ลักษณะของ cytokines และ cytotoxic mediators ที่หลั่งจาก T cells ในผู้ป่วยโรคข้อเข่าเสื่อม

นางสาวฐิติยา แซ่จุ้ง

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CHARACTERIZATION OF T CELL SECRETED CYTOKINES AND CYTOTOXIC MEDIATORS PROFILING IN KNEE OSTEOARTHRITIS PATIENTS

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ฐิติยา แซ่จุ้ง : ลักษณะของ cytokines และ cytotoxic mediators ที่หลั่งจาก T cells ในผู้ป่วยโรคข้อเข่าเสื่อม (CHARACTERIZATION OF T CELL SECRETED CYTOKINES AND CYTOTOXIC MEDIATORS PROFILING IN KNEE OSTEOARTHRITIS PATIENTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. พญ. ดร. รังสิมา เหรียญตระกูล, 67 หน้า.

โรคข้อเสื่อมเป็นโรคเรื้อรังที่เกี่ยวข้องกับความผิดปกติของข้อ เกิดจากการเสื่อมของเยื่อบุข้อ และสึกหรอของผิวกระดูกอ่อนที่หุ้มข้อต่างๆ ทำให้เกิดการอักเสบบริเวณข้อได้ โรคข้อเสื่อมเกิดในผู้สูงอายุและมีเหตุปัจจัยหลายๆ ปัจจัยที่มีผลต่อการเสื่อมสภาพของข้อได้ เช่น น้ำหนัก พันธุกรรม เพศ และ การบาดเจ็บ เป็นต้น ซึ่งมีผลทำให้เกิดอาการเจ็บปวดบริเวณข้อ และทำให้ความสามารถในการเคลื่อนไหวของข้อน้อยลง เช่น ข้อเข่า ข้อเท้า ข้อมือ ข้อสะโพก และ ข้อกระดูกสันหลัง โดยเฉพาะข้อเข่าซึ่งเป็นปัญหาสุขภาพที่สำคัญของคนไทย การเกิดโรคข้อเสื่อมมีความเกี่ยวข้องกับระบบภูมิคุ้มกันของร่างกาย เนื่องจากสามารถพบเซลล์ทางภูมิคุ้มกัน เช่น ทีเซลล์ (T cells), บีเซลล์ (B cells) และ แมโครฟาจ (macrophages) ได้ในบริเวณของเนื้อเยื่อที่ห่อหุ้มข้อเข่าและในน้ำไขข้อ โดยพบว่า ทีเซลล์ชนิดCD8+ เป็นเซลล์ที่มีบทบาทที่สำคัญในโรคข้อเข่าเสื่อม เพราะพบทีเซลล์ชนิด CD8+ เป็นปริมาณมากในเนื้อเยื่อบริเวณข้อเข่า รวมทั้งพบว่าปริมาณทีเซลล์ชนิดCD8+ จะแปรผันตรงกับอายุของผู้ป่วยโรคข้อเสื่อม โครงการวิจัยนี้เป็นการศึกษาดูการทำงานของทีเซลล์ชนิดCD8+ ในผู้ป่วยโรคข้อเข่าเสื่อม โดยวัดการสร้างไซโตไคน์และไซโตท็อกซิกแกรนูลจากทีเซลล์ชนิดCD4+ และCD8+ และวัดปริมาณทีเซลล์ชนิด CD4+ และCD8+ ที่มีการแสดงออกของตัวบ่งชี้การปล่อยสารออกจากเซลล์ (CD107a) ในเนื้อเยื่อไขมันใต้กระดูกสะบ้า (Infrapatellar fat pad; IFP), กระแสเลือด (peripheral blood) และน้ำไขข้อ (synovial fluid) ของผู้ป่วยโรคข้อเข่าเสื่อม และนำค่ามาวิเคราะห์หาความสัมพันธ์ระหว่างไซโตไคน์, ไซโตท็อกซิกแกรนูล และการแสดงออกของ CD107a ที่สร้างจากทีเซลล์ชนิดCD4+ และCD8+ กับความรุนแรงของโรค โดยใช้ระบบของ Kellgren-Lawrence (KL) เป็นเกณฑ์ (แบ่งเป็น 5 grades ตามความรุนแรง) ซึ่งจากการทดลองพบผู้ป่วยโรคข้อเข่าเสื่อมมีอายุมาก (ค่าเฉลี่ย=68.88) และเมื่อทำการวิเคราะห์ปริมาณของไซโตไคน์และไซโตท็อกซิกแกรนูลพบว่า TNF (Tumor necrosis factor) ที่สร้างมาจาก ทีเซลล์ชนิดCD8+ ในกระแสเลือดของผู้ป่วยสูงกว่าในคนปกติ อีกทั้ง IL-1b (Interleukin-1b), IL-6 (Interleukin-6) และ TNF ที่สร้างมาจากทีเซลล์ชนิด CD4+ และCD8+ พบเป็นปริมาณมากใน IFP ของผู้ป่วย และเมื่อทดสอบการแสดงออกของ CD107a บนทีเซลล์ชนิดCD8+ ในกระแสเลือดและ IFP พบว่ามีการแสดงออกของ CD107a ปริมาณสูงในผู้ป่วย นอกจากนั้นยังพบว่ามีการสร้างไซโตท็อกซิกแกรนูลคือ perforin/granulysin จากทีเซลล์ชนิด CD4+ และ perforin/granzymeB จากทีเซลล์ชนิด CD8+ ในปริมาณที่สูงจาก IFP ของผู้ป่วย ซึ่งไม่พบความสัมพันธ์ระหว่างไซโตไคน์, ไซโตท็อกซิกแกรนูลและการแสดงออกของ CD107a ที่สร้างมาจากทีเซลล์ชนิดCD4+ และCD8+ กับความรุนแรงของโรค จากการศึกษาสารละลายโปรตีนในน้ำไขข้อก็ยังพบ IL-6, sFas, granzyme A, perforin และ granulysin เป็นปริมาณมาก ซึ่งผลการศึกษานี้จะนำไปสู่ความรู้ความเข้าใจเกี่ยวกับ soluble factors สำหรับใช้ในการพยากรณ์โรคมากขึ้น รวมทั้งหา target molecule ที่ใช้ในการพัฒนาการผลิตยาที่จำเพาะกับโรคข้อเข่าเสื่อมต่อไป

