tregs produced low levels of IL-10 due to lack of cell surface expression of the T cell inhibitory receptor (60).

Even though, there is no obvious study describing antigen-driven immune response in OA, however, several candidate proteins have been postulated. There is evidence of the possible immune response to human cartilage glycoprotein-39 (HC gp-39) (62). The HC gp-39 at positions 263-275 peptide induced peripheral blood T cells from OA patients to have a higher proliferative index when compared with proliferation of normal controls (62). T cell responses to type II collagen was also observed in blood and synovial fluid of OA patients (58). Type II collagen peptide (p255-274) stimulation increased T cell proliferation in OA blood and synovial fluid. Also, anti-type II collagen IgG antibodies were also present in OA serum at high levels when compared to healthy controls (63). Another cartilage component that triggers immune responses in OA is proteoglycan (PG) aggrecan. Its turnover of PG can increase proteolytic process and release PG fragments into the joint (64). The aggrecan core protein 2 is commonly found in synovial fluid of OA patients, but not observed in rheumatoid arthritis (RA) patients (65). In addition, G1 domain located on aggrecan core protein contains several T cell epitopes and can stimulate peripheral blood T cells of both OA and RA patients to have a high proliferative (64). However, only PG regions at positions 16-31 (P16-31) and 263-280 (P263-280) can be recognized by T cells in peripheral blood of OA patients (16). The p263-280 PG peptide also stimulates T cell to produce IFN-γ and TNF-α (16). These studies suggest cartilage proteins acting as T cell epitopes recognized by T cells in individuals with OA (16).

Specific antigen-driven stimulation also results in clonal expansion of T cell clones that recognize the antigen. Stimulation of blood T cell with collagen type II generates four identical variable region of beta chain (BV) of T cell clones; BV13S1, BV12, BV1 and BV16; which are found in synovial T cell of RA patients. These T cell clones may recognize collagen type II in synovium (63). In OA, there were shared usage of T cell clones; BV14, BV15, BV27 and BV29, in infrapatellar fat pad from 3

patients, but these T cell clones could not be observed in peripheral blood. This study suggests that local stimuli in the joint may drive T cell recruitment and proliferation in OA (58, 66). Although these data indicate the ability of T cells to respond to specific antigens; however, recognition of specific antigens by T cells need to be presented on major histocompatibility complex (MHC) on antigen presenting cells (67).

Antigen presentation

As described above, there are T cells that responds to cartilage peptides found in blood of OA patients. These antigen recognition T cells relies on specific epitope sequence of unfolded proteins, therefore, antigens are necessarily processed and presented by antigen presenting cells (68). Although macrophages and dendritic cells infiltrate into synovium (56), but their role in OA pathogenesis has not been obviously identified. In addition to these professional antigen presenting cells, it is clear that cells that express MHC class I and II as well as co-stimulatory molecules on their surface can present antigen to T cells (68). Chondrocytes in OA joint have been reported to be able to present antigens (67, 69). Immunohistochemistry data show high expression levels of MHC class I and class II on chondrocytes of OA patients (70).

Antigen presentation via MHC-class I (endogenous antigens)

Although MHC class I and class II have the same function to present antigen to T cells; however, they recognize different pattern of antigens and stimulate different type of T cells (68). Target antigens that are processed and presented on MHC class I have been reported as intracellular or endogenous antigens, e.g. viral infection or intracellular bacteria transformation (71). The mechanism starts with digestion of endogenous antigens by protease enzymes in the proteasome. After protein digestion, digested peptides with a size of 4-20 amino acids are released and transported to endoplasmic reticulum (ER) by transporter for antigen processing (TAP) protein (72). In the ER, digested peptides with the size of 8-11 amino acid residues are folded and loaded on to MHC class I molecule by help of a chaperon, tapasin

(73). Antigen-MHC class I complexes are transported to the plasma membrane and the peptide loaded on MHC class I is presented to CD8+ T cell (cytotoxic T cells) (Figure 9) (72).

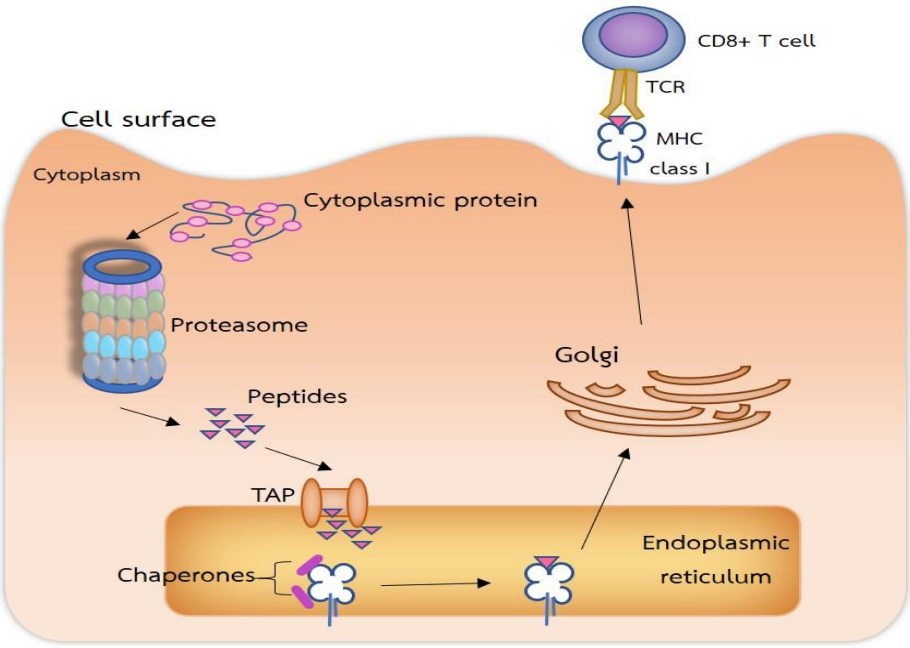


Figure 9 Schematic of antigen processing and presentation by MHC class I molecule on antigen presenting cells (adapt from Yewdell, J. W., et al., 2003)

Antigen presentation via MHC-class II (exogenous antigens)

MHC class II molecules are expressed by professional antigen presenting cells including dendritic cells, macrophages and B cells, but not observed on other cells (71). MHC class II binds peptides that are processed from extracellular proteins and present them to CD4+ T cells. The extracellular antigens are taken up into the cell by phagocytosis and form phagolysosomes by fusing phagosomes with lysosomes, which contain MHC class II (71). Due to the acidic pH of phagolysosome, several lytic enzymes are activated, resulting in digestion of antigens into small peptides with sizes of 13-25 amino acids (74). Protease enzyme such as cathepsin S and L are also activated and can cleave the invariant chain (Ii), which helps for stabilizing MHC class II molecules. However, a residue class II-associated Ii peptide (CLIP) is still bound to MHC class II molecule and is exchanged for the antigen peptide. The antigen

peptide-loaded MHC class II is transported to the cell membrane and presents antigen to CD4+ T cells (Figure 10) (74).

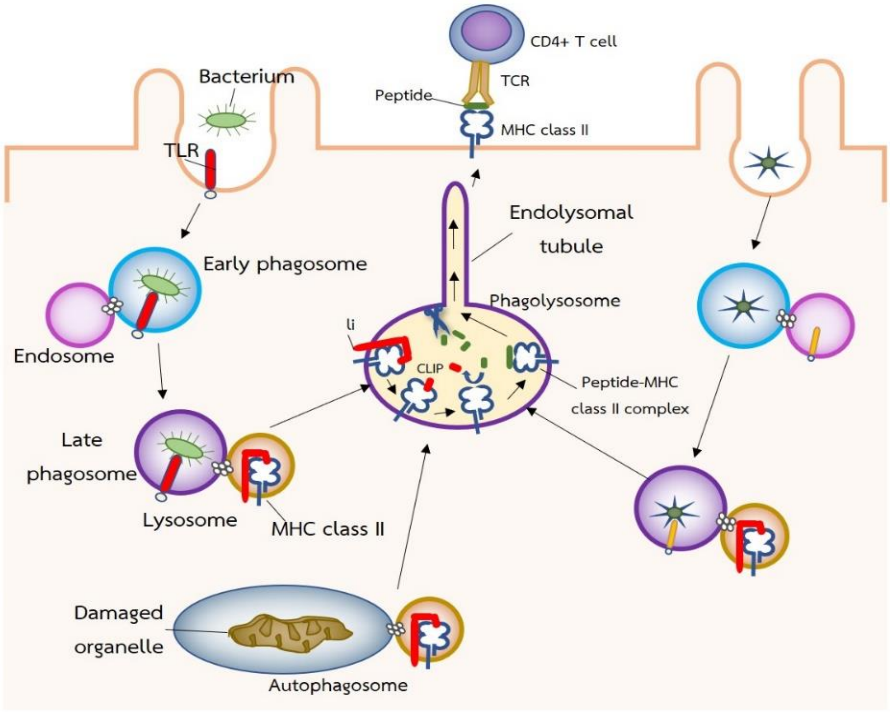


Figure 10 Schematic of antigen processing and presentation by MHC class II molecule on antigen presenting cells (adapt from Vyas, J. M., et al., 2008)

IFN-γ increase expression level of MHC class II molecule

Although MHC class II molecules are not expressed on other cells apart from dendritic cells, macrophages and B cells; however, IFN-γ stimulation can induce expression of MHC class II on non-professional APCs, including chondrocytes. Treatment of IFN-γ can increase expression level of MHC class II molecule via the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway as shown in Figure 11 (75). Binding of IFN-γ on its receptor results in self-phosphorylation on JAK proteins, followed by phosphorylation of the transcription factor, STAT1 (76). The heterodimerized STAT1 protein is translocated into the nucleus and binds on a promotor element, which controls expression of class II, major histocompatibility complex, transactivator (CIITA) gene, resulting in MHC class II

expression (76). This induction could be observed in mesenchymal stem cells, endothelial cells and epithelial cells (77-79)

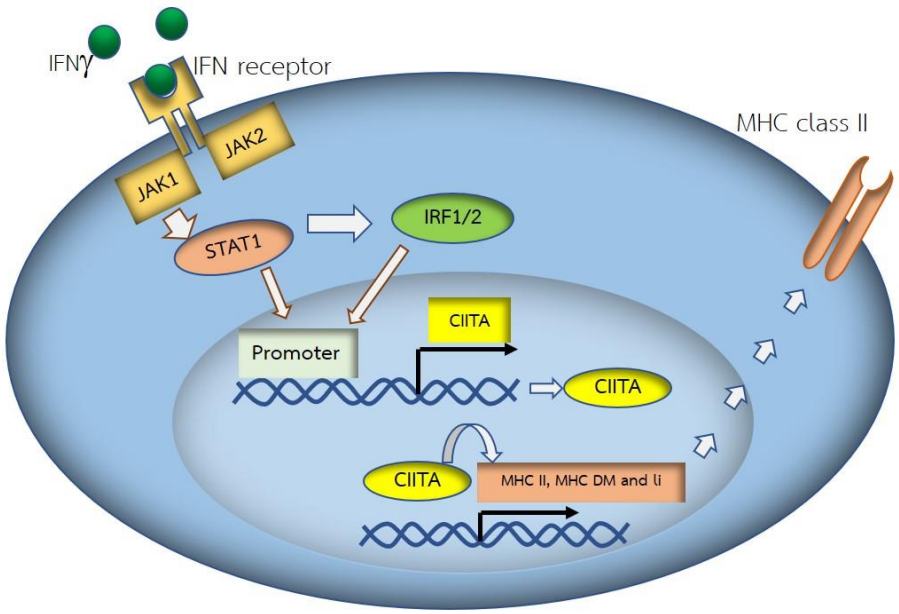


Figure 11 schematic of IFN-γ treatment induce expression of MHC class II molecules via JAK/STAT pathway (adapt from Ting, J.P.Y. and Trowsdale, J.J.C., 2002)

Role of chondrocytes in immunopathogenesis of OA

In normal cartilage, chondrocytes are surrounded with cartilage matrix that can protect chondrocytes from the immune cells, however, chondrocytes exposed to surrounding stimuli or other immune cells after trauma or cartilage broken can lead to immune responses to chondrocytes (80). After chondrocyte exposure to inflammatory mediators (51) e.g. IL-1β, TNF-α and IL-6; they can produce more proteolytic enzymes MMP-1, MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 and increase cartilage degradation (51, 58). Moreover, chondrocytes from OA patients have overexpression of the IL-1 receptor (IL-1R). Binding of IL-1β to its receptor increases reactive oxygen species (ROS) and nitric oxide synthase (NOS) production in chondrocytes, inducing cell apoptosis (51). In addition, IL-1β can increase expression of chemokines MIP-3, MIP-1β, IL-8 and MCP-1 on chondrocytes to recruit NK cells, neutrophils and macrophages into the joint (81). Furthermore, chondrocytes from OA patients express high levels of intracellular adhesion molecule-1 (ICAM-1), which is a

ligand for leukocyte associated antigen-1 (LFA-1) that is present on lymphocytes. ICAM-1 helps chondrocytes to interact with T cells and increase proliferation and cytotoxic function of T cells (82). In the lesion area of OA cartilage, chondrocytes show high levels of TLR2 and TLR4 expressions on their surface (83). These two receptors bind to DAMPs derived from ECM proteins in the cartilage, such as fibronectin and hyaluronan. The receptors are activated and this enhances inflammatory cytokine production, MMP expression, NO release and prostaglandin E_2 synthesis by chondrocytes (83, 84).

Antigen presentation function of chondrocytes

Chondrocytes have been reported to act as antigen presenting cells (80). Actually, expression of MHC class I molecules (HLA-A, HLA-B and HLA-C) could be observed on surface of every nucleated cells (85). However, IFN-γ can induce MHC class I expression (75). In ankylosing spondylitis (AS), IFN-γ treatment increased expression level of HLA-B27 on chondrocyte surface and this molecule is essential for presentation of the Epstein-Barr virus-derived peptide ($EBNA_{258-266}$) to CD8+ T cells. This activation increased IFN-γ, perforin and granzyme B production in CD8+ T cells (86). Also, cytotoxic T cell in AS mice recognized antigen peptides of influenza A virus nucleoprotein (NP) through MHC class I expression on chondrocytes. Recognition of specific cytotoxic T cells to NP-pulsed chondrocytes potentially increases chondrocyte lysis (87).

Not only MHC class I molecule, chondrocytes also present antigen via MHC class II. Antigen presentation function of chondrocyte via MHC class II is supported by the evidence that reported about phagocytosis ability of chondrocytes to phagocytosed collagen fragment and processed as exogenous antigen (67). Moreover, there are many studies reporting about expression of MHC class II molecules on chondrocyte surfaces (69, 87, 88). Expression of MHC class II molecules could be found on surface of normal rabbit and rat chondrocytes, while expression of these molecules could not be observed on healthy or unstimulated human chondrocytes (80). Similarly to MHC class I, expression of MHC class II molecules could be induced

by IFN-γ as well (75). Nasal chondrocytes treated with IFN-γ up-regulated their MHC class II on their surface and this expression increased the ability of chondrocytes to present tetanus toxoid antigen to autologous reactive T cells, resulting in a high proliferative rate of T cells (88). In contrast, T cells co-cultured with MHC class II negative chondrocytes showed low proliferation (88). Likewise, chondrocytes isolated from OA patients expressed MHC class II (HLA-DR, DP and DQ) at high levels on their cell surface and these expressions were able to stimulate autologous peripheral blood T cells from OA patients resulting in a higher proliferative index when compared to T cells from trauma patients (70). However, blocking of the MHC molecule reduces this proliferative response (70). This study suggests that chondrocytes activate autologous T cell in an MHC dependent manner (70). Additionally, expressions of co-stimulatory molecules, CD80 and CD86, are also observed on chondrocyte surface from OA patients, but not for healthy individual (70). These co-stimulatory molecules can bind to CD28 on T cells and this binding is necessary for T cell stimulation (67). However, the mechanism that induces co-stimulatory molecule expression in chondrocytes have not been clearly studied yet.

Taken this altogether, it could be suggested that chondrocytes can act as antigen presenting cells when they are under influence of inflammatory condition or exposed to stimuli. However, in OA, the antigen or factors that induce antigen presentation function of chondrocyte are still unclear.

RATIONALE

Chondrocytes are cartilage resident cells that play a predominant role in cartilage tissue biosynthesis and degeneration. We hypothesized that proteoglycan aggrecan produced from cartilage tissue may act as specific antigens to T cells and are presented to T cells by chondrocytes. Based on a previous study by de Jong *et al.*, proteoglycan aggrecan-specific T cells to P16-31 and P263-280 peptides were found in peripheral blood of OA patients. However, proteoglycan aggrecan are produced in the cytosol of chondrocytes and secreted to the extracellular matrix aggregating to hyaluronic acid (HA) on the cell surface. It is possible that chondrocytes may present these "self-synthesizing" fragments of proteoglycan aggrecan to T cells and in turn, specific T cells target chondrocytes by producing cytokines and cytotoxic molecules. Therefore, this study aims to look at the ability of chondrocytes to present proteoglycan aggrecan peptides to T cells and investigate the changes in chondrocytes after stimulation with proteoglycan aggrecan peptides.

RESEARCH QUESTIONS

Research questions of this study are:

1. What are the immunological changes in chondrocyte after stimulating with proteoglycan aggrecan peptides?
2. How do chondrocytes respond to proteoglycan aggrecan peptides when compared to other professional antigen presenting cells?
3. How do proteoglycan aggrecan-stimulated chondrocytes effect T cell function?

HYPOTHESIS

We hypothesized that chondrocytes stimulated with proteoglycan aggrecan peptides may have unique properties that allow for antigen presentation to T cells.

OBJECTIVES

The objectives of this study are:

1. To determine the effect of proteoglycan aggrecan peptides on antigen presentation markers expression and cytokine production in chondrocytes.
2. To determine gene expression pattern in chondrocytes in response to proteoglycan aggrecan peptides.
3. To investigate the ability of chondrocytes to present proteoglycan aggrecan peptides to autologous T cells from infrapatellar fat pads of OA patients and determine their T cell proliferation.

CHAPTER II

METHODOLOGY

Sample collection

Human articular cartilage and infrapatellar fat pad were harvested from knee joints of OA patients (ages 50-80 years) undergoing total or partial knee replacement at King Chulalongkorn Memorial Hospital. Blood samples (10 ml) were also taken in EDTA blood tube from OA patients and healthy donors from blood blank of The Thai Red Cross Society. Samples were obtained after informed consent and protocols were performed with approval from Institutional Ethics Committee of Chulalongkorn University (IRB 497/60).

Human articular chondrocyte isolation

Articular cartilages from OA patient were washed with phosphate buffer saline (PBS, Vivantis) for 15 minutes to remove red blood cells. Cartilages were cut into 2-3 mm^2 pieces and incubated with 1% (w/v) pronase (Sigma-Aldrich) in 10 ml of DMEM complete medium (contains 10% FBS, 100 µg/ml penicillin and streptomycin and 1% non-essential amino acids) (Gibco) at 37°C for 1 hour. Small pieces of cartilage were washed twice with 10 ml of complete medium and subsequently digested with 0.2% (w/v) type II collagenase (Worthington) in medium at 37°C for 16 hours. Isolated cells were filtered through a 40 µm filter to remove digested fragments. After washing twice with medium, cells were resuspended in DMEM complete medium and cultured at 37 °C, 5% CO_2. Experiments were started on day 4 after primary cells were completely attached on the cell culture plate.

Mononuclear cell isolation

Mononuclear cells were isolated from infrapatellar fat pads (IPFP) or blood of OA patients. After IPFP collection, IPFP tissue was washed twice by 15-20 ml of PBS, minced into 2-3 mm^3 pieces and digested with 3 µg/ml type IV collagenase (Worthington), 0.1 µg/ml DNase I (Worthington) and 5% FBS in 50 ml PBS by shaking at 200 rpm at 37 °C for 1-2 h. After incubation, supernatant was collected and

strained through a 40 μm nylon cell strainer to get rid of tissue debris. Cells were washed twice with 10 ml of RPMI 1640 (Gibco, Life Technologies) containing 10% FBS and collected by centrifugation at 1,500 rpm at 4°C for 5 minutes. Mononuclear cells were cryopreserved in freezing media (10% DMSO in FBS) for further experiments.

For blood samples, 10 ml of whole blood was diluted with 10 ml of RPMI 1640 containing 10% FBS and layered on to 10 ml Ficoll-Paque (ratio of media:blood:Ficoll-Paque is 1:1:1) and centrifuged at 1,500 rpm for 25 minutes at room temperature without deceleration force. PBMCs were collected and transferred into a new tube containing 10 ml of RPMI 1640 and centrifuged at 1,500 rpm at 4 °C for 5 minutes to wash the cells. PBMCs were cryopreserved in freezing media (10% DMSO in FBS) for further experiments.

Human monocyte derived dendritic cell (MoDC) culture

Isolated PBMCs from healthy donors were seeded onto 24 well culture plates at $5x10^6$ cells/well in 1 ml of IMDM supplemented with L-glutamine, 10% FBS and 100 U/ml Pen-Strep. Culture plate was incubated at 37°C, 5% CO_2 for 3 hours to allowing monocytes to completely attach to the plate. After incubation, supernatant was discarded to remove suspending cells. Monocytes were washed twice with 1 ml of PBS and 1 ml of dendritic cell (DC) condition medium containing 24 ng/ml rhGM-CSF and 25 ng/ml rhIL-4 (RPMI 1640 without L glutamine, 2mM glutamax, 100 U/ml Pen-Strep, 1 mM sodium pyruvate, 1X non-essential amino acid and 10% FBS) was added to each well. Monocytes were cultured at 37 °C, 5% CO_2 for 7 days and half of the medium was replaced by fresh condition medium containing 24 ng/ml rhGM-CSF and 25 ng/ml rhIL-4 every 2 days to induce dendritic cell differentiation (89). On day 7, half of the medium was removed and condition medium without supplemented cytokines was added and incubated for another 2 hours at 37 °C to rest the cells. DC maturation was induced by addition of four cytokines; IL-6 (50 ng/ml), IL-1β (10 ng/ml), TNF-α (20 ng/ml) and PGE2 (1 ug/ml), into the condition medium and cultured for 24 hours. For the control group, IFN-γ (50 ng/ml) was also added to induce DC maturation.

Peptide stimulation assay

Primary chondrocytes, mature DCs and primary keratinocytes (NHEK; ATCC® PCS-200-010™) were cultured in 48 well plates (5 x 10^4 cells/well) for 48 hours until cells were completely attached to the plate. Cells were treated with 10 µg/ml of synthetic proteoglycan aggrecan (PG) peptides; P16-31, P263-280 or P2379-2394 (Table 1) (16), with or without 50 ng/ml IFN-γ and cultured for 48 hours.

Table 1 Proteoglycan aggrecan (PG), ovalbumin and CEF peptide sequences

Peptide region	Amino acid sequences
P16-31	QPSPLRVLLGTSLTIP
P263-280	TTGHVYLAWQAGMDMCSA
P2379-2394	LQKRSSRHPRRSRPST
OVA 323-339	ISQAVHAAHAEINEAGR
CEF	GILGFVFTL (Influenza A)
	FMYSDFHFI (Influenza A)
	CLGGLLTMV (EBV)
	GLCTLVAML (EBV)
	NLVPMVATV (HCMV)

Determination of type II collagen expression via immunofluorescence microscopy

Isolated primary chondrocytes were cultured in 6 well plates at 1 x 10^5 cells/well at 37 °C. Attached chondrocytes were washed twice with 500 ml PBS and fixed with 4% formaldehyde for 10 minutes at 37 °C. Afterwards, cells were permeabilized with 500 ml of permeabilization buffer (PBS containing 0.1% Triton X-100) for 15 minutes. After washing twice, cells were treated with 500 ml of 2% BSA in PBS for 1 hour at room temperature. Then 1 µg/ml of purified mouse anti-human type II collagen antibody (Abcam) were added for 3 hours. After washing twice, goat anti-mouse IgG-Alexa Fluor 488 (Abcam) was added subsequently for 1 hour. Nuclei of cells were labeled with 4',6-diamidino-2-phenylindole (DAPI) for 1 hour. Cells were washed twice with 500 ml of PBS and imaging was visualized using an inverted

fluorescence microscope (Olympus) and analyzed with Soft Imaging System software (Olympus).