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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THITIYA SAE-JUNG: CHARACTERIZATION OF T CELL SECRETED CYTOKINES AND CYTOTOXIC MEDIATORS PROFILING IN KNEE OSTEOARTHRITIS PATIENTS. ADVISOR: RANGSIMA REANTRAGOON, M.D. Ph.D., 67 pp.

Osteoarthritis (OA) is a chronic disease associated with morphological joint changes that results in the breakdown of joint cartilage and underlying bone with erosive inflammation. Factors of OA are intrinsic factor and extrinsic factor. The pathogenesis of OA relates with immune responses and immune cells, T cells, B cells, and macrophages etc., T cells both CD4+ and CD8+ T cell are immune cells that have been suggested to play a role in OA. However, we found many finding in CD8+ T cells such as a high proportion of CD8+ T cell was observed in tissue of knee joint. In addition, a trend for higher frequency for CD8+ T cell with older in OA patients. In our study, we collected peripheral blood and infrapatellar fat pad (IFPs) from knee OA patients and measured their cytokine/cytotoxic granule production and the frequency of degranulating (CD107a+) from both CD4+ and CD8+ T cells. We determined the relation between, their cytokine (IL-1b, IL-17, IL-6, TNF and IFN-g), cytotoxic granules (perforin, granzymeB, granulysin) production and degranulating cells of CD4+ and CD8+ T cells with Kellgren-Lawrence (KL) radiographic gradings. We found that TNF-producing CD8+ T cells from peripheral blood of OA patients was a higher level than healthy individual. IL-1b, IL-6 and TNF-producing both CD4+ and CD8+ T cell were increased in IFP of knee OA patients. In addition, the presence of degranulating cell-producing CD8+ T cell both in peripheral blood and IFP from knee OA patients revealed a higher level than healthy individuals. Moreover, our data showed perforin/granulysin in CD4+ T cells and perforin/granzyme B in CD8+ T cell were a higher level in IFP of knee OA patients when compared to peripheral blood of knee OA patients. We observed no correlation between cytokines/cytotoxic granules and CD107a, with grade 3, grade 4. We were also detectable and a high level to IL-6, sFas, granzyme A, perforin and granulysin in synovial fluid of OA patients. This study provides a more understanding about the mechanism of knee OA pathogenesis and lead to the development of drug design for OA treatment and to find soluble factors as a prognostic marker for knee OA.