Determination of type II collagen expression via flow cytometer

Cells were fixed with 4% formaldehyde and incubated at 4°C for 1 hour before labeling with 1 µg/ml of purified mouse anti-human type II collagen antibody (Abcam) in PBS containing 5% FBS and 0.3% saponin for 1 hour. After washing with PBS containing 5% FBS, cells were incubated with goat anti-mouse IgG-Alexa Fluor 488 (Abcam) for 30 minutes at 4 °C. Chondrocytes were washed and acquired using the LSRII flow cytometer and analyzed using FlowJo software (Tree Star).

Determination of cell surface expression of antigen presentation markers

Chondrocytes, mature DCs and primary keratinocytes were cultured at $5x10^4$ cells in 48 well plates and then detached by flushing with cold PBS. After washing twice with PBS containing 5% FBS, cells were labeled with anti-human HLA-A, -B, -C-PE/Cy5, anti-human HLA-DR-AF700, anti-human CD40-PE, anti-human CD80-FITC and anti-human CD86-APC for 20 minutes at 4 °C in the dark. Cells were washed twice with PBS containing 5% FBS and acquired on the LSRII flow cytometer and analyzed using FlowJo software (Tree Star).

Cytokine production measurement by ELISA

After primary chondrocytes, mature DCs and primary keratinocytes (NHEK; ATCC® PCS-200-010™) were stimulated with 10 µg/ml PG peptides with or without 50 ng/ml of IFN-α for 48 hours, culture media was collected and ELISA was performed according to the manufacturer's instruction of IL-6, IL-8 and TNF-α ELISA kit (Invitrogen). Briefly, 50 µl of capture antibody against IL-6, IL-8 or TNF-α was coated on 96-well half-area plates (Corning) overnight at 4°C. After plate coating, the plate was washed 3 times with 200 µl of PBS containing 0.05% Tween-20. Then, 100 µl blocking buffer (PBS containing 2% BSA) was added and incubated at room temperature for 1 hour. Thawed supernatant was diluted with diluent buffer and 50 µl of the samples was added into each well. After two hours of incubation, samples were removed and the plate was washed 3 times with PBS containing 0.05% Tween-

20. Then, 50 µl of biotinylated antibody against IL-6, IL-8 or TNF-α was added and incubated for another 1 hour at room temperature. Plate was washed and streptravidin conjugated horseradish peroxidase (HRP) was subsequently added and incubated for 30 minutes. After washing the plate 5 times and blotting onto absorbent paper, 3,3',5',5'-Tetramethylbenzidine (TMB) substrate solution was then added into each well (50 µl/well) and incubated for 15 minutes. Then, 25 µl of 2 N H_2SO_4 was added to terminate the reaction. Plate was analyzed using multifunctional Microplate Reader (Vario Skan Flash, USA). Cytokine concentration was calculated by subtraction of $OD_{450\ nm}$ - $OD_{570\ nm}$. Standard curve of known concentration was generated.

RNA extraction

Chondrocytes stimulated with 50 ng/ml IFN-γ and 100 µg/ml P16-31, P263-280 or P2379-2394 for 6 hours were collected and 1 ml of TRIzol (Invitrogen, Carlsbad, CA) was added into the culture plate to lyse the cells (1 x 10^6 cells/well). Then, 100 ml of phase separation reagent, 1-bromo-3-chloropropane (BCP) (Sigma-Aldrich), was added into the tube containing TRIzol and tubes were vortexed. RNA phase was separated by centrifugation at 13,000 rpm for 15 minutes. RNA in aqueous phase was collected and precipitated by adding 500 µl of cold isopropanol at -20 °C for 2 hours. After centrifugation at 13,000 rpm, RNA pellet was collected and washed twice with 500 µl of 70% ethanol. RNA was preserved in 1 ml of absolute ethanol and stored at -80°C for further RNA microarray experiments. RNA quality and concentration were assessed by agarose electrophoresis and nanodrop spectrophotometer, respectively. In this experiment, RNA was also isolated from unstimulated chondrocytes and IFN-γ-treated chondrocytes as controls. In total, 15 extracted RNA samples (triplicate of unstimulated, IFN-γ-only, IFN-γ-P126-131, IFN-γ-P263-280 and IFN-γ-P2379-2394 treated chondrocytes) were sent to performed RNA microarray.

RNA microarray

RNA microarray was performed with Macrogen. Firstly, RNA integrity was measured by using an Agilent 2100 Bio-analyzer. QC-passed RNA samples were

converted to complementary RNA and labeled with cyanine 3 or 5 (Cy3 or Cy5) fluorescent dyes using Agilent's Quick Amp Labeling Kit (Agilent Technologies). Labeled cRNA was hybridized to SurePrint G3 Human Gene Expression 8x60K v3 Microarray (Agilent, Inc., Santa Clara, CA) and scanned using the Agilent SureScan Microarray Scanner. Raw data of array intensity was generated using Agilent Feature Extraction v11.0. Differential gene expression and hierarchical cluster analysis were calculated using R 3.5.1 software. Gene ontology annotation and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway pathways were created using Enrichr tool (90, 91) and expression levels of interested genes were chosen to validate the microarray results via quantitative-real time PCR.

Quantitative real time PCR

After RNA extraction, 500 ng of total RNA was converted to complementary DNA (cDNA) using SuperScript VILO cDNA Synthesis kit (Invitrogen). The reaction mix was incubated in a thermal cycler at 25°C for 10 minutes following 42°C for 60 minutes and 85°C for 5 minutes. cDNA (1 µg) was used as a template to amplify target genes. Template cDNA was added to SYBR Green PCR master mix (Applied biosystem) containing 10 µM forward and reverse primers (Genscript) (Table 2) for each gene. The samples were incubated at 95°C for 10 minutes, followed by 40 cycles of incubation at 95°C for 15 seconds and 60°C for 1 minute using Applied Biosystems 7500 Real-Time PCR System. In this study, the expression of glyceraldehyde-3-phosphat dehydrogenase (GAPDH) was used as an internal control. The expression level of target genes was standardized by the expression level of GAPDH and relative fold change of gene expression was calculated using $2^{-\Delta\Delta CT}$ equation.

Table 2 Oligonucleotide sequences of forward and reverse primers for RT-PCR

Genes	Primer sequences (5'-3')	
	Forward primer	Reverse primer
GAPDH	ACGAATTTGGCTACAGCAACAGGG	TCTACATGGCAACTGTGAGGAGG
hIL-6	ATGCAATAACCACCCCTGAC	AAAGCTGCGCAGAATGAGAT
NFkB1	AATGGTGGAGTCTGGGAAGG	TCTGACGTTTCCTCTGCACT
NFkB2	TAGCCACAGAGATGGAGGAG	CCGAGTCGCTATCAGAGGTA
RELA	GCACAGATACCACCAAGACC	TCAGCCTCATAGAAGCCATC
RELB	GGACATATTCAGCCTTGGCG	CTTCAAGTCTGGGGAGGGAG
JUN	TTTCAGGAGGCTGGAGGAAG	CTGCCACCAATTCCTGCTTT
MAP3K8	AAGAGGCTGCTGAGTAGGAAG	CGTTGCCTCTTGAGCATCTCA
TAP1	TGTCTTAGTGCTACAGGGGC	ATACCTGTGGCTCTTGTCCC
MYD88	CCAGCATTGAGGAGGATTGC	ATGAAGGCATCGAAACGCTC
TLR2	CTATGAATCAAGGCGGCCAC	AAAGATCCTGAGCTGCCCTT
CCL2	GCAGCAAGTGTCCCAAAGAAG	CTGGGGAAAGCTAGGGGAAA
COL2A1	AGCCTGGTGATGATGGTGAAG	ACTCTCACCCTTCACACCAG

Expansion of T cells from infrapatellar fat pad

Mononuclear cells isolated from infrapatellar fat pads (IPFPs) of OA patients were cultured in 96-U bottom plates and anti-CD3/CD28 dynabeads (Life technology) were added to each well. After 24 hours of stimulation, half-volume of culture medium was discarded and replaced with new RF10 medium containing 20 ng/ml IL-2. Cells were cultured for 7 days and the medium was changed every 48 hours. On day 8, anti-CD3/CD28 dynabeads were removed using magnetic separation. The proliferated cells were washed twice, resuspended in medium without IL-2 and cultured at 37 ºC for 48 hours to rest the cells.

CFSE labeling of IPFP expanded T cells.

Expanded T cells from IPFPs were labeled with 5 mM of carboxyfluorescein succinimidyl ester (CFSE, Biolegend) for 15 minutes at 37ºC in the dark. Cells were washed twice with RF10 medium. The labeled cells were count using hemacytometer and transferred into 96-U bottom plate ($1x10^5$ cells/well) for further co-culture experiment.

Chondrocyte-autologous T cell co-culture

Chondrocytes ($1x10^5$ cells/well) were cultured in 24-well plates and incubated for 48 hours. After cells completely attached to the plate, cells were treated with 50 ng/ml IFN-γ and cultured for 48 hours. Supernatant was removed from each well and cells were washed twice with PBS. IFN-γ -treated chondrocytes were transferred into 96-well U bottom plates ($1x10^4$ cells/well) and cells were pulsed with 10 μg/ml of P16-31, P263-280 and P2379-2394 for 3 hours. In this experiment, ovalbumin (OVA) or CEF peptides were used as positive peptide. After peptide stimulation, chondrocytes were washed twice with medium, and co-cultured with CFSE labeled autologous IFPF expanded T cells ($1x10^5$ cells/well) in 200 ml RF10 medium and centrifuged at 100 g for 2 minutes. T cells treated with anti-CD3/CD28 bead were used as positive control and T cells without treatment were used as a negative control. Chondrocyte and T cells were co-cultured at 37°C for 7 days.

Assessment of T cell proliferation

After 7 days of chondrocyte-T cell co-culture, cells were collected and transferred to 96-well V bottom plate. Cells were washed twice with PBS containing 5% FBS and the plate was centrifuged at 1,500 rpm for 5 minutes at 4°C. Cell pellets were collected and labeled anti-human CD3-APC, anti-human CD4-PE/Cy5 and anti-human CD8-AF700 for 15 minutes. After washing with PBS containing 5% FBS twice, the cells were acquired onto LSRII flow cytometer and were analyzed using FlowJo software (Tree Star).

MHC blocking assay

Chondrocytes ($1x10^5$ cells/well) were cultured in 24-well plates and incubated for 48 hours. After complete all attachments, cells were treated with 50 ng/ml IFN-γ and cultured for 48 hours. Supernatant was removed and cells were washed with PBS twice. Stimulated chondrocytes were transferred into 96-well U bottom plate ($1x10^4$ cells/well). MHC molecules on chondrocytes were blocked by addition of 10 mg/ml of Ultra-LEAF purified anti-human HLA-A, -B, -C (clone w6/32,

Biolegend) for MHC class I blocking or purified anti-human HLA-DR, DP, DQ (clone Tü39, Biolegend) for MHC class II blocking. Treated chondrocytes were incubated at 37 °C for 1 hour. After treatment, cells were treated with P16-31 or P263-280, and incubated at 37 °C for 3 hours. PG peptides were removed by washing the cells twice with DMEM complete medium (contains 10% FBS, 100 µg/ml penicillin and streptomycin and 1% non-essential amino acids) and centrifugated at 1,500 rpm for 5 minutes. Stimulated chondrocytes were subsequently co-cultured with CFSE labeled autologous T cells ($1x10^5$ cells/well) in 200 ml RF10 medium at 37 °C for 7 days. T cell proliferation was determined by flow cytometry as described earlier.

Statistical Analysis

RNA microarray analysis and visualization were conducted using R 4.0.2. Statistical significance of differential gene expression was determined using fold change and LPE statistical test. Hierarchical cluster analysis was performed using complete linkage and Euclidean distance to measure similarity. Statistical analysis of *in vitro* study was performed using Graph Pad version 7.0 software. Bonferroni correction was performed following one-way and two-way ANOVA to compare the expression level of chondrocyte surface markers in each group. Student's t-test was used to compare the data between two groups. Comparison of T cell proliferation between T cell co-cultured with unstimulated chondrocytes and PG peptides pulsed chondrocytes in each patient were evaluated using paired t-test. The data were illustrated as mean ± SEM and statistically significant data was considered at $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*), respectively.

CHAPTER III

RESULTS

PART I

(CHONDROCYTE STIMULATION BY PG PEPTIDE)

As mentioned above, chondrocytes are resident cell in the cartilage that play an important role in stabilizing cartilage structure by controlling metabolism of extracellular matrix in the cartilage (3). In OA condition, apart from chondrocytes losing their function in producing extracellular matrix, they also have immunological function when exposed to certain stimuli in the joint, such as inflammatory cytokines or cartilage fragment components (87). Proteoglycan aggrecan protein is a cartilage component that is found in synovial joints and may be able to stimulate chondrocytes (65). Therefore, this study will investigate the immunological function of chondrocytes after exposure to proteoglycan aggrecan.

Chondrocyte isolation and purity determination

Before investigating the immunological function of primary chondrocyte from OA patients, chondrocyte identification and purity evaluation were primarily performed. Because primary chondrocytes predominantly express intracellular type II collagen (Col II), therefore, the isolated cells from articular cartilage were labeled with fluorescence-conjugated anti-human type II collagen antibody and protein expression determined via flow cytometry to identify chondrocytes. Under the microscope, isolated cells from OA cartilage had a polygonal appearance and there were not any contaminant cells in this culture. After staining the cells with antibody against type II collagen and determining its expression by flow cytometry, we found that over 99% of cells expressed intracellular type II collagen (Figure 12). This finding indicated that isolated chondrocytes had high purity for our experiments.

Although the isolated cells from OA cartilage had a chondrocyte phenotype, however, this phenotype was unstable in long term *in vitro* cultures, leading to a fibroblastic phenotype (92). These transformed cells have a dendritic morphology and altered cell metabolism (93). Instead of type II collagen, these transformed cells produce high levels of type I collagen and may have a different response from chondrocytes to the stimuli in our experiments. Thus, we tested the optimum period for chondrocyte culture using expression of type II collagen as an indicator. In this experiment, chondrocytes were isolated from cartilage of OA patient and cultured for 6 days, 9 days and 3 days after the first passage by trypsinization and levels of type II collagen expression was determined via immunofluorescence spectroscopy and flow cytometry. Immunofluorescence showed that the cells stained green color for type II collagen could be highly observed on day 6 and day 9, while the cells at 3 days after the first passage showed lower amount of cells stained green color (Figure 13A). Similarly to immunofluorescence results, the percentage of type II collagen-expressing cells determined by flow cytometry increased to 96.5% on day 6 and decreased to 81.2% by day 9. After trypsinization, type II collagen-expressing cells observed were only 10.2% (Figure 13B). This data shows that human primary chondrocytes lose type II collagen expression by day 9. Therefore, in all experiments in this thesis, we used freshly isolated human primary chondrocytes that have been cultured for no longer than 8 days.

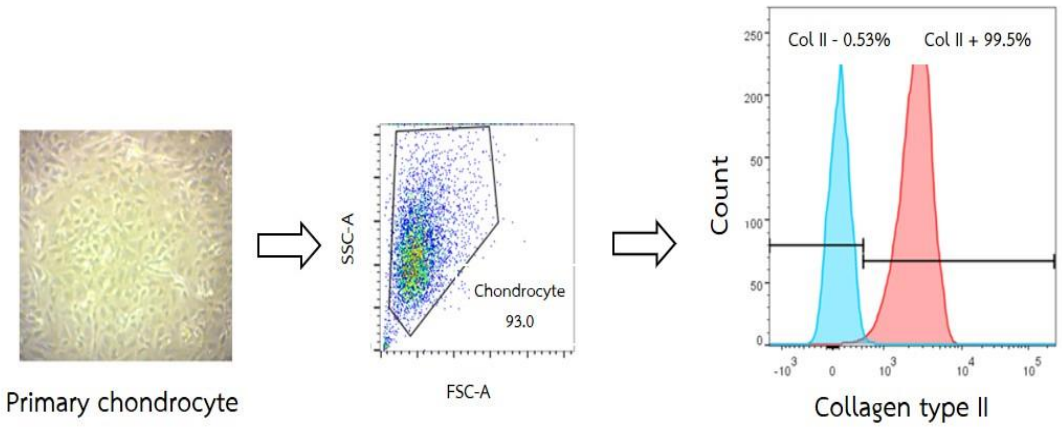


Figure 12 Identification of isolated primary chondrocyte intracellular collagen type II staining. Primary chondrocytes were visualized under microscope (left figure) and flow cytometer

(middle figure). Collagen type II expressed chondrocytes were shown in pink histogram compare with unstained cells in blue histogram (right figure).

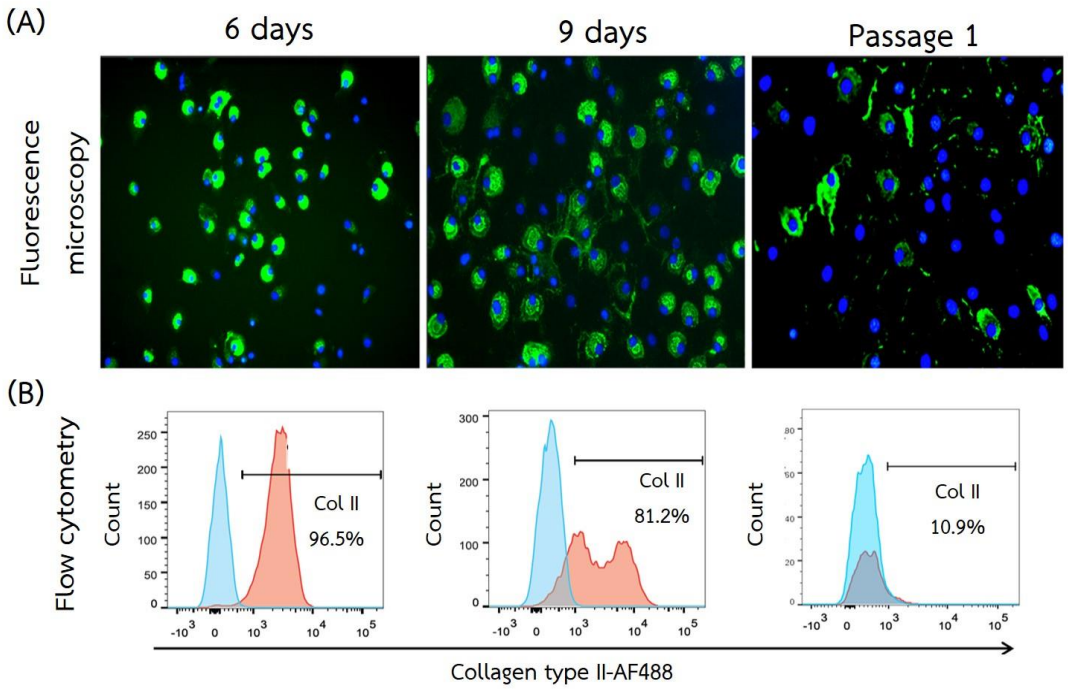


Figure 13 Time-course of collagen type II expression. Human articular chondrocytes isolated from knee OA patients and cultured for 6 days, 9 days and 3 days after the first passage were labeled with DAPI and anti-human type II collagen antibody. Protein expression was determined by (**A**) immunofluorescence spectroscopy (green and blue represent type II collagen and DAPI, respectively) and (**B**) flow cytometry (blue and pink histogram represent unstained cell and type II collagen-expressing cells, respectively).

Chondrocytes response to proteoglycan aggrecan peptides in inflammatory condition by increasing expression of antigen presentation markers

After chondrocyte isolation and culture period optimization, chondrocyte stimulation and immunological function were determined. Because chondrocytes have antigen presentation function when exposed to inflammatory cytokines or surrounding stimuli in the joint (87), we investigated the effects of proteoglycan aggrecan (PG) stimulation in the presence of IFN-γ on human primary chondrocytes isolated from knee OA patients. Firstly, isolated chondrocytes from OA cartilage were treated with PG peptides, which include P16-31, P263-280 and P2379-2394, with and

without IFN-γ for 48 hours. Expression of costimulatory molecules (CD40, CD80 and CD86), and MHC molecules (HLA-A, -B, -C and -DR) on cell surface chondrocytes were determined by flow cytometry. Data from one OA patient revealed that expression of CD40, CD80, CD86, HLA-A, -B, -C and -DR could be observed at low levels on unstimulated (no IFN-γ) chondrocyte surface (Figure 14). After cells were treated with IFN-γ, both HLA-A, -B, -C and HLA-DR increased expression level to 92.2% and 93.3%, respectively. For costimulatory molecules, CD40 expression, but not CD80 and CD86, was upregulated in response to IFN-γ stimulation. In the presence of PG peptides, only P263-280 with IFN-γ can increased the percentage of CD86+ chondrocytes up to 7.6% (Figure 14).

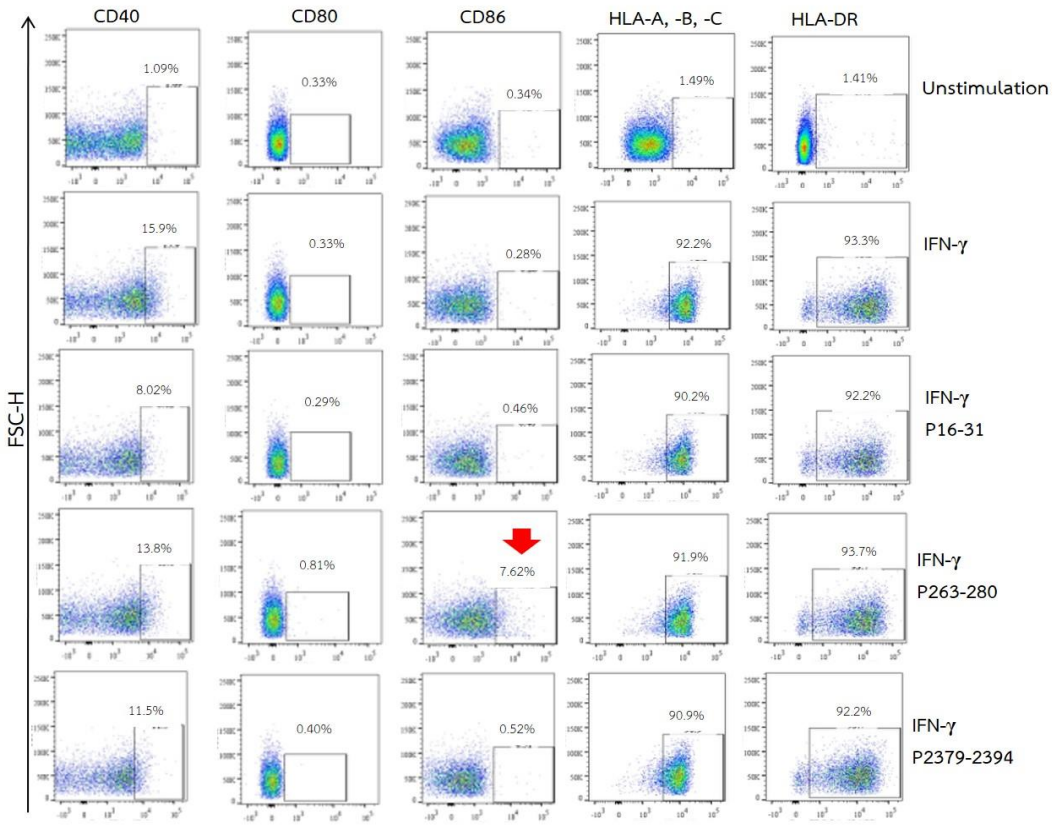


Figure 14 Flow cytometry dot plots showing expression of costimulatory and MHC molecules on cell surface of stimulated chondrocytes. Primary human chondrocytes isolated from knee OA patients were stimulated with 50 ng/ml of IFN-γ alone or in the presence of 10 mg/ml of PG peptides; P16-31, P263-280 or P2379-2394, for 48 hours. Expression of CD40, CD80, CD86, HLA-A, -B, -C and -DR on cell surface chondrocyte were determined by flow cytometry.