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

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LIST OF ABBREVIATIONS

Abbreviation	Term
APC	Antigen presenting cell
CBA	Cytometric bead array
CD107a	Cluster of differentiation107a
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CTL	Cytotoxic T lymphocyte
DAMPs	Damage-associated molecular patterns
ICRS	International cartilage repair society
ICS	Intracellular cytokine staining
IFN-γ	Interferon-γ
IFP	Infrapatellar fat pad
IL-1β	Interleukin-1β
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-18	Interleukin-18
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-6
KL	Kellgren-Lawrence

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NLRP3	The Nucleotide-binding domain, leucine rich family (NLR), pyrin containing 3
OA	Osteoarthritis
OSM	Oncostatin M
PBMC	Peripheral blood mononuclear cell
SF	Synovial fluid
SM	Synovial membrane
ST	Synovial tissue
Th1	T helper 1 cell
Th2	T helper 2 cell
TKA	Underwent total knee arthroplasty
TLR	toll-like receptor
TNF	Tumor necrosis factor

CHAPTER I

INTRODUCTION

Background information and rationale

One of the main community health issues worldwide is osteoarthritis (OA) (1). OA is a joint disease that results from the breakdown of joint cartilage and underlying bone and has an erosive inflammation profile (2). The clinical symptoms of OA include pain, stiffness, joint-line tenderness, muscle weakness, swelling, deformed joints, reduced range of motion and cracking and creaking sounds. Pathogenic features of OA include bone remodeling and sclerosis, cartilage breaking down, meniscal damage, synovial hypertrophy, and osteophytes (3). Knee OA is a chronic disease of the whole joint (articular cartilage, meniscis, ligaments, peri-articular muscles and adjacent adipose tissues) resulting from multiple pathophysiological mechanisms (4). Two major factors for OA are 1) intrinsic factor (age, gender, and genetic susceptibility) and 2) extrinsic factor (obesity, trauma and mechanical load) (5, 6).

The inflammation found in OA is chronic and low-grade in nature and involves both the innate and adaptive immune response (2). Adaptive immune responses in knee OA include both T cells and B cells. T cells are suggested to play an important role in the pathogenesis and progression of OA (7-9). A previous study showed that CD3+ T cells infiltrate into the synovial membrane of >50% of OA patients and that these infiltrations express early (CD69), intermediate (CD25, CD38), and late (CD45RO) activation antigens. This result suggesting that CD4+ and CD8+ T cells present have a memory and activated phenotype (8). Previous studies have found both CD4+ and CD8+ T cells in synovial tissue and Infrapatellar fat pad (IFP) (10, 11). The ratio of synovial tissue CD4+:CD8+ T cells was as 5:1 (12). However, peripheral blood CD8+ cells related with older OA patients (13). Furthermore, our group has previously found that activate CD8+ T cells were in a

higher level than activate CD4+ T cells in OA peripheral blood, further suggesting that CD8+ T cells may be an important player in knee OA (10).

Major factors of outcome of immune responses are T cell-derived cytokines (14). Many researchers found many cytokines, including IL-1β, TNF, IL-6, IFN-γ, TGF-β, IL-2, IL-4, IL-8, IL-10, and IL-18, in synovial tissues, synovial membranes and peripheral blood of OA patients (7, 11, 15). IL-1β and TNF are the most expressed in osteoarthritic inflammation. Two pro-inflammatory cytokines promote more catabolism and degradation of the cartilage in cartilage homeostasis process (7). In addition, IL-6 may play a role in cartilage loss in early stage OA and may be used as a diagnostic and prognostic biomarker (16). In addition, T cells are capable of releasing cytotoxic molecules in response to antigen stimulation (17). We hypothesize that T cell function may play a role in OA. We investigated cytokines and cytotoxic granules -producing T cells from peripheral blood and IFP. Moreover, we also determined cytokines, cytotoxic granules and transmembrane protein from synovial fluid. The soluble mediators from T cell can be used as biomarker and predict pathogenesis which will be very useful for OA patients.

Hypothesis

We hypothesize that T cell-mediated cytotoxicity may be one of the mechanisms playing an important role in OA. Thus, in this study, we aim to determine cytotoxic granules and cytokines that are produced from CD4+ and CD8+ T cells in peripheral blood and Infrapatellar fat pad. Moreover, we looked at the relative frequency of CD4+ and CD8+ T cells, cytokines and cytotoxic granules with radiographic grading and also molecules related to cytotoxicity from synovial fluid as well.