The effect of PG peptides in the presence of IFN-γ on costimulatory molecule expression on chondrocytes were investigated in 10 knee OA patients. The expression level of each marker is shown as fold change of fluorescence intensity which was calculated from mean fluorescence intensity of stimulated cell standardized by that of unstimulated cells. Without IFN-γ, chondrocytes treated with P16-31 showed higher levels of CD80 expression than unstimulated cells, while chondrocytes treated with P263-280 showed higher levels of both CD80 and CD86 expression than unstimulated cells and this was significantly higher than the irrelevant peptide (P2379-2394) (Figure 15A). However, these expression levels did not differ from chondrocytes treated with IFN-γ only. Expression of CD80 and CD86 were significantly increased if P263-280 and P16-31 stimulation was accompanied by IFN-γ (1.595 increase fold for CD80 and 1.811 increase fold for CD86 expression in P263-280 with IFN-γ stimulation, 1.247 increase fold for CD80 and 1.506 increase fold for CD86 expression in P16-31 with IFN-γ stimulation) when compared with unstimulated conditions and IFN-γ-only treatment (Figure 15B). The increase in CD80 and CD86 was not observed with P2379-2394 in both the absence and presence of IFN-γ. These PG peptide stimulations did not effect CD40 expression level as expression level did not differ from IFN-γ only condition, regardless of PG peptides.

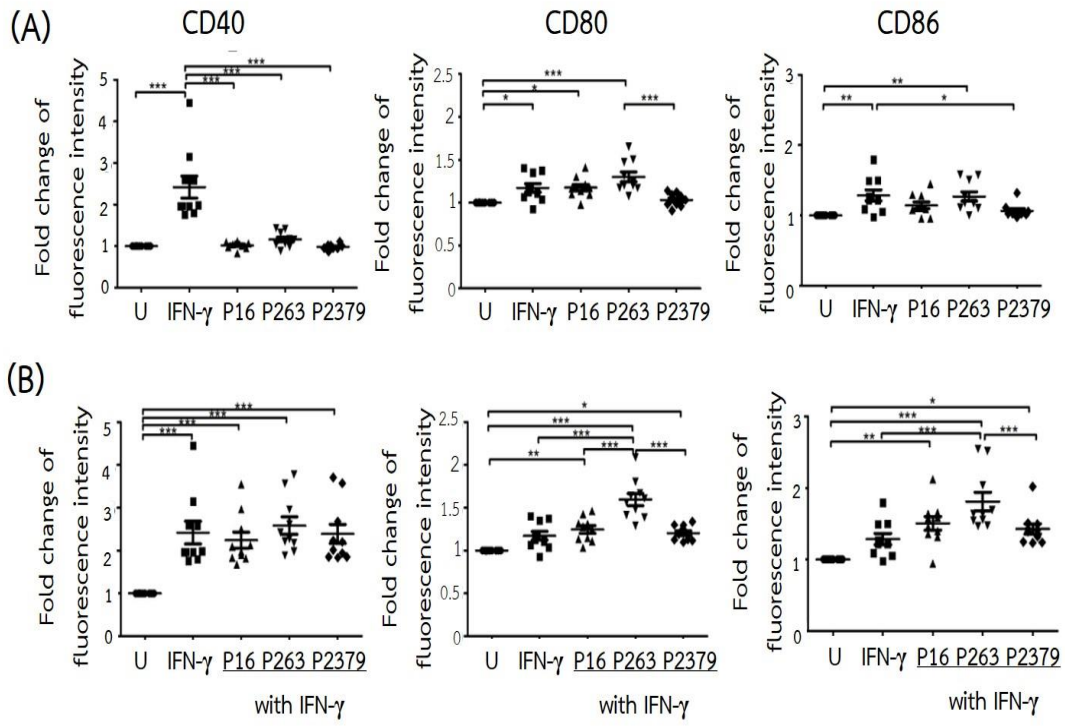


Figure 15 Comparison of costimulatory molecules expression on chondrocyte surface after stimulation with IFN-γ and PG peptides. Primary chondrocytes isolated from 10 OA patients were stimulated with 10 μg/ml of PG peptides; P16-31, P263-280 and P2379-2394, **(A)** without IFN-γ or **(B)** with 50 ng/ml of IFN-γ for 48 hours. Fold change of CD40, CD80 and CD86 expression were calculated from mean fluorescence intensity of stimulated cells divided by that of unstimulated cells. Each black dot represents each OA patient. Data are shown as mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ***, $p \leq 0.001$).

The effect of PG peptides on HLA-A, -B, -C and HLA-DR molecules expression on chondrocytes was also investigated. Results show that IFN-γ treatment significantly increased both expressions of HLA-A, -B, -C (by approximately 5-fold) and expression of HLA-DR (by approximately 30-fold) on cell surface chondrocytes when compared with unstimulated cells. However, these expressions were not observed when cells were stimulated with PG peptides alone (Figure 16A). The expression level of HLA-A, -B, -C and HLA-DR of chondrocytes when stimulated with PG peptides with IFN-γ did not differ significantly from cells treated with IFN-γ only (figure 16B).

From these results, we show that expression of HLA-A, -B, -C and HLA-DR on chondrocytes can be increased in IFN-γ treated conditions, but are not induced by

PG peptides. However, certain PG peptide stimulation, especially P16-31 and P263-280, but not P2379-2394, increased expression levels of costimulatory molecules (CD80 and CD86) on chondrocytes, which may lead to enhanced costimulatory signal for T cell activation.

To determine the dose response effect of PG peptides to chondrocytes, primary chondrocytes isolated from 3 OA cartilage were stimulated with IFN-γ along with various concentrations (0, 5, 10, 20, 50 and 100 μg/ml) of P16-31, P263-280 or P2379-2394 for 48 hours. The expression level of costimulatory and MHC molecules was determined by flow cytometry. From the results, expression level of CD40 was significantly increased more than 3-fold and 2-fold compared with unstimulated when cells were treated with the highest dose (100 μg/ml) of P16-31 and P263-280, respectively. Increasing concentrations of P16-31 and P263-280 peptides enhanced CD80 expression level in a dose-dependent manner (Figure 17). Moreover, at 100 μg/ml of P263-280 peptide stimulation, CD80 expression level was higher than when stimulated with P16-31 (more than 4-fold for P263-280 and approximately 3-fold for P16-31). Nevertheless, this dose-dependent effect of P16-31 and P263-280 peptides were not seen with CD86 (Figure 17). P2379-2394 peptide stimulation did not have any effect on the expression of CD40, CD80 and CD86 on chondrocytes. No increase in cell surface expression was observed (Figure 17).

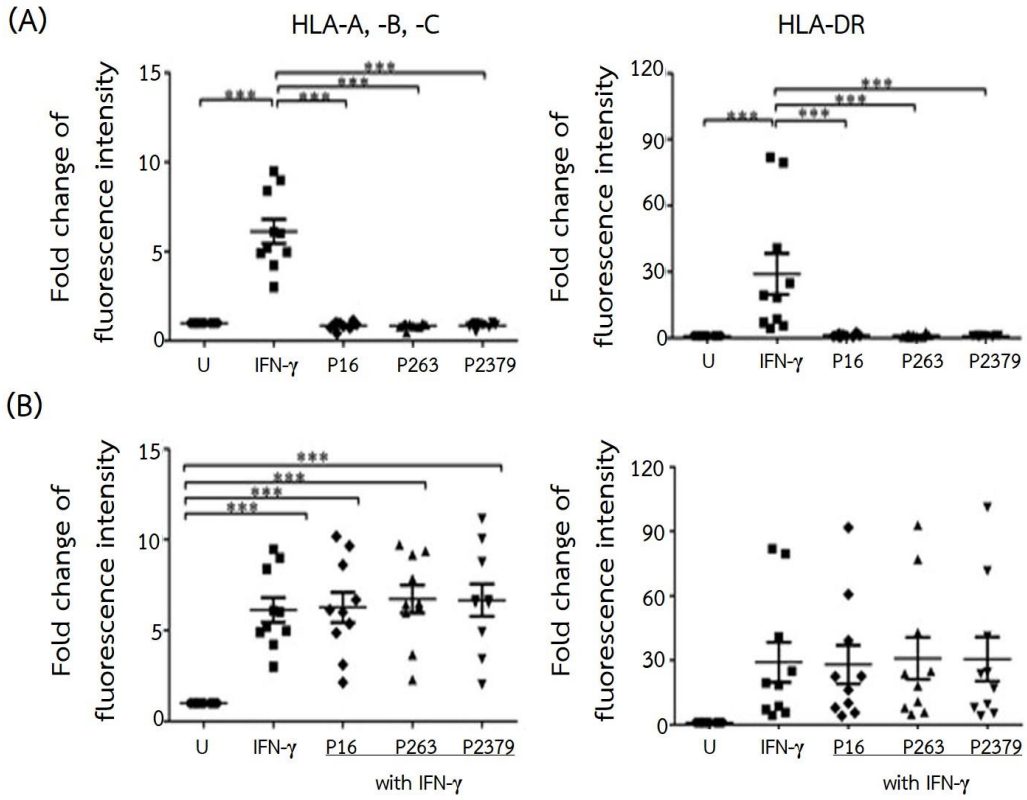


Figure 16 Comparison of MHC molecule expression on cell surface chondrocyte after stimulation with IFN-γ and PG peptides. Human primary chondrocytes isolated from 10 OA patients were stimulated with 10 μg/ml of PG peptides; P16-31, P263-280 and P2379-2394, **(A)** without IFN-γ or **(B)** with 50 ng/ml of IFN-γ for 48 hours. Fold change of HLA-A, -B, -C and HLA-DR expression were calculated from mean fluorescence intensity of stimulated cells divided by that of unstimulated cells. Each black dot represents each OA patient. Data are shown as mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ***, $p \leq 0.001$).

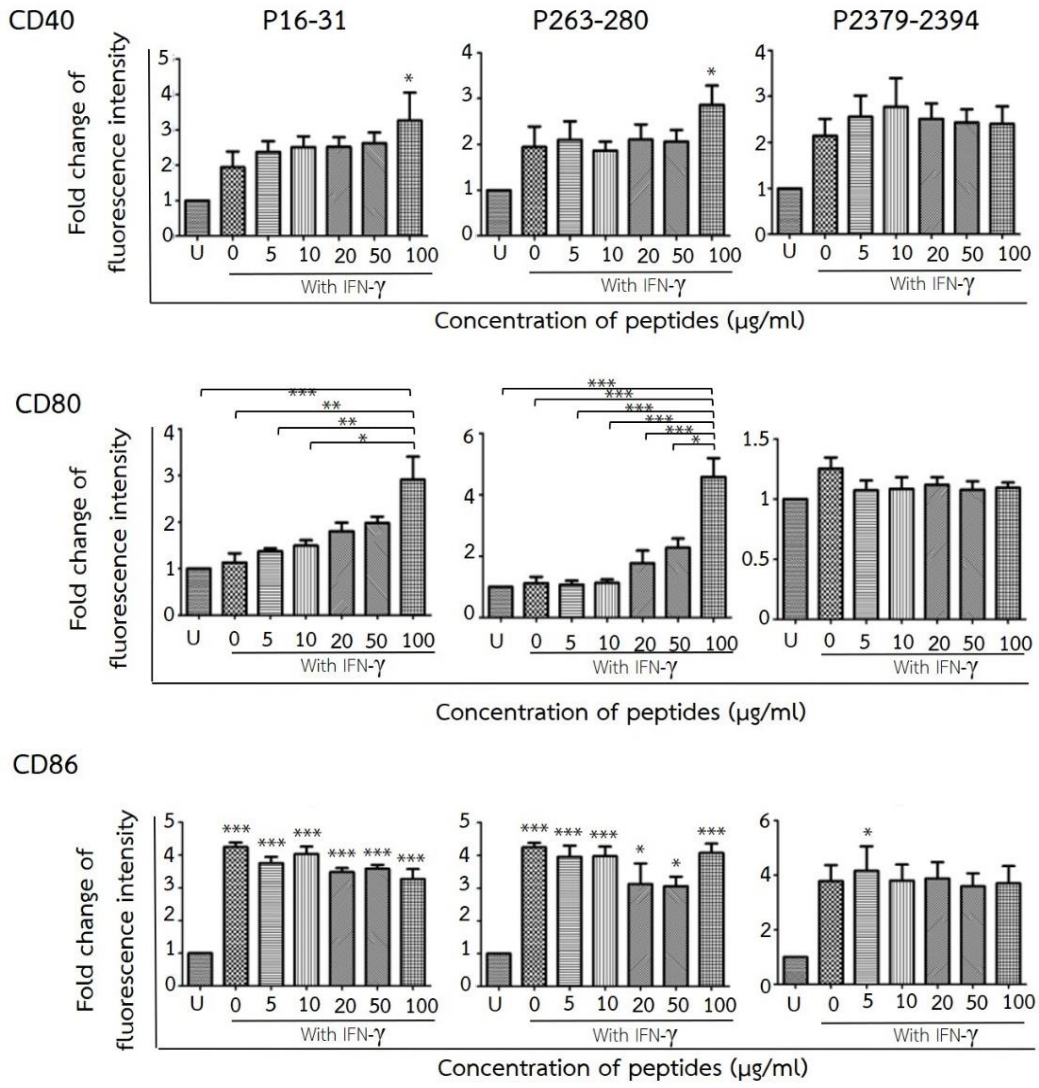


Figure 17 Expression of costimulatory molecules on cell surface chondrocytes after stimulation with IFN-γ and various concentrations of PG peptides. Human primary chondrocytes isolated from 3 OA patients were stimulated with 50 ng/ml of IFN-γ along with P16-31 (left panel), P263-280 (middle panel) and P2379-2394 (right panel) at concentrations of 0, 5, 10, 20, 50 and 100 μg/ml for 48 hours. Fold change of CD40, CD80 and CD86 expression were calculated from mean fluorescence intensity of stimulated cells divided by that of unstimulated cells. Data are shown as mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ***, $p \leq 0.001$).

PG peptide stimulation on MHC molecules showed that expression levels of both HLA-A, -B, -C and HLA-DR in PG peptide-treated conditions did not differ from IFN-γ-only conditions despite increasing concentrations of the peptides (Figure 18). From these results, it could be suggested that an increase in P16-31 and P263-280

concentration may increase activity of chondrocytes by upregulating co-stimulatory molecules and further contribute to T cell activation.

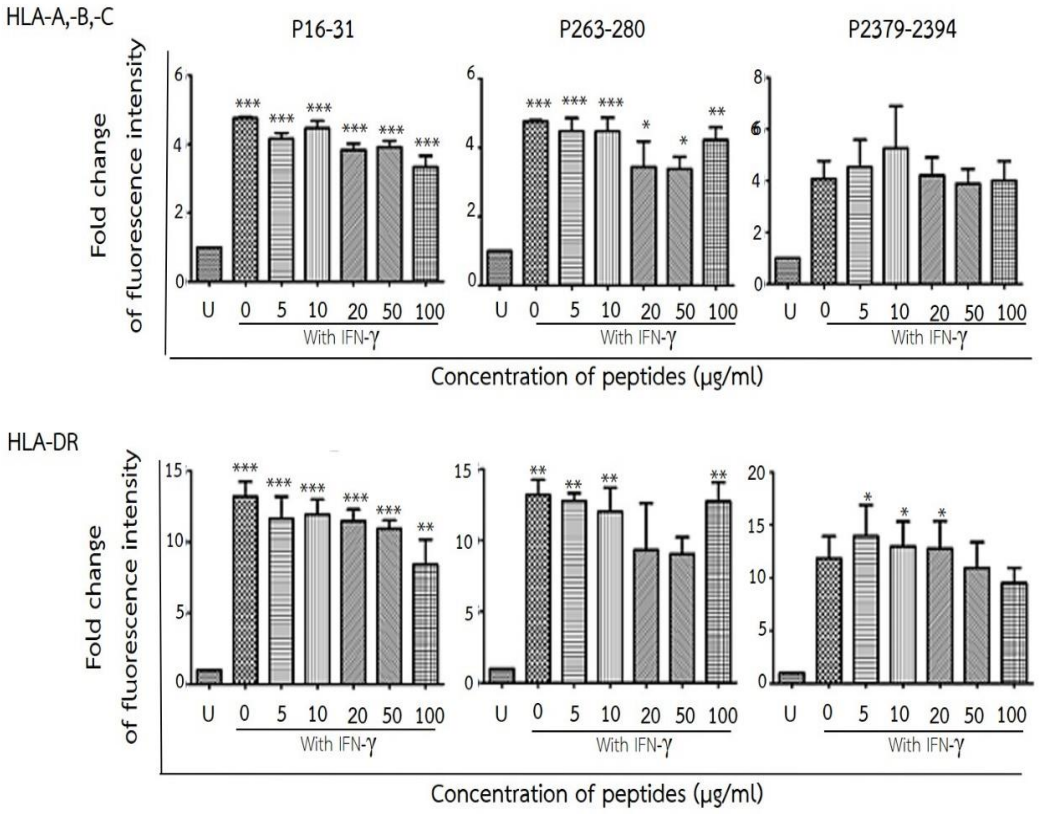


Figure 18 Expression of MHC molecules on chondrocyte surface after stimulation with IFN-γ and various concentrations of PG peptides. Human primary chondrocytes isolated from 3 OA patients were stimulated with 50 ng/ml of IFN-γ along with P16-31 (left panel), P263-280 (middle panel) and P2379-2394 (right panel) at concentrations of 0, 5, 10, 20, 50 and 100 μg/ml for 48 hours. Fold changes of HLA-A, -B, -C and HLA-DR expression were calculated from mean fluorescence intensity of stimulated cells divided by that of unstimulated cells. Data are shown as mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ***, $p \leq 0.001$).

Induction of antigen presentation markers by p16-31 and p263-280 proteoglycan aggrecan peptides is chondrocyte-specific

To investigate whether the ability of P16-31 and P263-280 to increase expression levels of antigen presentation markers was specific to chondrocytes, we compared PG peptide stimulation in chondrocytes with other cell types, including primary keratinocytes and human monocyte derived dendritic cells (MoDCs). Primary

keratinocytes (NHEK; ATCC® PCS-200-010™) were chosen to represent epithelial cells that responded to IFN-γ by expressing of MHC molecules on their surface and MoDCs were chosen to represent professional antigen presenting cells. MoDCs was generated from peripheral blood monocytes of 3 healthy donors. Monocytes were stimulated with IL-4 and GM-CSF for 7 days until they differentiated to immature dendritic cells. Maturation of dendritic cells was then induced by further treating with TNF-α, IL-1β, IL-6 and PGE2 to generate conventional dendritic cells. In parallel, immature dendritic cells were treated with IFN-γ only as a control group.

After generating MoDCs, their purity and maturation were evaluated by detecting expression levels of CD1a, CD80, CD86, HLA-A, -B, -C and HLA-DR on their cell surface. Immature dendritic cells, conventional dendritic cells and IFN-γ treated dendritic cells expressed CD1a of 86.4%, 76.7% and 72.2%, respectively, indicating that differentiation was successful with high purity of the dendritic cells. We show that immature dendritic cells expressed only high levels of HLA-DR, while expression of HLA-A, -B, -C, CD80 and CD86 were not comparable to conventional dendritic cells. In conventional dendritic cells and IFN-γ-treated dendritic cells, all these antigen presentation markers were expressed in over 90% of the population (98.5% of CD80, 99.3% of CD86, 96.5% of HLA-A, -B, -C and 100% HLA-DR expressed on conventional dendritic cells and 92.3% of CD80, 91.6% of CD86, 90.3% of HLA-A, -B, -C and 100% of HLA-DR on IFN-γ treated dendritic cells), reflecting similar phenotypes between these two types of MoDCs (Figure 19).

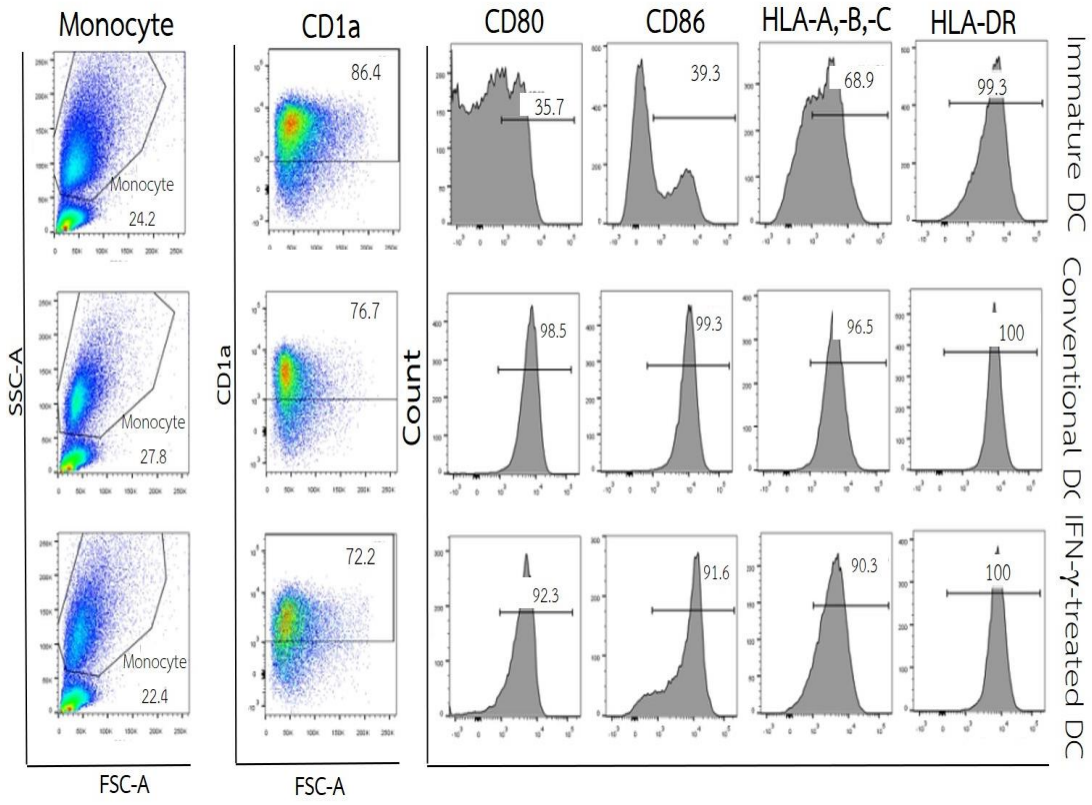


Figure 19 Expression levels of antigen presentation markers on human monocyte-derived dendritic cells (MoDCs). Human monocytes were isolated from peripheral blood of healthy donors and treated with 24 ng/ml rhGM-CSF and 25 ng/ml rhIL-4 to induce differentiation into immature dendritic cells (immature DCs) (upper panel) for 7 days. On day 8, the immature DCs were treated with IL-6 (50 ng/ml), IL-1β (10 ng/ml), TNF-α (20 ng/ml) and PgE2 (1 μg/ml), or IFN-γ (50 ng/ml) to induce the cells into conventional dendritic cells (conventional DCs) (middle panel) or IFN-γ-treated dendritic cells (IFN-γ-treated DCs) (lower panel), respectively. Flow cytometry dot plots showing monocyte population gating and percentage of CD1a+ cells. Fluorescent intensity histogram shows expression level of CD80, CD86, HLA-A, -B, -C and HLA-DR on MoDCs.

Next, we treated primary keratinocytes, MoDCs and primary chondrocytes with IFN-γ-only or IFN-γ with either P16-31, P263-280 or P2379-2394 for 48 hours, and determined cell surface expression of antigen presentation markers on these cells via flow cytometry. Chondrocytes treated with P263-280, but not P16-31 and P2379-2394, with IFN-γ increased CD86 expression level when compared with unstimulated and IFN-γ-only treated cells (Figure 20A). For keratinocytes, HLA-A, -B, -C and HLA-DR showed high expression level on keratinocytes treated with IFN-γ in the absence and presence of either P263-280, P16-31 or P2379-2394 peptides when compared to unstimulated condition, while upregulation of CD80 and CD86 could be not observed in all conditions (Figure 20B). For MoDCs, upregulation of only CD86 expression level was observed on IFN-γ-only and IFN-γ with P263-280, P16-31 or P2379-2394 peptides when compared with unstimulated MoDCs (immature DCs); regardless of PG peptides used to stimulate cells. HLA-A, -B, -C, HLA-DR and CD80 were highly expressed on unstimulated DCs (immature DCs) and these expressions did not change when cells were treated with IFN-γ-only or IFN-γ with P263-280, P16-31 or P2379-2394 peptide (Figure 20C).

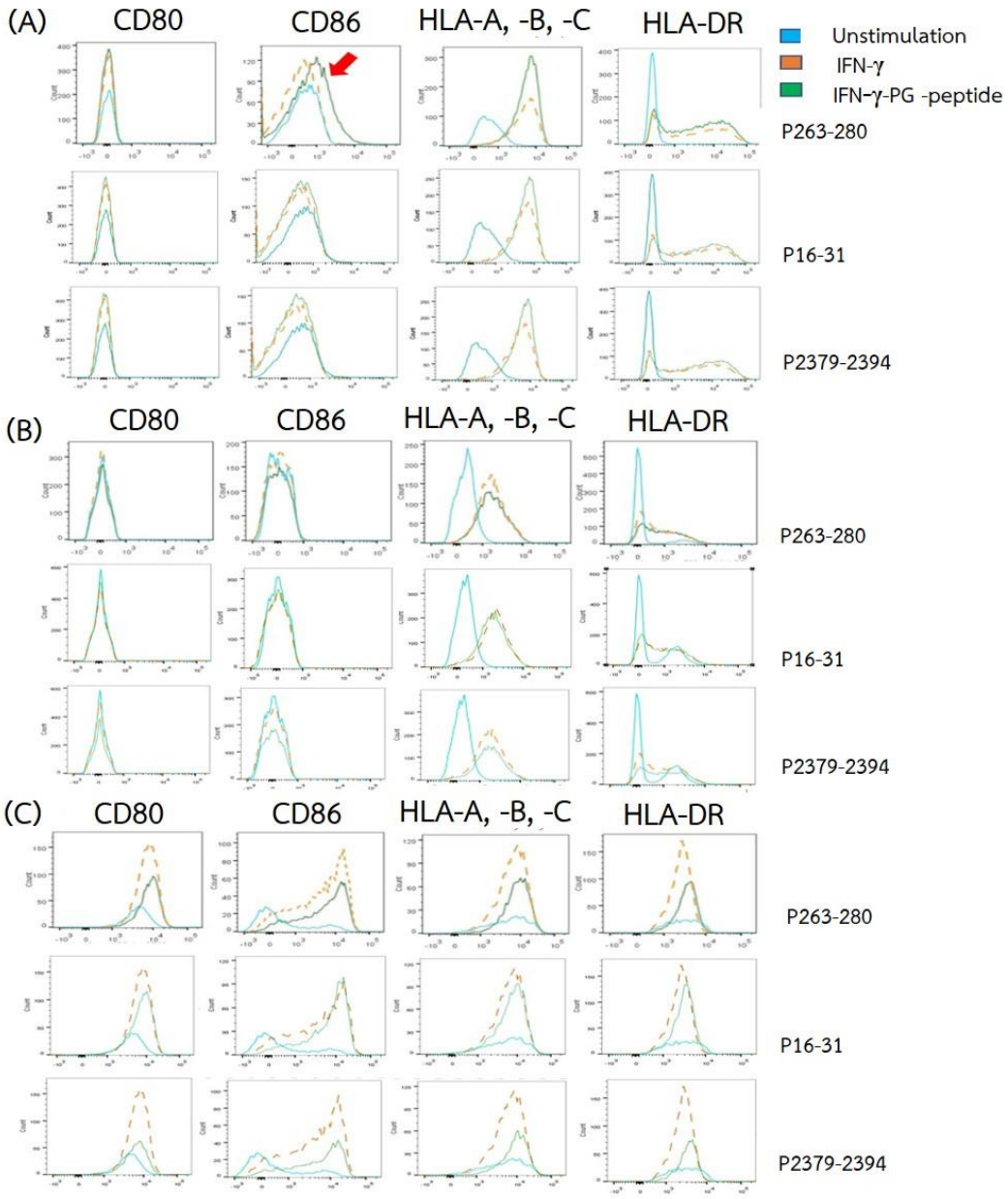


Figure 20 Histogram of costimulatory and MHC molecules expressed on surface of chondrocytes, keratinocytes and MoDCs. A, Primary chondrocytes from OA patient **B,** primary keratinocytes (ATCC) and **C,** IFN-γ-treated DCs were stimulated with IFN-γ along with P16-31 (upper panel), P263-280 (middle panel) and P2379-2394 (lower panel) for 48 hours. Expression of CD80, CD86, HLA-A, -B, -C and HLA-DR were investigated by flow cytometry. The expression of surface markers on unstimulated cells, IFN-γ treated cells and IFN-γ along with PG peptides-treated cells were shown as fluorescent intensity which were colored by blue, orange and green histogram, respectively.

Cell surface CD80 and CD86 expression levels were high in both conventional dendritic cells and IFN-γ-treated dendritic cells when compared with chondrocytes (Figure 21A). However, PG peptide treatment did not further increase CD80 and CD86 expression levels as no difference in expression levels among PG peptide and IFN-γ -treated condition and IFN-γ only-treated condition and untreated condition was observed (Figure 21A). For MHC molecules, both conventional dendritic cells and IFN-γ-treated dendritic cells showed expression level of HLA-A, -B, -C and HLA-DR higher than chondrocytes (Figure 21B). However, PG peptide treatment did not increase HLA-A, -B, -C and HLA-DR expression levels in both MoDCs and chondrocytes (Figure 21B).

In contrast to MoDCs, keratinocytes showed low expression levels of costimulatory molecules and MHC molecules and was similar to levels of unstimulated chondrocytes. Chondrocytes expressed significantly higher levels of CD80 when stimulated with P16-31 peptide and significantly higher levels of CD86 when stimulated with all peptides; when compared to peptide-treated keratinocytes (Figure 22A). P16-31 and P263-280 treatment induced higher levels of CD86 expression on chondrocytes when compared to treatment with the control peptide, P2379-2394 (Figure 22A). Keratinocytes did not respond to these peptide treatments nor IFN-γ treatment (Figure 22A and Figure 22B).

From these results, we show that chondrocytes expressed antigen presentation markers higher than keratinocytes, but not comparable to MoDCs. However, expression of CD80 and CD86 increased with P16-31 and P263-280 peptide stimulation were seen in chondrocytes. These results suggest that these proteoglycan aggrecan peptide stimulation may be unique to chondrocytes, despite not achieving levels of activation as high as professional antigen-presenting cells (MoDCs).

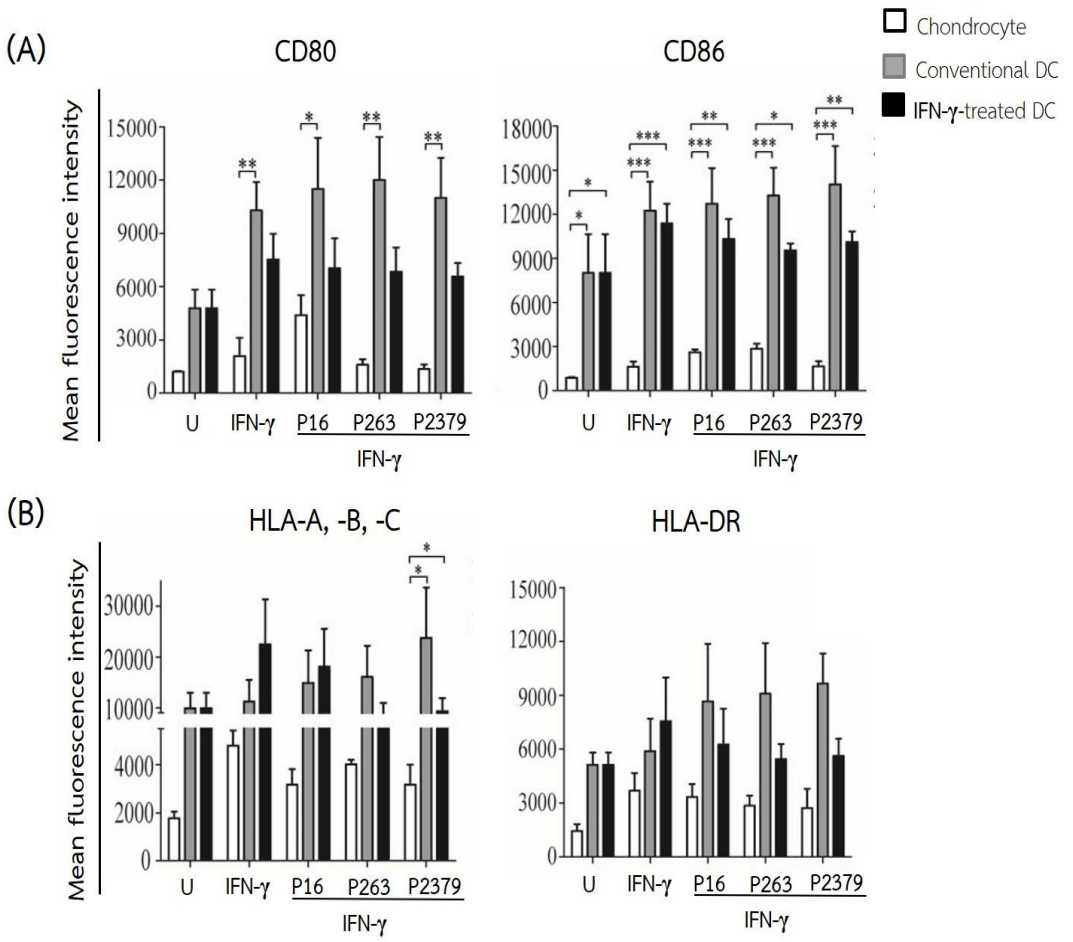


Figure 21 Comparison of antigen presentation marker expression on OA chondrocyte and MoDc after treatment with IFN-γ and PG peptides. Knee OA chondrocytes (N=3) and human monocyte-derived dendritic cells (MoDC, N=3) were treated with 50 ng/ml of IFN-γ and 10 μg/ml of PG peptides (P16-31, P263-280 or P2379-2394) for 48 hours. Expression of **(A)** co-stimulatory molecules; CD80 and CD86, and **(B)** MHC molecules; HLA-A,B,C and HLA-DR were determined by flow cytometry. The expression data are quantified by mean fluorescence intensity comparing between OA chondrocytes (white bar) and MoDCs, which was differentiated by cytokine cocktail treatment (gray bar) or 50 ng/ml IFN-γ treatment (black bar). Data were shown as mean ± SEM and statistical significance were calculated by two-way ANOVA with bonferroni post hoc test (*, $p < 0.05$; **, $p < 0.01$; *** $p \leq 0.001$).

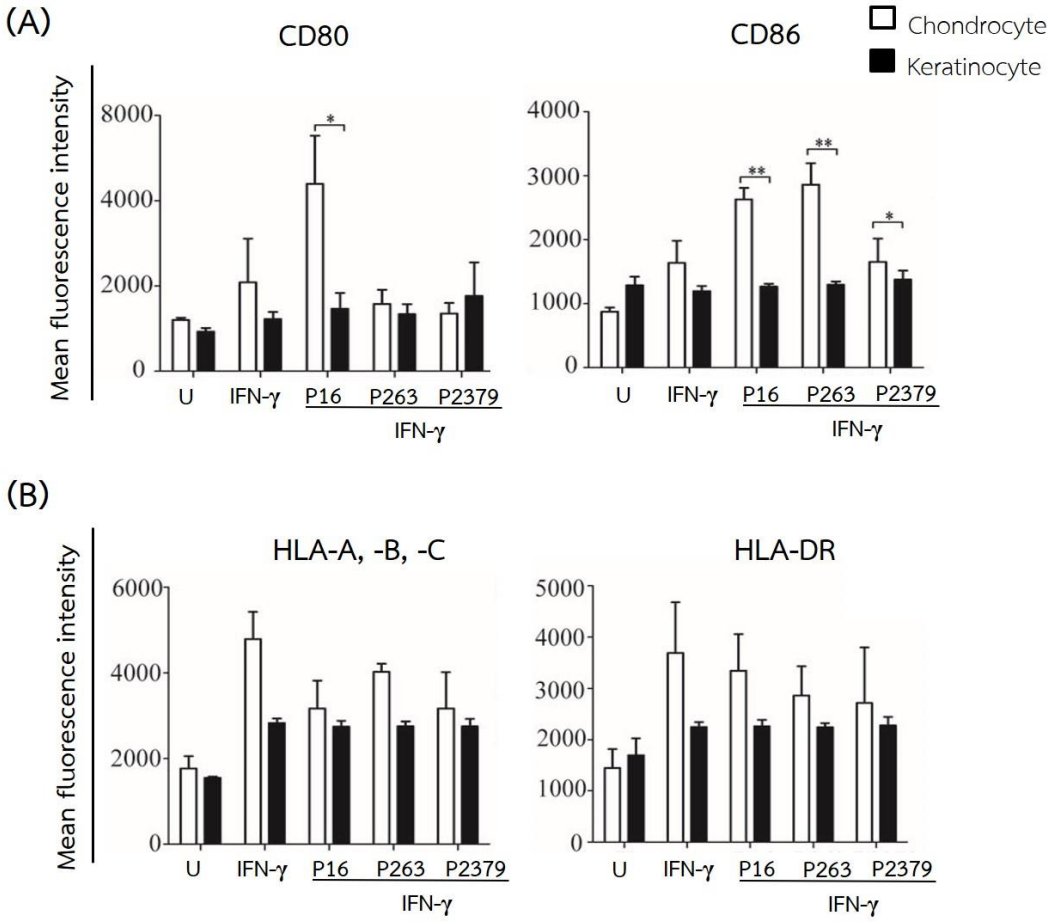


Figure 22 Comparison of antigen presentation marker expression on OA chondrocyte and primary keratinocyte after treating with IFN-γ and PG peptides. Knee OA chondrocytes (N=3) and primary keratinocytes (ATCC) (N = 1, 3 experimental replications) were treated with 50 ng/ml of IFN-γ and 10 μg/ml of PG peptides (P16-31, P263-280 or P2379-2394) for 48 hours. Expression of **(A)** co-stimulatory molecules; CD80 and CD86, and **(B)** MHC molecules; HLA-A,-B,-C and HLA-DR were determined by flow cytometry. The expression data are quantified by mean fluorescence intensity comparing between OA chondrocytes (white bar) and primary keratinocytes (black bar). Data were shown as mean ± SEM and statistical significance were calculated by two-way ANOVA with bonferroni post hoc test (*, $p < 0.05$; **, $p < 0.01$; *** $p \leq 0.001$).

P263-280 peptide requires IFN-γ to stimulate pro-inflammatory cytokine production in chondrocytes

OA is a local inflammatory disease with IL-6, IL-8 and TNF-α present in the knee joint (94). Chondrocytes are capable of producing these cytokines (95).

Therefore, we investigated the effect of PG peptides on IL-6, IL-8 and TNF-α production from chondrocytes. Chondrocytes isolated from cartilage of 3 knee OA patients were treated with PG peptides with or without IFN-γ for 48 hours. Cytokine production was measured by ELISA. Results shows that chondrocytes produced low levels of IL-6, IL-8 and TNF-α when cells were treated with IFN-γ-only or PG peptide-only and these levels did not differ from unstimulated conditions. When peptide stimulation was accompanied by IFN-γ treatment, only P263-280 was able to induce chondrocytes to secrete significantly high levels of IL-6 (7,500 pg/ml) and IL-8 (600 pg/ml) and also slightly increased TNF-α production (28 pg/ml) (Figure 23).

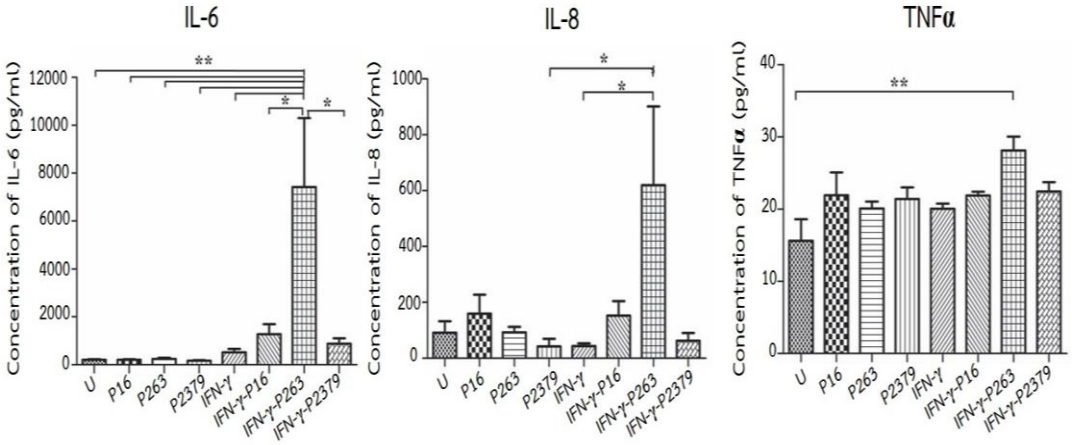


Figure 23 Pro-inflammatory cytokine production in OA chondrocytes in response to proteoglycan aggrecan peptide stimulation with IFN-γ treatment. IL-6, IL-8 and TNF-α production from 3 OA chondrocytes were determined by ELISA after cells were treated with 10 μg/ml of PG peptides (P16-31, P263-280 or P2379-2394) with or without 50 ng/ml of IFN-γ for 48 hours. Y axis represents concentration of cytokines in pg/ml. Data is shown as mean ± SEM and statistical significance were calculated by one-way ANOVA with bonferroni post hoc test (*, $p < 0.05$; **, $p < 0.01$; *** $p \leq 0.001$).

When we tested the effect of PG peptide stimulation on cytokine production in MoDCs, we found that IL-6 and TNF-α was strikingly high in conventional DCs and peptide stimulation did not further increase any cytokine production (Figure 24A). For IFN-γ treated dendritic cells, no increase in cytokine production as a result of peptide stimulation was observed (Figure 24B). When compared MoDC cytokine production

with of chondrocytes, we found that MoDCs secreted IL-6, IL-8 and TNf-α higher than chondrocytes. However, P263-280 treatment with IFN-γ induced high IL-6 secretion in chondrocytes to nearly comparable levels as conventional dendritic cells (Figure 24C). This finding suggests that certain proteoglycan aggrecan peptides stimulates substantial amounts of cytokine production from chondrocytes.

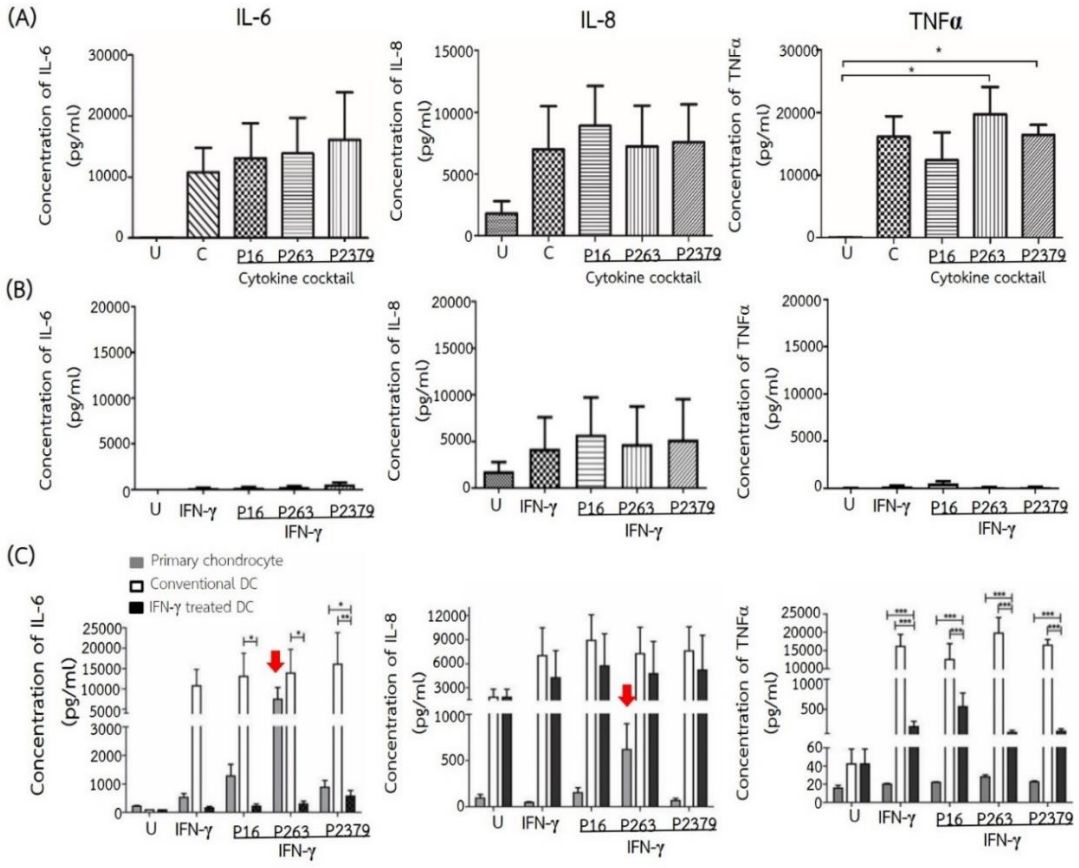


Figure 24 Comparison of pro-inflammatory cytokine production between OA chondrocytes and MoDCs after treatment with IFN-γ and PG peptides. A and **B,** Cytokine production of IL-6, IL-8 and TNF-α from MoDCs (N=3) stimulated with 10 μg/ml of PG peptides (P16-31, P263-280 and P2379-2394) along with cytokine cocktails (IL-1β, IL-6, TNF-α and PgE2) or IFN-γ, respectively, was determined by ELISA. **C,** Concentrations of IL-6, IL-8 and TNF-α secreted from stimulated conventional dendritic cells (white bar) and IFN-γ-treated dendritic cells (black bar) were compared with cytokines secreted from stimulated OA chondrocytes (gray bar). Error bar represents mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; *** $p \leq 0.001$).

Unstimulated and peptide only-treated keratinocytes expressed low levels of IL-6, IL-8 and TNF-α (Figure 25A). IFN-γ treatment, regardless of the presence of peptides, resulted in higher IL-6 and IL-8 production than unstimulated and peptide-only treated conditions (Figure 25A). When cytokine production from primary keratinocytes was compared with chondrocytes, the concentrations of IL-6 and IL-8, but not TNFa, secreted from chondrocytes was significantly higher than from keratinocytes, especially when stimulated with the P263-280 peptide (Figure 25B).