Research question

- What is the role of CD8+ T cells (and/or CD4+ T cells) in OA?
- What is the correlation between the frequency of CD107a+ of CD3+, CD4+ and CD8+ T cells, cytokines and cytotoxic granules with radiographic grading?
- Are any predominant cytotoxic molecules found in synovial fluid of knee OA patients?

Research Objective

- To compare the frequency of CD4+ and CD8+ T cells in healthy individuals and OA patient.
- To study the cytokine and cytotoxic granule released from CD4+ and CD8+ T cells in peripheral blood and infrapatellar fat pad and synovial fluid of OA patient.
- To determine the correlation of the frequency of CD107a+ of CD3+, CD4+ and CD8+ T cells, cytokines and cytotoxic granules with radiographic grading.

Application

This study will provide a more understanding about the mechanism of knee OA pathogenesis and lead to the development of drug design for OA treatment and to find soluble factors as a prognostic marker for knee OA.

CHAPTER II

LITERATURE REVIEW

Introduction to osteoarthritis

Osteoarthritis (OA) is a joint disease that results from the breakdown of joint cartilage and underlying bone and has an erosive inflammation profile, which is characterized by inflammation in joints. The inflammation observed in OA is chronic and low-grade, and involves the innate and adaptive immunity (2). OA is the most common articular disease affecting millions of people worldwide (1). OA can affect any joint, but it most often occurs in the knees, hips, small joints of the fingers, the thumb and big toe (18).

Knee OA is a major health problem in Thai people (5). Knee OA mostly often affects middle-aged to elderly people, reducing their quality of life and provides a financial burden to those affected due to the high cost of treatment (4, 19). A research of this disease in 2002 collected from 392 elderly people in Thailand found that most knee OA patients range from not having any symptoms to having moderate severe symptoms. Those with symptoms have poor levels of quality of life (5).

The clinical symptoms of OA include usage-related joint pain, stiffness due to inactivity of short duration, joint-line tenderness, muscle weakness, swelling, deformed joints, reduced range of motion, loss of use of the joint, and cracking and creaking sound (4). In part of pathogenic features of OA include bone remodeling and sclerosis, joint space narrowing, cartilage breaking down, meniscal damage, synovial hypertrophy, and osteophytes (4) (Figure 1).

Currently, OA is incurable but only treatable by controlling the symptoms. The treatment is based on local or oral analgesics and nonsteroidal anti-inflammatory drugs in combination with exercise therapy and physiotherapy techniques (5). The treatment

method will vary for each patient depending on many factors. A research of knee OA in 2002 in Thailand found that OA patients were treated with prescription drugs, medication pot, herbal medicine, physical therapy and modified behavior (5).

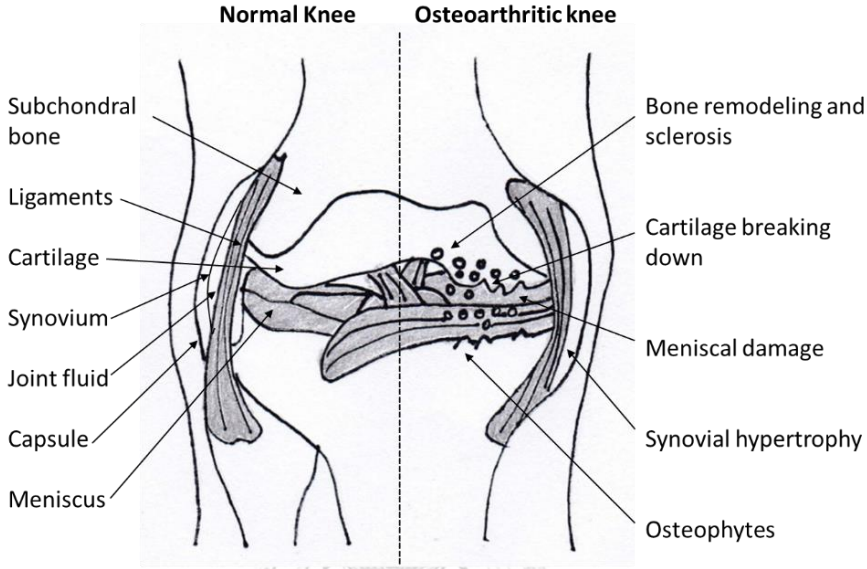


Figure 1 Pathogenic features of osteoarthritis

Knee Anatomy

The knee joint consists of the femur, patella and tibia surrounded by a ligamentous structure called the joint capsule (Figure 2). Inside this capsule is a specialized membrane known as the synovial membrane, which provides nourishment to all the surrounding structures. The capsule is strengthened by surrounding ligaments (20). Other structures around the knee joint include the Infrapatellar fat pad (IFP) and bursa, which function as cushions to exterior forces that impact the knee (21).