These results suggest that PG peptides may stimulate chondrocytes specifically and that certain PG peptide fragments (P16-31 and P263-280) in the presence of IFN-γ stimulate chondrocytes to secrete high levels of IL-6 and IL-8.

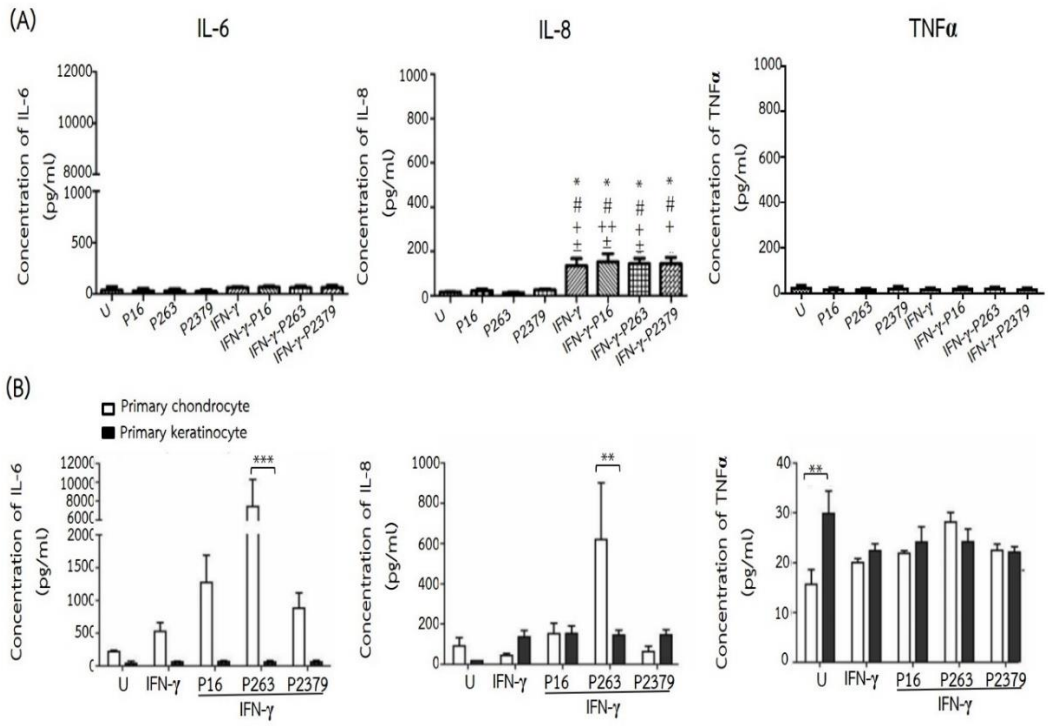


Figure 25 Comparison of pro-inflammatory cytokine production in OA chondrocytes and primary keratinocytes after treatment with IFN-γ and PG peptides. A, Cytokine production of IL-6, IL-8 and TNF-α from primary keratinocytes (ATCC) (N=1, 3 experimental replications) stimulated with 10 μg/ml of PG peptides (P16-31, P263-280 and P2379-2394) with or without IFN-γ for 48 hours was determined by ELISA. **B,** Concentrations of IL-6, IL-8 and TNF-α secreted from primary keratinocytes (black bar) were compared with cytokines secreted from stimulated OA chondrocytes (white bar). Error bar represents mean ± SEM (*; compared with unstimulation, #;

compared with P16-31 treatment, +; compared with P263-280 treatment, ±; compared with P2379-2394 treatment) (*, $p < 0.05$; **, $p < 0.01$; *** $p \leq 0.001$).

In this part, we show that PG peptides, P16-31 and P263-280 peptides, increased expression levels of antigen presentation markers and increased production of inflammatory cytokines in chondrocytes in the presence of IFN-γ. These responses were seen only in chondrocytes, but not in keratinocytes and MoDCs. These results could suggested that P16-31 and P263-280 peptides may stimulate chondrocytes specifically.

PART II

(RNA MICROARRAY AND GENE VALIDATION)

In addition to investigating the effects of PG peptides on antigen presentation markers expression and cytokine production, we sought to determine gene expression patterns in chondrocytes in responsive to various PG peptides.

PG peptides induce different gene expression in OA chondrocyte

According to earlier results, we found that chondrocytes secreted high levels of IL-6 when stimulated with IFN-γ and PG peptides for 48 hours. In order to study gene level expression in chondrocytes, we tested for various time points in which chondrocytes start responding to peptide stimulation. Therefore, we treated primary chondrocytes with high doses (100 μg/ml) of P16-31, P263-280 and P2379-2394 peptide fragments in the presence of IFN-γ and determined both IL-6 mRNA expression and IL-6 secretion at 6, 24 and 48 hours after stimulation. Our results show that expression of IL-6 at the transcriptional level peaked at 6 hours and decreased gradually by 48 hours, whilst IL-6 at the protein level peaked at 48 hours after stimulation. (Figure 26). Therefore, in further experiments, we chose the timepoint of 6 hours of stimulation to evaluate mRNA expression levels.

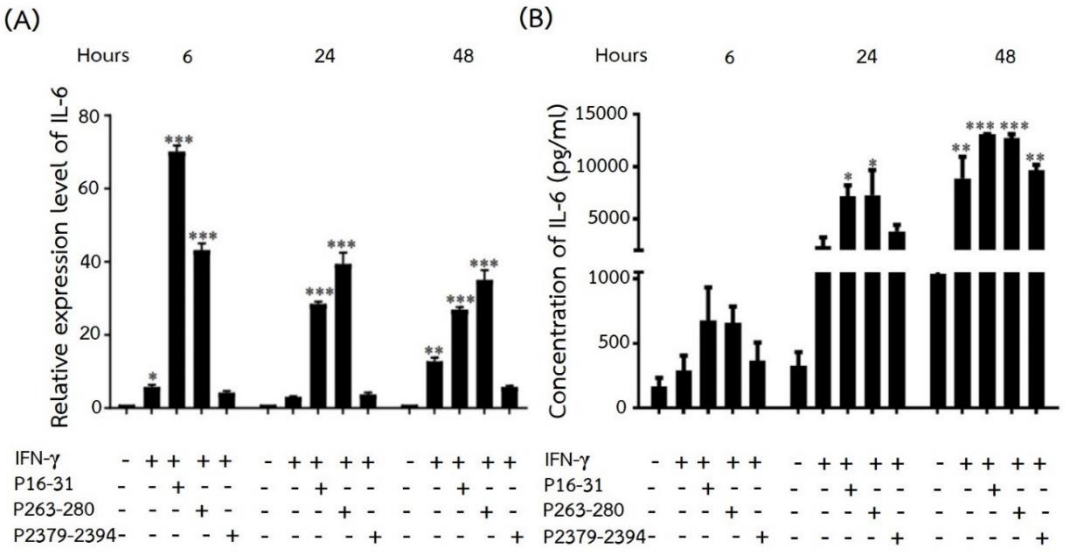


Figure 26 IL-6 production at various time points following stimulation with PG peptides and IFN-γ A, Expression of IL-6 mRNA expression in OA chondrocytes (N = 3) was investigated by real-time PCR after treatment with 50 ng/ml of IFN-γ and 100 µg/ml of PG peptides (P16-31, P263-280 or P2379-2394) at 6, 24 and 48 hours after stimulation. mRNA expression was standardized with expression levels of GAPDH and shown as relative expression level on y-axis. **B,** IL-6 protein levels from OA chondrocytes (N = 3) was determined by ELISA at 6, 24 and 48 hours after stimulation. Data is shown as mean ± SEM and statistical significance was calculated by one-way ANOVA with bonferroni post hoc test (*, $p < 0.05$; **, $p < 0.01$; *** $p \leq 0.001$).

Next, mRNA expression in stimulated chondrocytes were investigated using mRNA microarray. In this experiment, primary chondrocytes isolated from 3 knee OA patients were stimulated with IFN-γ in the absence and presence of P16-31, P263-280 and P2379-2394 for 6 hours and total RNA extraction was performed. The quality of extracted RNA was assessed via gel electrophoresis. We found that the extracted RNA from all samples revealed two bands of 28s and 18s ribosomal RNA, indicating good quality of RNA (Figure 27A). Moreover, IL-6 production was measured by ELISA to validate successful stimulation of chondrocytes by PG peptides. The pattern of IL-6 protein levels amongh the different stimuli conditions among the 3 samples were similar, suggesting reproducibility of the response (Figure 27B). The results also show that IL-6 production was highest when chondrocytes were stimulated with P16-31 (and IFN-γ) stimulation (Figure 27B).

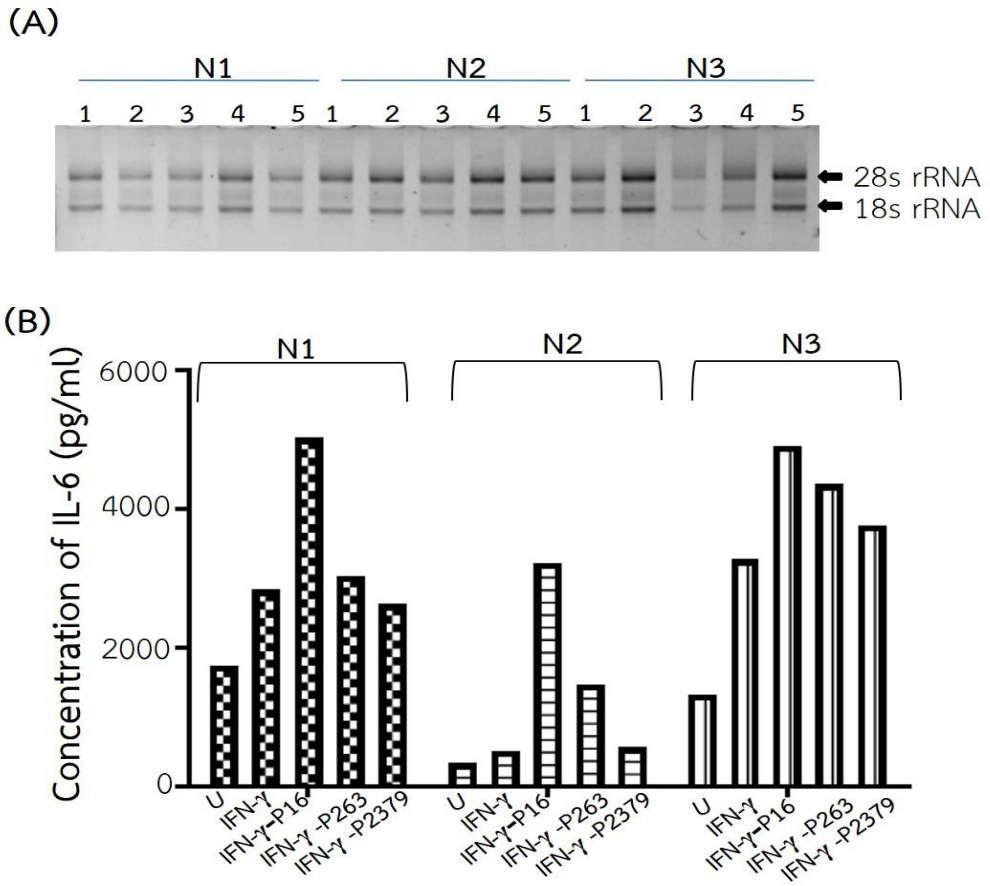


Figure 27 Total RNA extraction and validation of IL-6 production from peptide-stimulated chondrocytes. Chondrocytes from 3 knee OA patients were stimulated with 50 ng/ml of IFN-γ and 100 μg/ml of PG peptides (P16-31, P263-280 or P2379-2394) for 6 hours and collected for total RNA extraction. **A,** Quality of extracted RNA were assessed by gel electrophoresis. Numerical labeling represents each stimuli conditions (1, unstimulated; 2, IFN-γ only; 3, P16-31 and IFN-γ; 4, P263-280 and IFN-γ and 5, P2379-2394 and IFN-γ). Upper bands represent 28 rRNA and lower bands represent 18 rRNA (arrow). **B,** IL-6 production from OA chondrocytes were determined by ELISA at 6 hours after stimulation with IFN-γ with and without P16-31, P263-280 and P2379-2394. Concentration of cytokine is shown as pg/ml on X-axis.

Extracted total RNA from 15 samples (5 conditions from three individuals) was delivered to perform mRNA microarray on a Human Gene Expression 8x60K v3 platform (Agilent, Inc., Santa Clara, CA). Results of the number of upregulated and downregulated gene expressions are shown in Figure 28. Among the stimuli conditions, stimulation with P16-31 and IFN-γ induced the most number of uniquely

upregulating and downregulating genes (Figure 28). Compared to unstimulated chondrocytes, 1,628 differential expressed genes (DEGs) (value of fold change > 1.5 and p-value < 0.05) was found in stimulated chondrocytes. Chondrocytes treated with IFN-γ induced 504 DEGs (403 upregulated genes and 101 downregulated genes); with IFN-γ and P16-31 peptide induced 1,478 DEGs (944 upregulated genes and 534 downregulated genes); with IFN-γ and P263-280 peptide induced 846 DEGs (542 upregulated genes and 304 downregulated genes); and with IFN-γ and P2379-2394 peptide induced 676 DEGs (493 upregulated genes and 183 downregulated genes) (Figure 28). DEGs of chondrocytes stimulated with IFN-γ and the control peptide, P2379-2394, were approximate with DEGs of chondrocytes stimulated with IFN-γ only; and no differences was observed. Overlapping DEGs among the various conditions are shown in Figure 28.

To determmine the effect of direct peptide stimulation, DEGs in PG peptide and IFN-γ -stimulated chondrocytes were also compared with chondrocytes treated with IFN-γ only as well. We found that IFN-γ with P16-31 treatment induced 169 DEGs (156 upregulated genes and 13 downregulated genes), IFN-γ with P263-280 treatment induced 45 DEGs (39 upregulated genes and 6 downregulated genes) and IFN-γ with P2379-2394 treatment induced only 3 DEGs, in which all were upregulated genes (Figure 28). These results show IFN-γ with P16-31 treatment induced the most number of DEGs and that both IFN-γ with P16-31 and IFN-γ with P263-280 treatments effecte mRNA gene expression more profoundly than IFN-γ with P2379-2394 treatment.

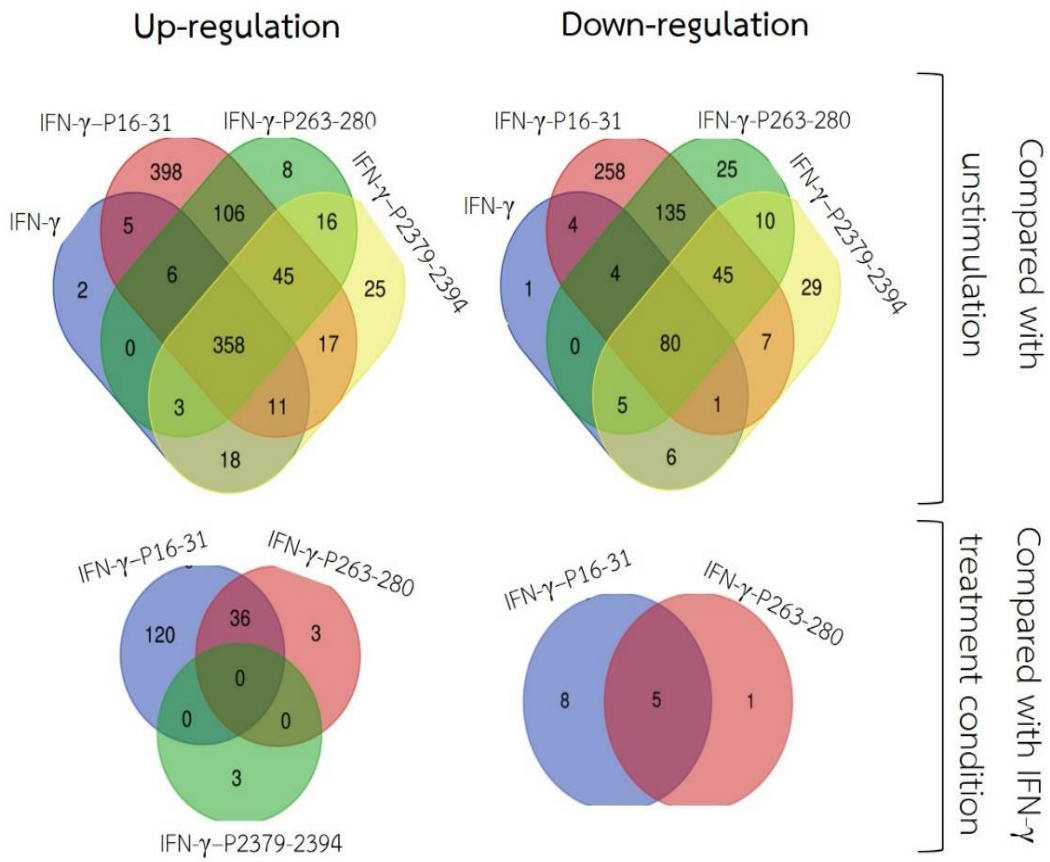


Figure 28 Differential gene expression analysis of human primary chondrocytes treated with various proteoglycan aggrecan peptides with IFN-γ. Venn diagrams showing overlapping upregulated and downregulated genes of chondrocytes stimulated with IFN-γ with and without PG peptides (P16-31, P263-280 and P2379-2394) (N=3 for each condition) compared with unstimulated (upper panel) or IFN-γ only stimulated conditions (lower panel).

Next, we analyzed the DEGs identified in chondrocytes treated with IFN-γ and either with P16-31 or P263-280 using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway to identify their functional biological roles. In total, these DEGs were categorized into 76 pathways ($p < 0.05$). However, only 9 out of 76 pathways were related to antigen processing and presentation, IL-6 production and inflammation.

The 9 pathways that we focused on consist of antigen processing and presentation, proteasome, phagosome, ubiquitin mediated proteolysis, protein processing in endoplasmic reticulum, NF-κB, TNF, JAK-STAT and MAPK signaling pathways. Ranking of these pathways was ordered according to the number of DEGs

found in each pathway. Among the 9 pathways, the majority of DEGs found in IFN-γ-treated chondrocytes as well as IFN-γ with P2379-2394-treated chondrocytes were in the antigen processing and presentation, following by TNF, JAK-STAT, phagosome and NF-κB pathways. On the contrary, the majority of DEGs found in IFN-γ with either P16-31 or P263-280-treated chondrocytes were categorized in TNF signaling, following by NF-κB signaling pathway, antigen processing and presentation, JAK-STAT, phagosome, proteasome and MAPK signaling pathways, indicating similar effects of P16-31 and P263-280 on gene expression in TNF and NF-kB pathways, but different from P2379-2394 peptide stimulation (Figure 29). Therefore, the first three pathways (antigen processing and presentation, TNF and NF-κB signaling pathway) which contained the most number of DEGs after stimulating with P16-31 and P263-280 peptides were collected to determine the details of expressing genes.

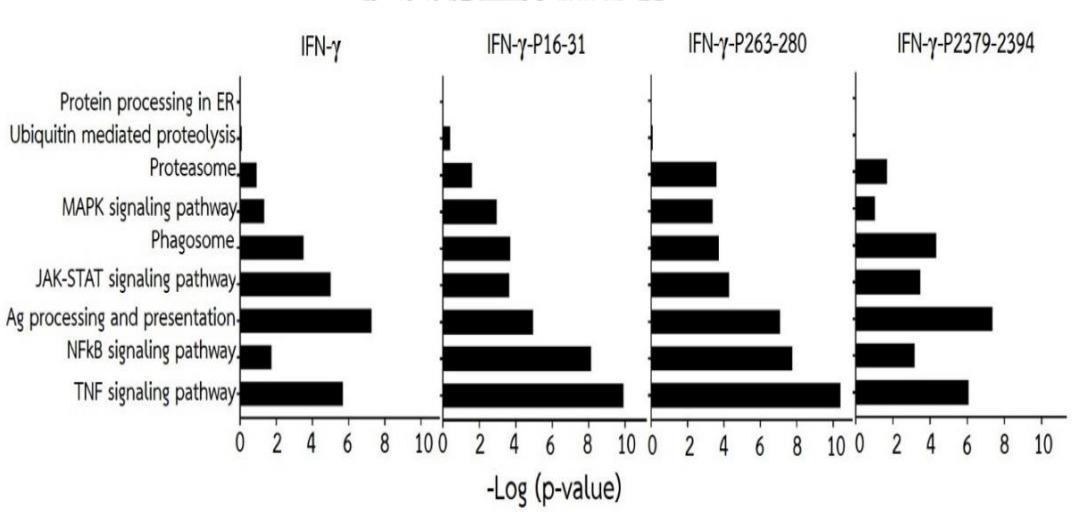


Figure 29 Categorization of differential expressed genes (DEGs) in chondrocytes stimulated with IFN-γ in the absence or presence of PG peptides. Classification of KEGG pathway for differential expressed genes in chondrocytes after IFN-γ and PG peptide treatment were generated by Enrichr tool. Y axis represents biological pathways and X axis represents -Log (p-value).

To investigate the expression level of genes in TNF signaling, NF-κB signaling, antigen processing and presentation pathways, we generated heatmaps of gene expression according to their fold change after comparing with unstimulated

condition (Figure 30). The DEGs found in phagosome and proteasome pathways were analysed with the antigen processing and presentation pathway. From the results, we found 68 DEGs (p-value < 0.05 and fold change > 1.5) involving in antigen processing and presentation, phagosome and proteasome. Among the 68 genes, there were 8 genes showing downregulation and 60 genes showing upregulation in IFN-γ with P16-31 or P263-280-treated chondrocytes when compared with unstimulated chondrocytes. Expression level of DEGs in IFN-γ with P16-31 or P263-280-treated chondrocytes is higher than IFN-γ-only or IFN-γ with P2379-2394 treated chondrocytes (Figure 30A).

Expression level of DEGs in antigen processing and presentation, phagosome and proteasome of chondrocytes treated with IFN-γ and PG peptides were also compared with chondrocytes treated with IFN-γ-only to determine the effects of PG peptides. We found that there were only 3 genes, inculuding *TLR2* (Toll like receptor), *B2M* (Beta-2 microglobulin) and *LAMP2* (Lysosome-associated membrane protein 2 or CD107b), showing upregulation in IFN-γ with P16-31 or P263-280-treated chondrocytes, while IFN-γ with P2379-2394-treated chondrocytes showed downregulation of these genes (Figure 30B, Table 3).

For NF-κB signaling pathway, there were 28 DEGs (p-value < 0.05 and fold change > 1.5) found in this pathway. P16-31 and P263-280 induced up regulation of 26 out of 28 genes and these expression levels were higher than IFN-γ-only and IFN-γ with P2379-2394 treated chondrocytes (Figure 30A). When compared with IFN-γ-only treatment, we found that 12 genes, including *NFKB1, NFKB2, REL-B, BIRC2, ICAM1, VCAM1, CCL4L2, NFKBIA, TRAF1, PTGS2, BIRC3* and *TNFAIP3,* were upregulated (Table 3) in IFN-γ with either P16-31 or P263-280 treated conditions. For IFN-γ with P2379-2394 treatment, there were no difference in gene expression level from IFN-γ-only treatment (Figure 30B).

For TNF signaling pathway, there were 35 DEGs found in this pathway when compared with unstimulated chondrocytes. In total, 25 genes were up regulated in IFN-γ with either P16-31 or P263-280 treated chondrocytes and these expression levels were higher than that of IFN-γ-only or IFN-γ with P2379-2394 treated

chondrocytes (Figure 30A). When compared with IFN-γ-only treatment, both IFN-γ with P16-31 and IFN-γ with P263-280 treatments upregulated 15 genes (*CCL20, IL-6, LIF, BIRC2, BIRC3, TNFAIP3, CXCL1, PTGS2, NFKB1, CCL2, ICAM1, VCAM1, NOD2, NFKBIA* and *TRAF1*) when compared to IFN-γ-only treatment, while, the difference of expression level of these genes could not be found in IFN-γ with P2379-2394 treatment (fold change < 1.5) (Figure 30B, Table 3).

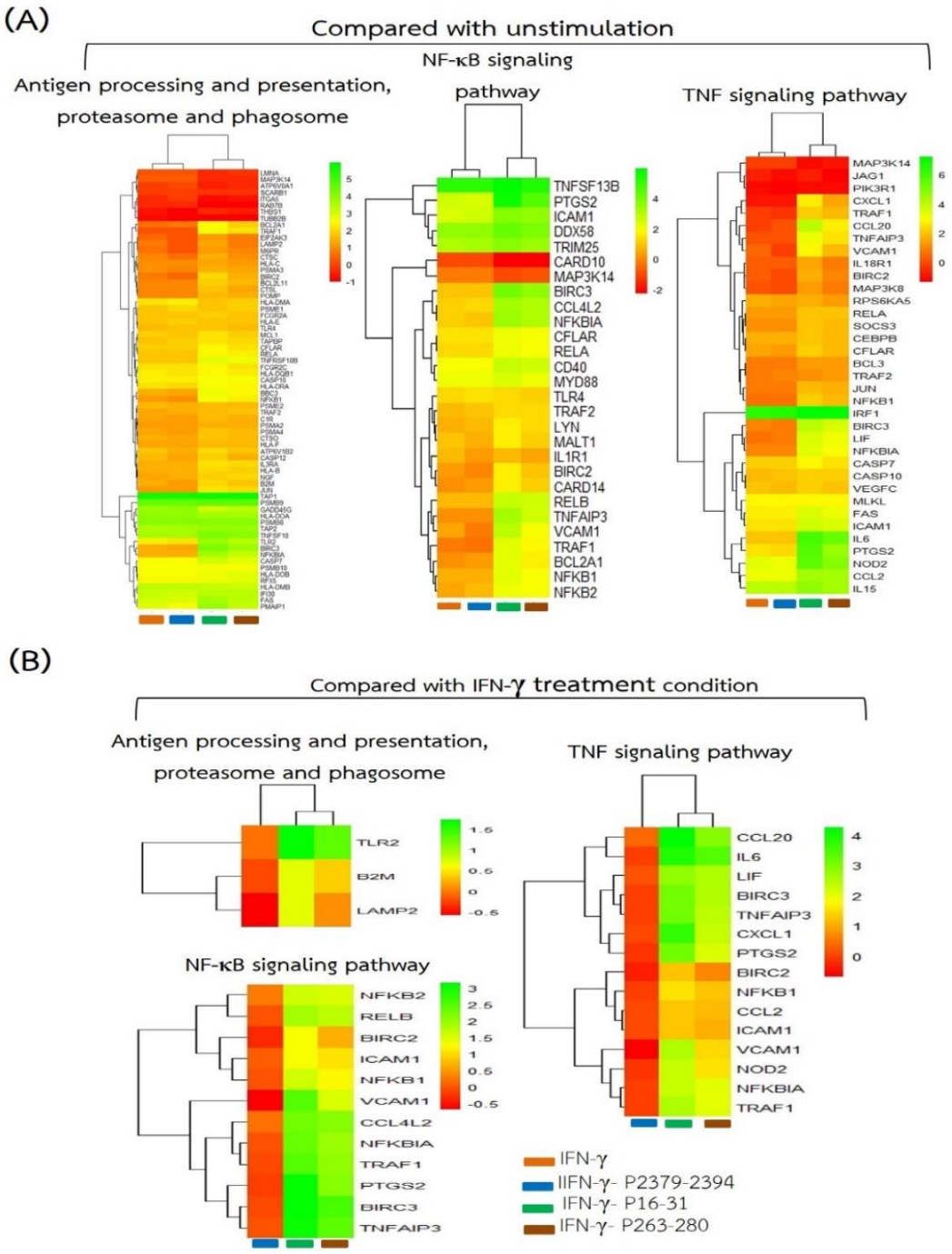


Figure 30 ***Heatmaps illustrating differential gene expression (DEGs) related to antigen presentation, NF-κB and TNF signaling pathways. A,*** *Heatmap of differential expressed genes in IFN-γ -only and IFN-γ with PG peptides stimulated chondrocytes compared with unstimulated chondrocytes.* ***B,*** *Heatmap of differential expressed genes in IFN-γ with P16-31, P263-280 and P2379-2394 stimulated chondrocytes compared with IFN-γ-only stimulated chondrocytes. The map contains log_2 (fold change) ratio that are color coded with red (down regulation) and green (up regulation).*

Table 3 Expression level and function of DEGs of PG peptide- IFN-γ -treated condition compared with IFN-γ-only treated condition

Gene names	Fold change (Compared with IFN-γ-only treatment)			Gene function
	IFN-γ P16-31	IFN-γ 263-280	IFN-γ P2379-2394	
Antigen processing and presentation, phagosome and proteasome				
TLR2	3.331125	2.470675	-1.033920	Pathogen recognition and innate immunity activation
B2M	1.608368	1.278467	-1.182088	Component of MHC class I molecule
LAMP2	1.605029	1.072684	-1.489385	Function on maintaining of integrity, pH and catabolism in lysosome
NF-kB and TNF signaling pathway				
REL-B	3.731663	3.731663	-1.042940	Induce non-canonical NF-kB pathway
CCL4L2	4.834336	4.304353	1.163804	Chemoattractant for natural killer cells, monocytes and variety of immune cells
NFKB2	2.966950	2.903252	1.191440	Transcription factor control gene expression in NF-kB pathway
NFKB1	2.980197	2.311079	-1.044738	Transcription factor control gene expression in NF-kB pathway
BIRC2	2.393013	1.589184	-1.276374	Inhibition of apoptosis
BIRC3	8.801910	5.916575	-1.051193	Inhibition of apoptosis
ICAM1	2.382048	2.064606	1.045496	Mediate the adhesion of leukocytes to endothelial cells
VCAM1	5.634096	2.792565	-1.601540	Mediate the adhesion of lymphocytes, monocytes or eosinophils and basophils to vascular endothelium
NFKBIA	5.102938	4.006501	-1.024998	Inhibition of transcription factor expression in NF-kB pathway
TNFAIP3	8.534879	5.307826	-1.004103	Inhibit TNF mediated apoptosis
TRAF1	5.726347	4.127761	-1.047411	Induce activation of MAPK8/JNK and NF-kB pathway
PTGS2	9.015040	4.285524	-1.136038	Production of prostaglandin H2 in inflammation
CCL20	19.159498	7.507919	1.303211	Responsible for DCs, effector or memory T cells and B cells recruitment in inflammatory condition
IL-6	15.137315	10.387211	-1.002753	Stimulation of acute phase protein synthesis and support B cell growth
LIF	6.961494	5.842411	1.111980	Promote cell growth and cell differentiation
CXCL1	12.768829	5.051317	1.041438	Chemoattractant for neutrophils and other immune cells and regulate immune and inflammatory response
CCL2	2.392677	2.177750	1.023285	Monocytes, memory T cells and DC recruitment
NOD2	4.917219	2.982187	-1.123128	Recognize bacterial molecules and stimulates immune system

To validate the accuracy of RNA microarray, we chose 12 genes from the antigen processing and presentation, phagosome, proteasome, NF-κB and TNF signaling pathways and peformed quantitative real time PCR to determine 5 their mRNA expression levels in 5 knee OA patients. We chose four genes (*IL-6, MAP3K8, CCL2* and *JUN*) from TNF signaling pathway, five genes (*NFKB1, NFKB2, REL-A, REL-B* and *MYD88*) from NF-κB pathway, and two genes (*TAP1* and *TLR2*) from antigen processing and presentation. We also collected *COL2A* (type II collagen alpha chain) as a representative downregulated gene. From the results, 9 out of 12 genes, including *IL-6, CCL2, JUN, MAP3K8, NFKB1, NFKB2, REL-A, REL-B* and *TLR2*, in IFN-γ with either P16-31 or P263-280 treated chondrocytes showed significant upregulation when compared with IFN-γ-only and IFN-γ with P2379-2394 treated chondrocytes (Figure 31). Expression level of *MYD88, TAP1* and *COL2A* genes in IFN-γ with either P16-31 or P263-280 treated chondrocytes did not differ from that of IFN-γ-only and IFN-γ with P2379-2394 treated chondrocytes. These validation results show that the levels of gene expression in each stimulated condition correlated with the RNA microarray results, indicating reliability of gene expression data.

In addition to antigen processing and presentation, NF-κB and TNF signaling pathways, the effect of PG peptides on proteolytic enzymes production in chondrocytes was also evaluated. Proteolytic enzymes found in this study consisted of 16 metrix metalloprotenases (MMPS) and 25 disintegrin and metalloprotenase with thrombospondin (ADAMTS). Among 41 proteolytic enzyme expressed genes, IFN-γ with either P16-31 or P263-280 treated chondrocytes showed a tendency to increase expression level of 14 genes, including *MMP1, MMP2, MMP3, MMP9, MMP10, MMP12, MMP13, MMP19, MMP25, ADAMTS4, ADAMTS5, ADAMTS9, ADAMTS7P1* and *ADAMTSL3* (Figure 32A). To determine the proteolytic enzyme gene expression that respond to PG peptides, we also compared gene expression levels in IFN-γ-PG peptide treated chondrocytes with IFN-γ-only treated chondrocytes. IFN-γ-P16-31 treated chondrocytes expressed both MMP and ADAMTS at high levels, particularly MMP1, MMP12 and MMP13; which upregulated more than 1.5-fold when compared with IFN-γ-only treated chondoryctes. For ADAMTS, IFN-γ-P16-31 and IFN-γ-P263-280 treated chondrocytes up-regulated expression levels of ADAMTS4 and ADAMTS9 higher than

1.5-fold when compared with IFN-γ-only treated chondoryctes. Upregulation of these genes were not observed in IFN-γ-P2379-2394 treated chondroyctes (Figure 32B). These findings indicate that both P16-31 and P263-280 peptides increased expression level of proteolytic enzymes in chondrocytes, in which may effect cartilage degradation in OA.

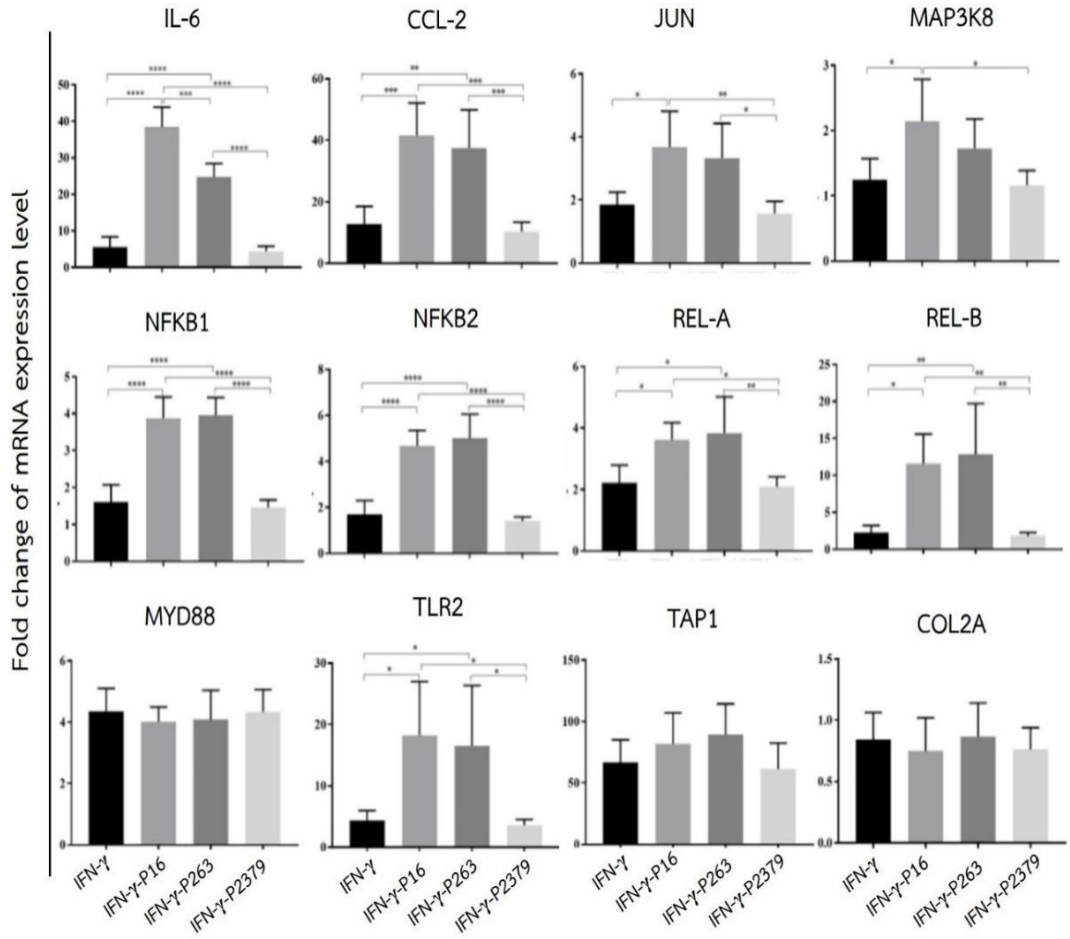


Figure 31 ***Gene expression validation.*** *Validation of RNA microarray results with quantitative real-time PCR. Twelve selected genes were tested and gene expression level was compared using one way-ANOVA and Bonferroni correction (*, p < 0.05; **, p < 0.01; ***, p ≤ 0.001).*

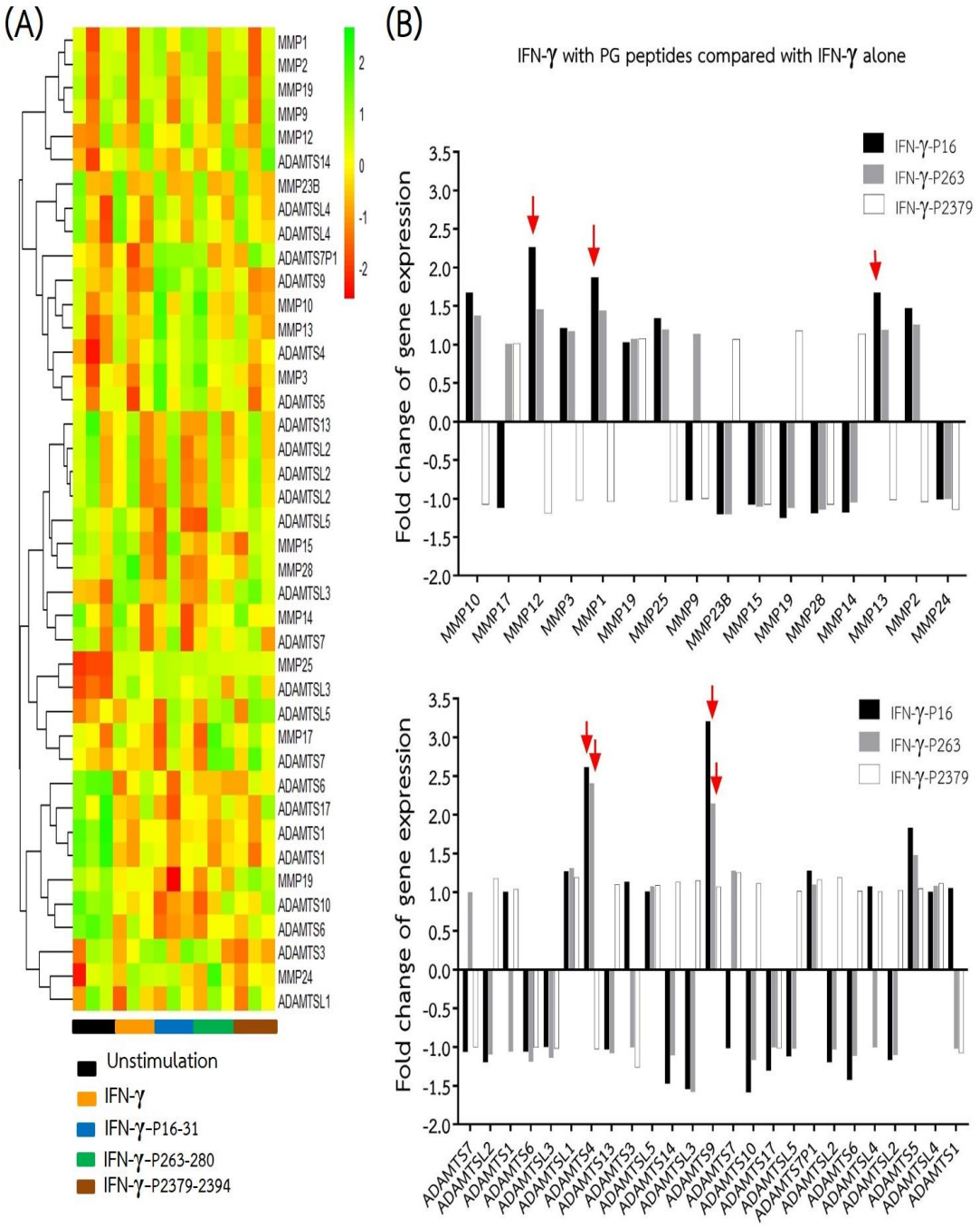


Figure 32 Gene expression of proteolytic enzymes in PG peptide stimulated chondrocytes. **A,** Heatmap showing expression level of MMPs and ADAMTS genes in unstimulated, IFN-γ-only and IFN-γ with eighter P16-31, P263-280 or P2379-2394 peptides treated chondrocytes from 3 OA patients. Gene expression level in each row was showed as Z-score that are color coded with red (low expression level) and green (high expression level). **B,** Graph showing fold change of MMPS and ADAMTS genes expression in IFN-γ with eighter P16-31, P263-280 or P2379-2394 peptides treated chondrocytes compared with IFN-γ-only treated chondrocytes.

The data from this part showed that PG peptides, P16-31 and P263-280, might induce IL-6 production through stimulation of TNF signaling pathways. Also, P16-31 and P263-280 along with IFN-γ might stimulated chondrocytes from OA patients upregulates genes in the antigen processing and presentation, and NF-κB signaling pathway. These two pathways can promote expression of co-stimulatory molecules and antigen presentation and supports the cell surface upregulation data of CD80/CD86 on chondrocytes in the previous chapter. Moreover, P16-31 and P263-280 stimulated chondrocytes increased MMPs and ADAMTS expression in OA chondrocytes.

PART III

(ANTIGEN PRESENTATION FUNCTION OF CHONDROCYTES)

After determining the effects of IFN-γ and PG peptides on chondrocytes, we also wanted to know whether these stimuli can induce chondrocytes to stimulate T cells. In this part, we investigated the ability of chondrocytes to present PG peptides to autologous T cells from infrapatellar fat pads (IPFPs) of OA patients and determined their T cell proliferation.

PG peptide presentation of chondrocytes to T cells induces T cell proliferation

We started this experiment by isolating mononuclear cells from IPFPs. Because the number of T cells that we obtained were not enough for experiments, cells were expanded *in vitro* by stimulating with anti-CD3/anti-CD28 dynabeads with IL-2 for 7 days. Before using the cells, anti-CD3/anti-CD28 dynabeads were removed and cells were rested for 2 days until expression level of activation markers (CD25 and CD69) decreased to basal level to eliminate background of cell stimulation. The percentage of CD3+ T cell population and expression level of activation markers was determined by flow cytometry. We found that isolated cells from IPFPs before anti-CD3/anti-CD28 dynabead stimulation (day 0) contained CD3+ T cells 56.3% of mononuclear cell lymphocyte gating, but after stimulation, the number of CD3+ T cells increased and the percentage reached 98% (day 7) (Figure 33). We also found that CD69 and CD25 expression level increased in the CD3+ T cell population (5.49% and 88.9%, respectively) (Figure 33). After cell resting (day 9), percentage of the cells that expressed CD69 and CD25 was reduced, while the percentage of CD3+ T cells was still high (95.2%). This indicates that the expanded T cells had high purity and cells were at resting state when used in experiments.

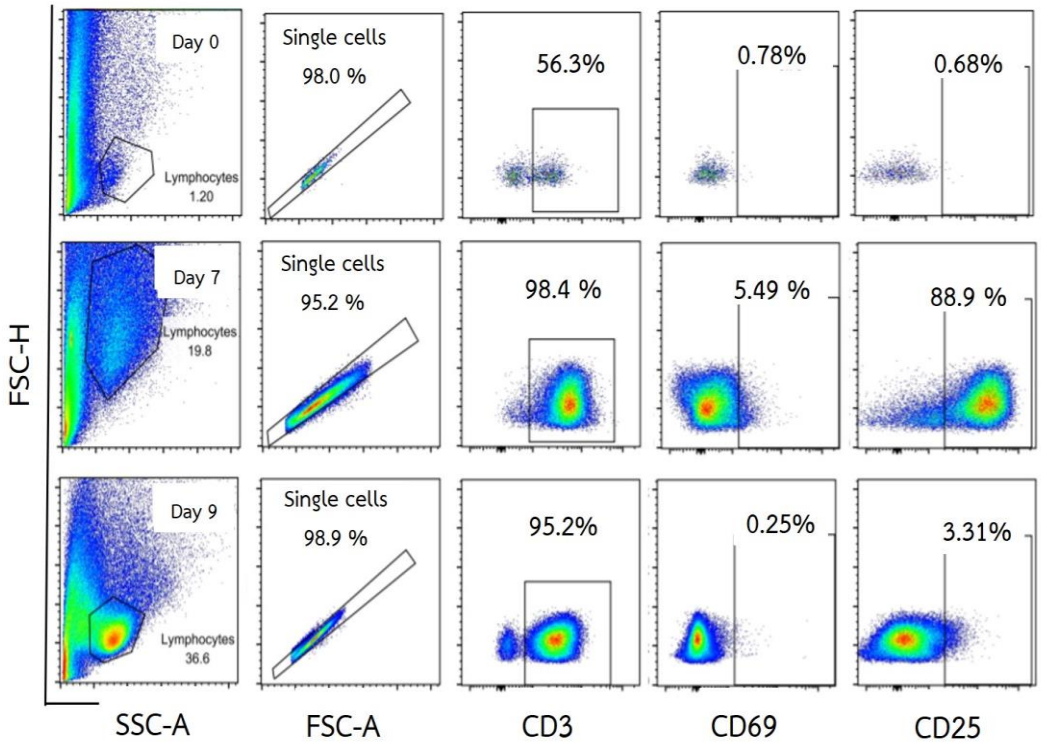


Figure 33 FACS plot showing expansion of T cells from IPFPs of knee OA patients. Mononuclear cells isolated from IPFPs of knee OA patients were stimulated with anti-CD3/anti-CD28 beads with 20 ng/ml of IL-2 and cultured for 7 days. After stimulation, anti-CD3/anti-CD28 beads were removed and cells were rested for 2 days (day 9). Expression of CD3 and activation markers, including CD69 and CD25, were determined on day 0, day 7 and day 9 by flow cytometry.

Next, we test antigen presentation function of chondrocytes to present PG peptides to expanded autologous T cells. In this experiment, chondrocytes isolated from knee OA patients were treated with IFN-γ and pulsed with P16-31, P263-280, P2379-2394, or positive control peptides, including CEF and OVA peptides. Expanded T cells were labeled with CFSE. The stimulated chondrocytes were co-cultured with the expanded T cells at a ratio of 1:10 in the presence of IL-2 for 7 days. T cells alone and anti-CD3/anti-CD28 dynabead-stimulated T cells were used as negative and positive controls, respectively. The decrease in fluorescence intensity of CFSE-labeled cells detected by flow cytometry represents proliferating T cells. Net T cell proliferative level in each condition was calculated from subtraction of percentage of

T cell-alone proliferation from percentage of stimulated T cell proliferation of each individual patient. From a representative experiment, the percentage of T cell proliferation after co-culture with unstimulated chondrocytes or IFN-γ only-treated chondrocytes showed an increase up to 34.3% and 38.6%, respectively, from 33.4 % (T cells alone condition) (Figure 34). T cell proliferation showed an increase in proliferation when co-cultured with P16-31 and P263-280 pulsed chondrocytes (56.5% and 50.8%, respectively). This level of proliferation was similar to the positive control conditions (T cells co-culturing with CEF peptide-pulsed chondrocytes or T cells stimulated with anti-CD3/anti-CD28 dynabeads had a 58.2% and 56.9 %, respectively) (Figure 34).

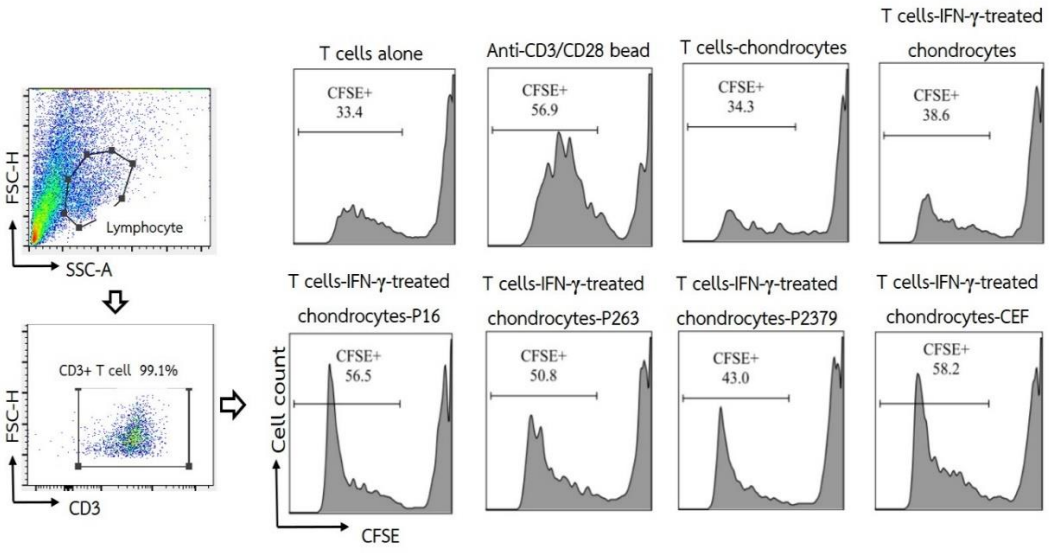


Figure 34 FACS plot and histogram showing T cell proliferation when co-cultured with chondrocytes with different stimuli. Primary chondrocytes were stimulated with 50 ng/ml of IFN-γ and pulsed with 10 μg/ml of PG peptides (P16-31, P263-280 or P2379-2394) or control peptide (OVA and CEF peptides) for 3 hours before co-culturing with CFSE-labeled autologous T cells from IPFPs (ratio chondrocytes : T cell = 1:10) for 7 days. T cells stimulated with anti-CD3/anti-CD28 beads were used as a positive control. The decrease in fluorescence intensity of CFSE-labeled cells detected by flow cytometry represents proliferating T cells. Net T cell proliferative level in each condition was calculated from subtraction of percentage of T cell-alone proliferation from percentage of stimulated T cell proliferation of each individual patient.

We found that IFN-γ-treated chondrocytes significantly increased the percentage of T cell proliferation when compared to T cells co-cultured with unstimulated chondrocytes (Figure 35A). This increase in T cell proliferation level was correlated with the level of T cell proliferation when stimulated with anti-CD3/anti-CD28 dynabeads. The proliferation level of T cells was increased when co-cultured with P16-31 and P263-280 pulsed chondrocytes, but not P2379-2394 pulsed chondrocytes. However, only P263-280 pulsed chondrocytes induced significantly higher T cell proliferation when compared with IFN-γ only-treated chondrocytes (p = 0.0104) (Figure 35B). These results show that chondrocytes can present P263-280 peptide to autologous T cells and induce significant increase in T cell proliferation, suggesting a significant stimulation from P263-280 peptide-pulsed chondrocytes.

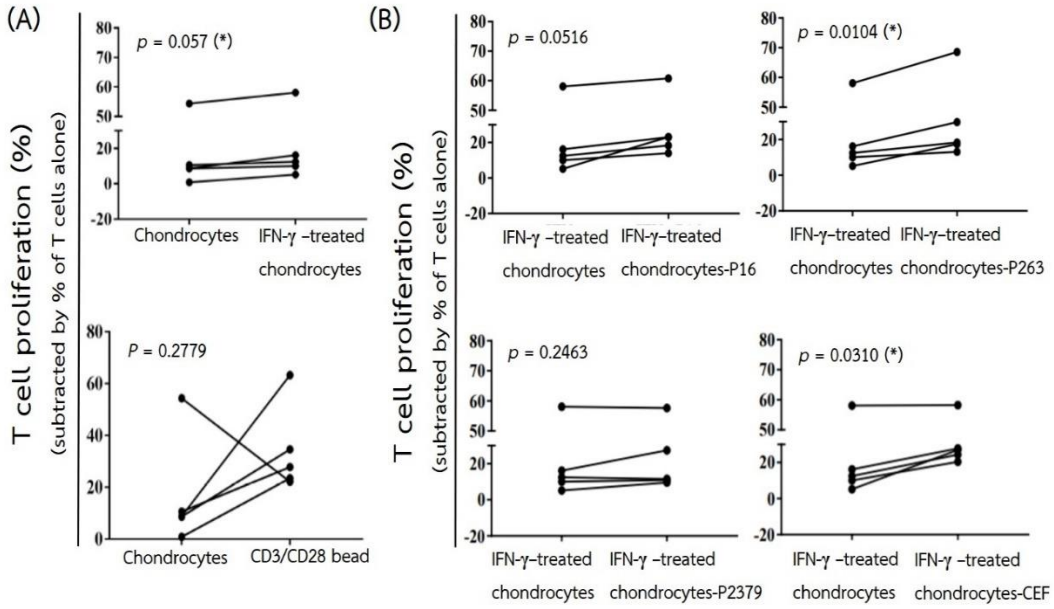


Figure 35 Analysis of T cell proliferation after co-cultured with stimulated chondrocytes. Primary chondrocytes (N=5) were stimulated with 50 ng/ml of IFN-γ and pulsed with 10 μg/ml of PG peptides (P16-31, P263-280 or P2379-2394) or control peptide (OVA and CEF peptides) for 3 hours before co-culture with CFSE-labeled autologous T cells from IPFPs for 7 days. **A,** Comparison of T cell proliferation between co-culture with IFN-γ only-treated chondrocytes and unstimulated chondrocytes in each individual. T cells stimulated with anti-CD3/anti-CD28 beads were used as a control. **B,** Comparison of T cell proliferation between co-culture with PG peptide pulsed-chondrocytes and IFN-γ -treated chondrocytes in each individual. Y axis represents percentage of T cell proliferation and X axis represents chondrocyte stimuli conditions. Net T cell

proliferative level in each condition was calculated from subtraction of percentage of T cell-alone proliferation from percentage of stimulated T cell proliferation of each individual patient. Differences of T cell proliferation was calculated by paired-T test (*, $p < 0.05$; **, $p < 0.01$; ***, $p< 0.001$).

To characterize the population of T cells that responded to PG peptide stimulation, we also investigated the proliferation of CD4+ and CD8+ T cells from CD3+ proliferating T cells (N=5). From the results, we found that the proliferated CD3+ T cells contained population 33-46% of T cells were CD4+ T cells and 25-35% of T cells were CD8+ T cells (Figure 36A). The population of CD4+ proliferating T cells increased when cells were co-cultured with IFN-γ-only, IFN-γ with P263-280, IFN-γ with CEF and IFN-γ with ovalbumin (OVA) treated chondrocytes. In contrast, CD8+ proliferating T cells decreased after co-culture with IFN-γ and P263-280 treated chondrocytes (Figure 36A).

We also determined the proportion of proliferation level in the CD4+ T cell and CD8+ T cell population in response to the different stimuli conditions. The percentage of proliferation of CD8+ T cells was overall higher than CD4+ T cells and increased when T cells were co-cultured with IFN-γ-treated chondrocytes. For peptide-pulsed chondrocytes, the percentage of CD8+ T cell proliferation was at similar levels as when co-cultured with unstimulated chondrocytes. On the contrary, percentage of proliferation of CD4+ T cells increased when T cells were co-cultured with IFN-γ-only, IFN-γ with P16-31, CEF and OVA peptide-pulsed chondrocytes. Although, there was no significant difference among T cell proliferation in each condition, however, P263-280 peptide-pulsed chondrocytes had the highest proliferation level of T cells than other conditions (Figure 36B). These findings suggest that chondrocytes can present certain PG peptides to T cells and the responsive T cell population may likely be CD4+ T cells.

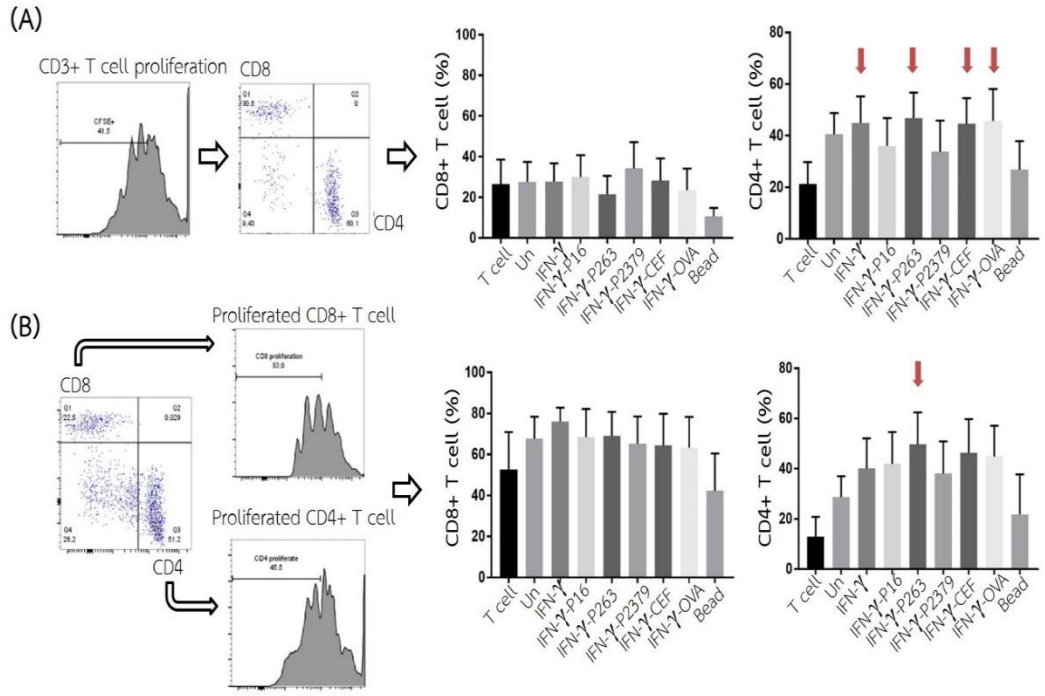
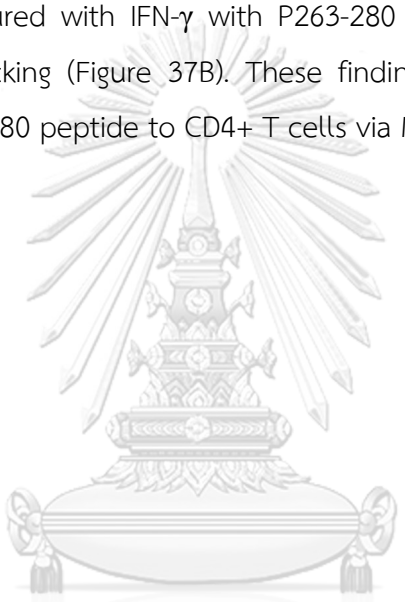


Figure 36 Determination of proliferating T cell subsets after co-culture with PG peptide-pulsed chondrocytes. A, FACS plot and bar graph showing percentage of CD4+ and CD8+ T cells found in population of proliferating CD3+ T cells after co-culture with unstimulated, IFN-γ -only or combination of IFN-γ and peptide-treated chondrocytes. **B,** FACS plot and bar graph showing the proportion of proliferation level of CD8+ T cells and CD4+ T cells after co-culture with unstimulated, IFN-γ-only or combination of IFN-γ and peptide-treated chondrocytes.

Effect of MHC blocking on antigen presentation function of chondrocytes to present PG peptides to T cells.

Next, we would like to investigate whether peptide presentation by chondrocytes to T cells was dependent on MHC. In this experiment, chondrocytes were treated with IFN-γ and incubated with ultra-LEAF purified anti-human HLA-A, B, C (clone w6/32) antibodies for MHC class I blocking or purified anti-human HLA-DR, DP, DQ (clone Tü39) antibodies for MHC class II blocking, before pulsing with PG peptides and co-culture with CFSE-labeled autologous T cells. Because we found that proliferation of T cells increased when T cells were co-cultured with P16-31 and P263-280 pulsed chondrocytes, therefore, this experiment focused on effect of MHC blocking only on P16-31 and P263-280 peptide stimulation conditions. Our results show high percentage of T cell proliferation in T cells co-cultured with IFN-γ -treated

chondrocytes and this was further increased when chondrocytes were pulsed with P263-280 peptide (Figure 37A). MHC class I blocking did not reduce T cell proliferation in condition of IFN-γ only, IFN-γ with P16-31 and IFN-γ with P263-280 treated chondrocytes when compared with nonblocking condition. For MHC class II blocking, T cells from all OA patients showed an increase in proliferation level when co-cultured with IFN-γ -only treated chondrocytes, but had no effect on T cell proliferation when co-cultured with IFN-γ and P16-31 peptide-treated chondrocytes. The percentage of T cell proliferation had a tendency to decrease in all 6 OA patients when co-cultured with IFN-γ with P263-280 peptide-treated chondrocytes with MHC class II blocking (Figure 37B). These findings suggest that chondrocytes perhaps present P263-280 peptide to CD4+ T cells via MHC class II.



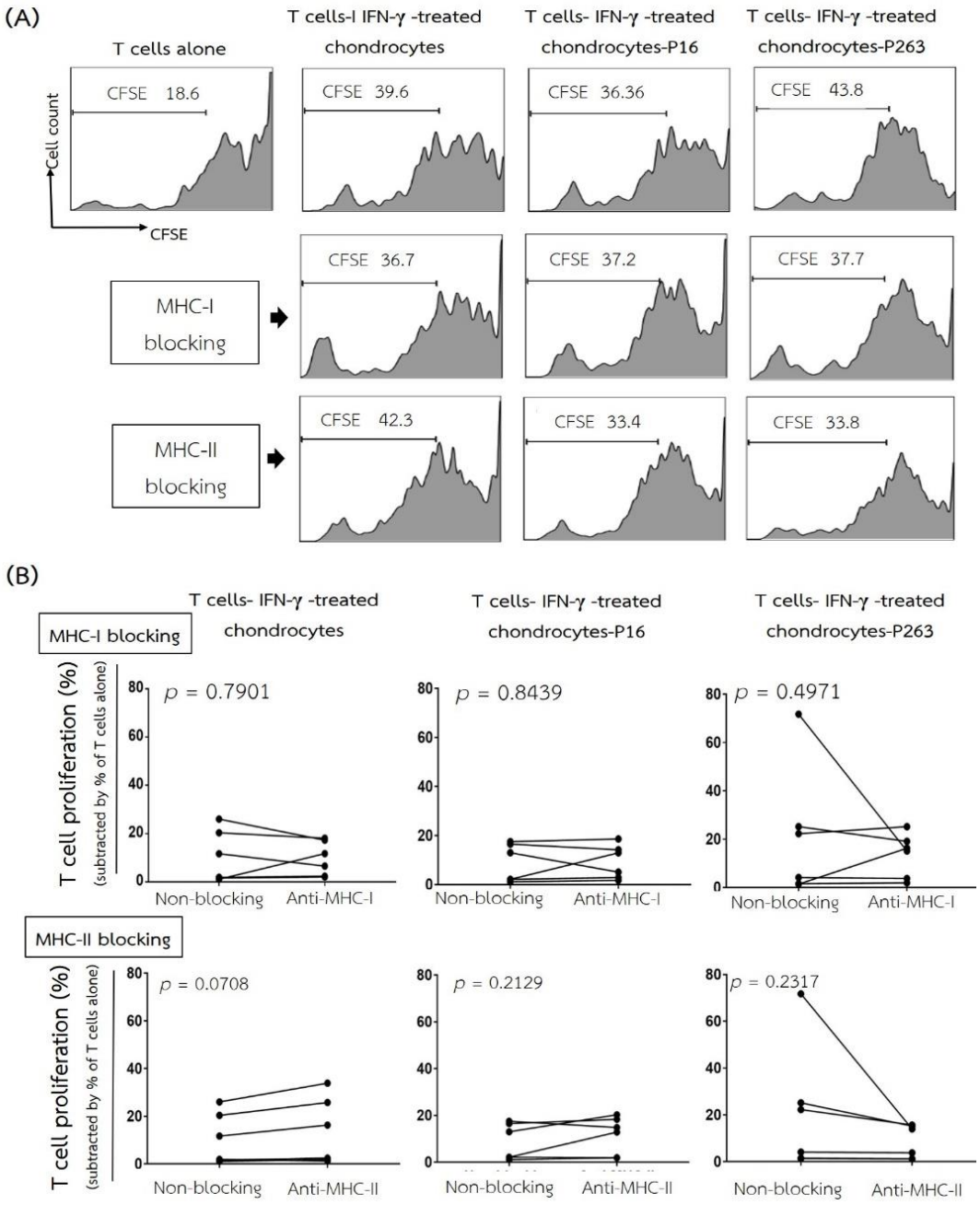


Figure 37 Effect of MHC blocking on T cell proliferation. IFN-γ-treated **c**hondrocytes were pre-treated with 10 µg/ml of ultra-LEAF purified anti-human HLA-A, -B, -C (clone w6/32) or purified anti-human HLA-DR, DP, DQ (clone Tü39) for 1 hours before pulsing with P16-31 and P263-280 peptides and co-culture with CFSE-labeled autologous T cells for 7 days. **A,** Histograms of fluorescence intensity of CFSE showing percentage of T cell proliferation after co-culture with PG peptide-pulsed chondrocytes. **B,** Comparison of T cell proliferation between co-culture with non-blocking and MHC-I and MHC-II blocking on PG peptides pulsed-chondrocytes in each individual patient (N=6). Y axis represents percentage of T cell proliferation and X axis shows condition of MHC blocking on chondrocytes. T cell proliferation was ca Net T cell proliferative

level in each condition was calculated from subtraction of percentage of T cell-alone proliferation from percentage of stimulated T cell proliferation of each individual patient. Differences of T cell proliferation was calculated by paired-T test (*, $p < 0.05$; **, $p < 0.01$; ***, $p< 0.001$).

In this part, we show that chondrocytes carry antigen presentation function when exposed to stimuli, such as IFN-γ, and can stimulate T cells to proliferate. When chondrocytes were exposed to certain PG peptides, they were able to present those PG peptides, especially P16-31 and P263-280 peptides, to autologous T cells. Our data suggests the P263-280 peptide can be presented to CD4+ T cells via MHC class II that is expressed on chondrocytes, resulting in an increase in CD4+ T cell proliferation.

CHAPTER IV

DISCUSSION

In this study, we investigated antigen presentation function of chondrocytes in stimulating IPFP T cells of OA patients. The cartilage is an immunological privileged site with chondrocytes separated from the outer environment by extracellular matrix (ECM). Therefore, chondrocytes are not exposed to any stimuli and are thought to not have immunological function in normal condition (96). However, in OA, chondrocytes can secrete several inflammatory cytokines and express antigen presentation markers on the cell surface (69, 97). We hypothesized that cartilage is broken down exposing chondrocytes to several stimuli in synovium. In our study, we interrogated stimuli that can activate and induce antigen presentation function of chondrocytes in OA.

There are many studies reporting about antigen presentation function of chondrocytes (69, 86). Actually, these antigen presentation molecules, apart from MHC class I, could be rarely detected in normal human chondrocytes (96). However, expression of MHC class II can be induced by IFN-γ. Induction of MHC molecules by IFN-γ could be detected in human nasal chondrocytes and in transgenic mice (87, 98). These evidences related to our *in vitro* results of OA chondrocytes that revealed upregulation of MHC class I, MHC class II and co-stimulatory molecules (CD40, CD80 and CD86) expression after treatment with IFN-γ. On the contrary, chondrocytes isolated from cartilage of ovalbumin immunized rabbits showed high expression of MHC class II (99). In 2009, Sakata M. and his colleagues found that chondrocytes isolated from OA patients also expressed both MHC class I and MHC class II on their surface, but these molecules could not be observed on normal chondrocytes (69). These evidences indicate that human chondrocytes may require inflammation to upregulate antigen presentation molecules.

Expression of MHC I and II on chondrocytes is likely the main feature that can stimulate T cells. In OA, T cells responded to autologous chondrocytes by

proliferating, in which proliferation can be blocked by antibodies against MHC molecules (69). Activation of T cells by chondrocytes seems likely through MHC/antigen/T cell receptor complex; however, the stimulating antigen in OA is not clearly identified. Many previous studies mentioned that the antigen might be produced by chondrocytes. Evidence that support this hypothesis is the presence of autoantibodies against collagen and osteopontin in serum of OA patients (100, 101). Proteomic studies also revealed accumulation of proteoglycan aggrecan (PG) core protein, which is a hallmark of cartilage degradation, in synovial fluid of OA patients (65). G1 domain in PG core protein contained several arthritogenic T cell epitopes, especially P16-31 and P263-280 peptides, in which can stimulate autoreactive T cells in peripheral blood of OA patients (16). Although, OA is not classified as an autoimmune disease; however, T cell responses to autoantigens may be a secondary phenomenon that occur due to extensive degradation of cartilage fragment.

Because PG fragments could be found in synovial fluid of OA patients, there is a high chance that chondrocytes are exposed to them. Therefore, PG fragments may stimulate and induce antigen presentation function in chondrocytes. de Jong *et al*. found specific T cell epitope region on PG fragment, including P16-31 and P263-280 (16). Therefore, we treated these peptides to OA chondrocytes and investigated chondrocyte response. After exposure to PG peptides, MHC molecules did not increase on chondrocyte cell surface, but, interestingly, both P16-31 and P263-280 treatment induced higher expression level of co-stimulatory molecules, CD80 and CD86. Moreover, expression level of CD80 and CD86 increased when the concentration of PG peptides increased. The expression of co-stimulatory molecules after stimulating with cartilage protein was also observed on chondrocytes isolated from collagen induced arthritis (CIA) mice, resulting in increasing of susceptibility to arthritis (102). In other antigen presenting cells, collagen injected mice also expressed CD80 and CD86 on cell surface of macrophages and inhibition of these molecules reduced the severity of arthritis (103).

Both CD80 and CD86 have predominant function in activation of immune response by binding to its cognate receptor, CD28, on T cells, resulting in T cells

activation, proliferation and differentiation (104). On the other hand, the absence of CD80 and CD86 engagement prohibits T cell activation and proliferation, leading to T cell anergy and apoptosis (105). The mechanisms of antigen inducing co-stimulatory molecules expression in chondrocyte is not known. However, regulation of CD80 or CD86 expression was studied in other antigen presenting cells. In B cells, co-stimulatory molecules were up-regulated upon engagement of IL-4 receptor, B cell receptor and toll like receptor 4 (TLR4) (106-108). Also, binding of glycoprotein 39 to CD40 increased expression level of CD86 on B cells (109). Moreover, X- irradiation could induce expression of CD80 on lipopolysaccharide (LPS) stimulating B cells through NF-κB activation (110). Expression of CD80 after exposure to X-irradiation was also observed in dendritic cells, but this expression was not controlled by NFκB pathway (110). These reports show that expression of co-stimulatory molecules on each antigen presenting cell are activated and controlled by different pathways. Our study showing upregulation of CD80 and CD86 in P16-31 and P263-280 treated chondrocytes, but not for P16-31 and P263-280 treated keratinocyte and MoDCs, may provide preliminary insight into how co-stimulatory molecules are regulated in chondrocytes.

Cytokine production is a key feature used to identify activated cells. In OA, inflammatory cytokines, including IL-1α, IL-1β, IL-6, IL-8 and TNF-α and IFN-γ could be observed both in synovial fluid and cartilage tissues. Chondrocytes in cartilage tissue could produce these cytokines (97). Elevated levels of cytokine production of chondrocytes could be induced by several stimuli. Stimulating chondrocyte with synovial fluid of OA patients increased the concentration of IL-6, IL-8 and MCP-1 (Monocyte chemoattractant protein-1, CCL2) (95). Also, chondrocytes responded to factors such as substance P and bradykinin, neurovascular peptides found in synovial fluid of OA patients, by increasing IL-6 and proteolytic enzyme (ADAMTS) production (111). These findings correlated with our results that high concentrations of IL-6 and IL-8 was secreted from IFN-γ and P263-280 treated chondrocytes. Both IL-6 and IL-8 play an essential role in knee OA. IL-6 promotes inflammation in the local joint and increase of this cytokine correlates with severity of the disease (112). IL-8 recruits

neutrophil and other granulocytes into the inflamed joint and induces cartilage destruction (113).

Gene expression profile in chondrocyte treated with combination of IFN-γ and either P16-31 or P263-280 showed upregulation of a number of genes in the antigen processing and presentation, TNF signaling and NF-κB pathways when compared with unstimulated chondrocytes. However, when compared with IFN-γ treated chondrocytes, the number of differential expressed gene could be found mostly in TNF and NF-κB signaling pathways. It means that TNF and NF-κB signaling are more likely to be from the effects of the P16-31 and P263-280. TNF and NF-κB signaling pathways play a pivotal role in cytokines, chemokines and immune cell regulation, leading to inflammation and immune responses (114). In OA, NF-κB signaling pathway is very essential, not only for cytokine production, but it also regulates ECM production and degradation (114). Extracellular matrix fragments such as fibronectin can stimulate chondrocytes to secrete high levels of IL-6, IL-8 and MCP-1 and cytokine production was decreased after blocking the NF-κB signaling pathway (115). Upregulation of the transcription factors NFKB1 and NFKB2 in NF-κB signaling genes was also previously seen in IL-1β treated chondrocytes. Upregulation of these two transcription factors correlated with an increase of IL-6 and IL-1β gene expression (116). NF-κB signaling pathway is also essential to regulate expression of antigen presentation molecules in human dendritic cells and blocking of this pathway by specific inhibitors can inhibit expression of HLA-DR, CD83, CD86, 80 and CD40 on their surface (117, 118). These evidence supports the essential function of NF-κB signaling in cell maturation and antigen presentation that might correlate with expression of co-stimulatory molecules in our study.

Chondrocytes have been reported to phagocytose cartilage components such as collagen and possess antigen presentation function (119). In ankylosing spondylitis (AS), chondrocytes presented Epstein-Barr virus-derived peptide, (EBNA) 258-266, to HLA-B27 restricted CD8+ T cells and induced IFN-γ, perforin and granzyme B production (86). This antigen presentation function of chondrocytes was also observed in mice. IFN-γ treated chondrocytes presented influenza A virus

nucleoprotein derived peptide to HLA-B27 restricted CD8+ T cell, resulting in chondrocyte lysis (87). The ability of chondrocytes to present antigens to T cells was also observed in our study. We observed significantly higher proliferative responses of IPFP T cells co-cultured with IFN-γ and P263-280 treated chondrocytes when compared with T cells co-cultured with IFN-γ-only or IFN-γ with other PG peptides treated chondrocytes. Moreover, P263-280 peptide increased proliferation in CD4+T cell subset by present via MHC class II. Also, there were CD4+ T cells in blood of OA and AS patients that could respond to the G1 domain fragments by producing inflammatory cytokines (16, 120). In OA, besides peripheral blood, P263-280 specific T cell population (16) were also found in IPFPs because they could respond to P263-280 presentation by chondrocytes. The G1 domain of proteoglycan aggrecan was reported to contain several T cell epitopes which showed high theoretical binding score with MHC class II, particularly HLA-DR1 and HLA-DR4, by motif-prediction (16). In addition, these peptide sequences, especially P16-31 and P263-280, in the G1 domain were T cell epitopes identified in HLA-DR2, DR3, DR4 and DQ8-transgenic mice immunized with human proteoglycan aggrecan (121). These finding supports our data which found that chondrocytes may present PG peptides, P16-31 and P263-280, to autologous T cells via MHC molecules. T cell proliferation had a slight reduction when they were co-cultured with MHC class II-blocked chondrocytes. Dramatic reduction of T cell proliferation was observed in only one patient, while others showed insignificant reduction levels. Our findings indicate that presentation of these PG peptides by chondrocytes to T cells may be restricted to MHC molecules. Further investigation is still required to evaluate the role of MHC molecules in the presentation of these PG peptides.

In this study, we found an abundant IL-6 production in PG peptide-stimulated chondrocytes and IL-6 has been reported to promote differentiation of Th17 cells (61). PG peptide-stimulated chondrocytes may increase Th17 population in OA patients. Th17 cells produce IL-17, which stimulates osteoclastogenesis and bone destruction in arthritis (58). In addition to Th17 cells, synovial fluid and synovial tissue of OA patients also contained a number of Th1 cells (58). Several evidences showed that Th1 cytokines, such as IL-2 and IFN-γ, could be detected in synovial membrane

and synovial fluid at both the gene and protein levels, while Th2 cytokines, such as IL-4 and IL-5, could be observed at lower levels in OA patients (122-124). Therefore, we also expect that Th1 cells might also respond to PG peptide-treated chondrocytes and increase the severity of OA pathogenesis by increasing proinflammatory cytokines. Not only cytokine production, chondrocyte-T cell interaction also enhances the ability of proteolytic enzyme production in chondrocytes (125). In OA, chondrocytes co-cultured with autologous T cells could produce MMP1 and MMP13, which predominantly digest collagen type II in cartilage (125, 126). Moreover, there were studies that found that chondrocytes in OA patients can respond to cartilage fragment such as fibronectin by increasing proteolytic enzyme (MMP1, 3 and 13) production (127). These findings correlate with our RNA microarray study that found transcripts of proteolytic enzymes, including MMPs (MMP1 and MMP13) and ADAMTS (ADAMTS4), in PG peptide-IFN-γ treated chondrocytes. These data suggest that PG peptides might affect OA pathogenesis by increasing proteolytic enzyme production in chondrocytes, resulting in cartilage destruction.

Although our study showed a new aspect of chondrocyte response to PG peptides by increasing co-stimulatory molecule expression and inflammatory cytokine production in OA patients; however, this study still has limitations regarding investigating these responses in normal healthy chondrocytes, due to ethical reasons. Also, this study showed that chondrocytes could present cartilage protein or peptide and induced autoreactive T cells in joint of OA patients. However, the question that why activation of autoreactive T cells could be observed in OA remains to be solved. One hypothesis for this question is that digestion of cartilage protein by proteolytic enzymes might generate neoepitope that can induce immune respond in OA. Although OA is mild condition of autoimmunity, but cartilage is continuously damaged. Therefore, understanding about mechanism of autoantigen generation and role of chondrocyte to present autoantigen to T cells is essential for further developing treatment.

CHAPTER V

CONCLUSION

In this study, we found that chondrocytes from OA patients possessed antigen presentation function and responded to IFN-γ and PG peptide stimulation. IFN-γ treatment can increase expression level of MHC class I and class II and co-stimulatory molecules, CD40, CD80 and CD86. After treating with PG peptides, P16-31 and P263-280 treated chondrocytes increased both CD80 and CD86 expression levels and expression level of these two molecules showed more increasing in combination of IFN-γ and P16-31 or P263-280 treatments. Moreover, both P16-31 and P263-280 upregulated co-stimulatory molecules, in particular CD80, in a dose-dependent manner. This data supports the hypothesis that PG peptides can stimulated chondrocytes by inducing co-stimulatory molecule expression.

IFN-γ and P16-31 or P263-280 treatment increased CD80 and CD86 expression on OA chondrocytes specifically, but not on keratinocytes. IFN-γ and PG peptides induced antigen presentation function in chondrocytes higher than keratinocytes; albeit, still lower than of dendritic cells. Moreover, we observed induction of IL-6, IL-8 and TNFα production by combination of IFN-γ and P263-280 treated chondrocytes. This P263-280 peptide did not have any effect on cytokine production in keratinocytes and dendritic cells, suggesting specificity of PG peptide stimulation in OA chondrocytes.

We also found that PG peptide stimulation effected on gene expression profile in OA chondrocytes. RNA microarray analysis revealed that a number of genes related to antigen processing and presentation, TNF signaling and NF-κB signaling pathways were upregulated in combination of IFN-γ and P16-31 or P263-280 treated chondrocytes. This finding suggests the potential influence of immunological function induction by IFN-γ and PG peptides in OA chondrocytes.

Chondrocytes treated with IFN-γ and PG peptides, especially P263-280, stimulated autologous T cells to proliferate at higher levels in OA patients and analysis suggested that the responsive T cell subset was CD4+ T cell. The percentage of T cell proliferation had a tendency to reduce after blocking MHC class II molecules on chondrocytes. This indicates that chondrocytes may potentially present PG peptides to T cells in an MHC-restricted manner.

Taken together, this study proposes that PG peptides may be candidate antigens that induce immunological function of chondrocytes under inflammatory condition of OA to perpetuate the pathogenesis severity by increasing proinflammatory cytokine production and stimulating T cells via presenting PG peptides on MHC class II molecules (Figure 38).

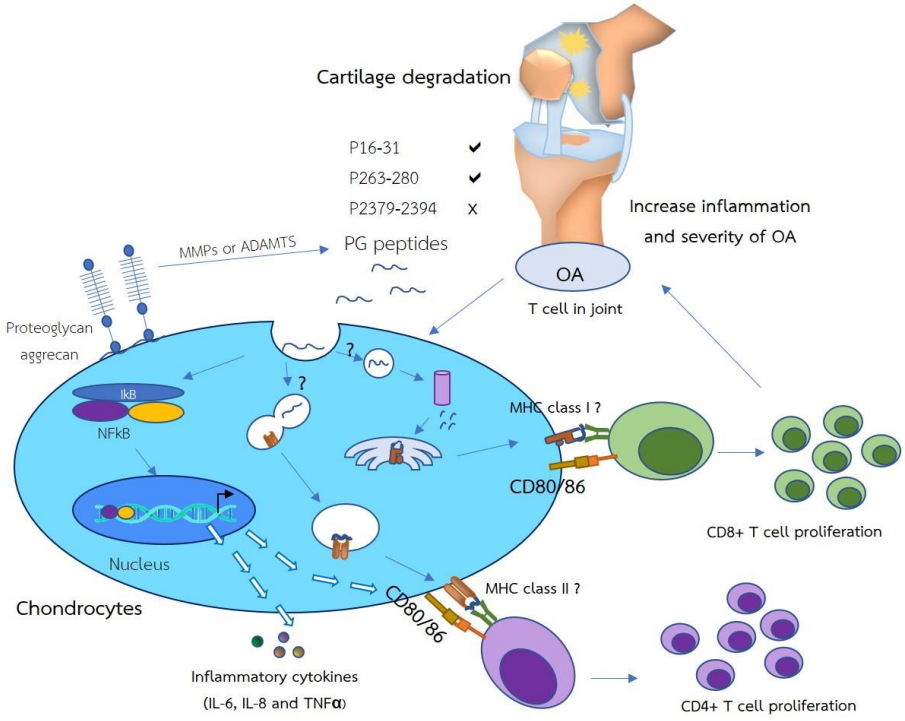


Figure 38 Illustration of the proposed antigen presentation function of chondrocyte. Chondrocyte from OA patients might phagocytose cartilage fragment peptides, process and present these peptides to T cells via MHC class II or cross-present via MHC class I. Stimulated T cells in joint might produce cytokines or cytotoxic molecules to destroy cartilage and increase severity of the disease.

APPENDIX

1. Endotoxin test (Using HEK-Blue LPS Detection Kit (Invivogen))

1.1. HEK-Blue LPS Detection Kit

HEK-Blue cells which were engineered to become stably express TLR4 and extremely sensitive to LPS was used to detect endotoxin contamination. LPS contaminant will bind to TLR4 and increase expression of NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP). Activity of this enzyme will be detected by addition of substrate solution, QUANTI-Blue, and color of culture media will become purple that can measure absorbance at 620 nm.

1.2. Reagents

1.2.1. Growth medium (DMEM high glucose supplemented with 10% FBS, Penicillin-Streptomycin and 1X normocin)

1.2.2. Selection medium (Growth medium supplemented with 1X HEK-Blue Selection)

1.2.3. Test medium (Growth medium supplemented with 10% FBS)

1.2.4. QUANTI-Blue solution

1.3. Methodology

The HEK-Blue cells were thawed and resuspended in growth medium. Cells were cultured at 37ºC for 48 hours until the cell completely attached to bottom flask. At 50-80% confluency, cells were trypsinized with 0.05% Trypsin containing EDTA and were further grown in selection medium in 96 well-plate at cell concentration of $1x10^5$ cells/well. HEK-Blue cells were treated with PG peptides, including P16-31, P263-280 and P2379-2394, at various concentration of 10 µg/ml or 50 µg/ml and incubated at 37ºC for 18 hours. The HEK-Blue cell supernatant collected from each well was mixed with QUANTI-Blue solution and the mixer was incubated at 37ºC for 6 hours. Endotoxin contamination was measured the absorbance at wavelength of 620 nm. HEK-Blue cell treated with

either 1 ng/ml of lipopolysaccharide (LPS) or culture medium without any stimuli were used as positive and negative control, respectively.

1.4. Result

This study was performed to check endotoxin contamination in synthetic peptides that might affect chondrocyte stimulation. The absorbance at 620 nm, which represented level of endotoxin contamination, of PG peptides (P16-31, P263-280 or P2379-2394) treated HEK-Blue cells could be detected as low as that of unstimulated HEK-Blu cells (culture medium treatment), while positive control (LPS treatment) showed significantly higher level of this endotoxin (Figure 39). It could be indicated that these synthetic peptides did not have endotoxin contamination.

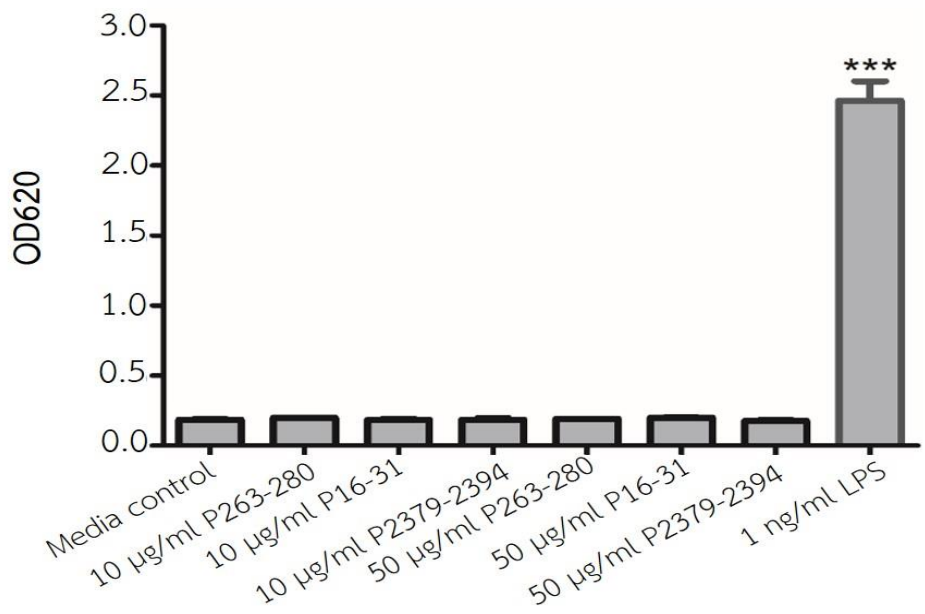


Figure 39 Endotoxin test of synthetic peptides. HEK-Blue cells were treated with PG peptides, P16-31, P263-280 and P2379-2394, at concentration of 10 µg/ml and 50 µg/ml and cultured for 24 hours. Supernatant was collected to test endotoxin contamination. LPS treated HEK-Blue cells was used as positive control and culture medium treated cells was used as negative control. Endotoxin contamination was detected by measuring the absorbance at 620 nm. Statistical significance was calculated from mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ***, $p< 0.001$).

2. Reagent preparation

2.1. Cell culture

Supplemental Complement (SC)

RPMI	320	ml.
Non-essential amino acid (NEAA)	100	ml.
Penicillin Streptomycin Solution	80	ml.
HEPES	11.9	g.
L-glutamine	100	ml.
β-mercaptoethanol	35	µl.

Filtered by 0.2 µm and stored at 20°C

RF10 medium

RPMI	500	ml.
Supplemental complement	30	ml.
Fetal bovine serum	60	ml.

Freezing media

Fetal bovine serum	9	ml.
Dimethyl sulfoxide	1	ml.

Plating medium for MoDC

IMDM with L-glutamine	26.7	ml
Fetal bovine serum	3	ml
Penicillin Streptomycin Solution	300	µl

Condition medium for MoDC

RPMI	8.6	ml
2 mM Glutamax	100	µl
Penicillin Streptomycin Solution	100	µl
1 mM Sodium pyruvate	100	µl
Non-essential amino acid (NEAA)	100	µl

Fetal bovine serum	1	ml
GM-CSF (25 ng/ml)	5	µl
Recombinant IL-4 (25 ng/ml)	5	µl

Digestion buffer for infrapatellar fat pad

Phosphate buffer saline	47.25	ml.
Fetal bovine serum	2.5	ml.
3 mg/ml Collagenase type IV (Final conc 3 µg/ml)	50	µl.
1 mg/ml DNase I (Final conc. 0.1 µg/ml)	5	µl.

Digestion for cartilage

Pronase solution

DMEM	500	ml.
Supplemental complement	30	ml.
Fetal bovine serum	60	ml.
Pronase (1% w/v)	0.15	g.

Freshly prepared and filtered by 0.2 µm

Collagenase type II solution

DMEM	500	ml.
Supplemental complement	30	ml.
Fetal bovine serum	60	ml
Collagenase type II (0.3% w/v)	0.05	g.

Freshly prepared and filtered by 0.2 µm

2.2. Flow cytometry

FACS buffer

Phosphate buffer saline	95	ml.
Fetal bovine serum (final conc. Is 5% v/v)	5	ml.

Fixation buffer

Phosphate buffer saline	92.2	ml.
Fetal bovine serum (final conc. Is 5% v/v)	5	ml.
Formaldehyde	2.8	ml.

2.3. Immunofluorescence

Blocking buffer

Phosphate buffer saline	100	ml.
Bovine serum albumin (final conc. Is 2% w/v)	2	g.

Fixation buffer

Phosphate buffer saline	48	ml.
Formaldehyde	2	ml.

Permeabilization buffer

Phosphate buffer saline	50	ml.
Triton X-100 (Final conc. Is 0.1% v/v)	50	µl.

3. **Chemical and reagents**

1-Bromo-3-chloropropane (BCP) (Sigma-Aldrich)	Germany
4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Applichem)	Germany
4', 6'-Diamidino-2-Phenylindole (DAPI) (Biolegend)	USA
Anti-CD3/CD28 dynabeads (Gibco)	USA
Anti-CD3-APC (clone: UCHT1) (Biolegend)	USA
Anti-CD40-PE (Biolegend)	USA
Anti-CD4-APC-Cy7 (clone: RPA-T4) (Biolegend)	USA
Anti-CD80-FITC (Biolegend)	USA
Anti-CD86-APC (Biolegend)	USA
Anti-CD8-AF700 (clone: SK1) (Biolegend)	USA
Anti-collagen II antibody (Abcam)	UK
Anti-HLA-A, -B, -C-FITC (Biolegend)	USA
Anti-HLA-DR-AF700 (Biolegend)	USA
Beta-mercaptoethanol (Sigma-Aldrich)	Germany
Bovine serum albumin (BSA) (Serva)	Germany
Carboxyfluorescein succinimidyl ester (CFSE, Biolegend)	USA
Dimethyl sulfoxide (DMSO) (Applichem)	Germany
DNase I (Worthington)	USA
Dulbecco's Modified Eagle Medium (DMEM) (Gibco)	USA
Fetal bovine serum (FBS) (Gibco)	USA
Ficoll (GE Healthcare)	Sweden
Goat-anti-mouse IgG linked AF488 (Abcam)	UK
IL-6 Human uncoated ELISA Kit (Invitrogen)	USA
IL-8 Human uncoated ELISA Kit (Invitrogen)	USA
Improved Minimum Essential Medium (IMEM) (Gibco)	USA
L-Glutamine solution (Gibco)	USA
Non-essential amino acid (NEAA) (Gibco)	USA
Penicillin Streptomycin Solution (Pen-Strep) (Gibco)	USA
Phosphate buffer saline (PBS) (Serva)	Germany

Power Up™ SYBR™ Green Master Mix (Applied biosystems)	USA
Pronase (Sigma-Aldrich)	Germany
Prostaglandin E2 (PeproTech)	USA
Purified anti-human HLA-A, -B, -C antibody (Clone W6/32) (Biolegend)	USA
Purified anti-human HLA-DR, DP, DQ antibody (Clone Tü39) (Biolegend)	USA
Recombinant human IFN-γ (R&D systems)	USA
Recombinant human IL-1β (PeproTech)	USA
Recombinant human IL-2 (Biolegend)	USA
Recombinant human IL-6 (PeproTech)	USA
Recombinant human TNF-α (PeproTech)	USA
Roswell Park Memorial Institute (RPMI) (Gibco)	USA
Saponin (Sigma-Aldrich)	Germany
SuperScriptTM VILOTM cDNA Synthesis Kit (Invitrogen)	USA
synthesized nucleotide sequences (primers) (GenScript)	USA
Synthesized peptides (Genscript)	USA
TNF-α Human uncoated ELISA Kit (Invitrogen)	USA
Triton X-100 (Applichem)	Germany
TRIzol reagent (Invitrogen)	USA
Type II collagenase (Worthington)	USA
Type IV collagenase (Worthington)	USA

4. Equipment

CO_2 incubator (Thermoscientific, USA)

Flow cytometer (LSRII, BD Bioscience)

Hemacytometer

Biological Safety Cabinet class II

Multifunctional Microplate Reader (Vario Skan Flash, USA)

Phase contrast fluorescence microscope (Olympus)

QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, USA)

Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA)

Vortex mixer (Scientific industries, USA)

Water bath (Grant Instruments Ltd., UK)

5. Software and programs

GraphPad Prism 7.0

EndNote X9

FlowJo V10

RStudio 4.0.2

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AWARD RECEIVED

1. The Outstanding Master Thesis Award 2015 from the Graduate School, Kasetsart University (Title of thesis is “Functional and Structural Characterisation of Calmodulin from Black Tiger Shrimp, Paneus monodon”.)

2.The best academic performance award at the M.Sc. level (Biochemistry Discipline) from The Professor Dr.Tab Nilanidhi Foundation