The IFP has the typical histological structure of adipose tissue that is interposed between the joint capsule and the synovial membrane of the knee joint (21). Adipocytes make up most of the cell population within the IFP. Other cells include fibroblasts, which

produce the extracellular matrix, and immune cells such as macrophages, mast cells, natural killer (NK) cells, NKT cells, T cells, and B cells which depend on adiposity of the individual (22).

There are many roles of the IFP in OA including source of cytokine/adipokines, source of lipid mediators and site of inflammatory cells (21). IFP can release free fatty acids (oleic, linoleic, palmitic and palmitoleic acids) and display immune modulatory properties. It can detect of levels of mono-hydroxylated derivatives of arachidonic acid and 5-, 12-, and 15-HETE as well as PGE2 and the novel pro-resolving lipid mediator PD1 from IFP (21). These results indicate that IFP of OA patients are a source of various soluble mediators with pro- as well as anti-inflammatory properties. Investigation of IFP in end stage patients showed that adipose tissue was a site of energy storage and also exhibit inflammatory profile (21).

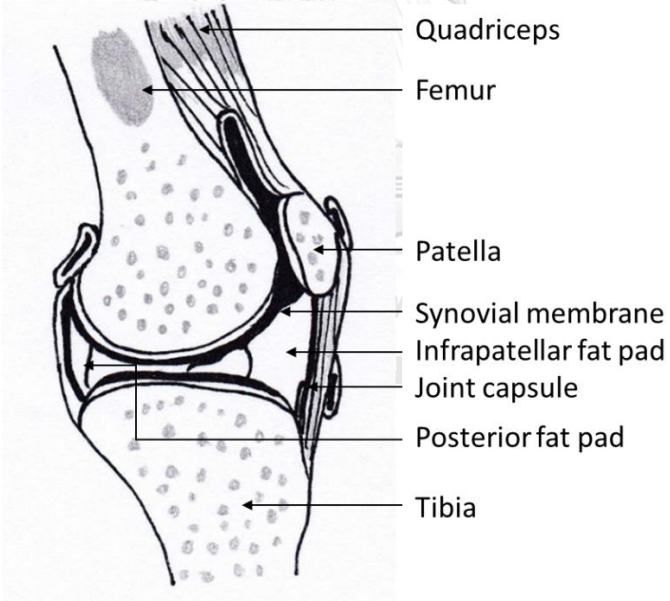


Figure 2 Sagittal view of the knee

Factors related to osteoarthritis

OA results from two factors that induce disease progression: (i) intrinsic factors (age, gender, and genetic susceptibility) and (ii) extrinsic factors (obesity, trauma and mechanical load), followed by activation of inflammatory response involving the interaction of the cartilage, subchondral bone, and synovium (4, 23).

Intrinsic factors

Age is a risk factor for knee OA. When individuals age, biological changes such as cartilage thinning, decrease proprioception, weak muscle, and oxidative damage in the body lowers the ability of joints to withstand external forces (24). In many previous studies OA patients are most often in the elderly population (4, 5, 23, 25).

OA is more common in female than male due to the difference in gender biology and hormones. There are reports in Thai people showing that knee OA patients were 78.1% female and 21.9% male (5). This is due to lower elastic in female than male. The female hormones, estrogen, also prevent inflammation of the cartilage. Thus, people with menopause have a higher risk of OA (9).

Another important intrinsic factor is the genetic susceptibility to OA (26). Several genes have been reported to be involved with OA (Table 1). These include genes related with i) the structure of joint and strength of connective tissues (COL2A1), ii) chondrocytes (BMP5, FRZB, IL-4Rα), iii) bone density (vitamin D receptor (VDR)), iv) hormones (estrogen receptor alpha (ESR1)), v) HLA region (HLA-A1, B8, and HLA-DR2) and vi) inflammatory cytokines (IL-1, IL-10, TGFB1, IL-6, TNF-α) (26-28).

Table 1 Genes implicated in osteoarthritis

Genes implicated in OA	
Vitamin D receptor (VDR) (26, 28)	CRTM (cartilage matrix protein) (26)
Col2A (26, 27)	CRTL (cartilage link protein) (26)
Aggrecans (AGC1) (26, 28)	A1ACT (26)
Insulin-like growth factor 1 (IGF-1) (28)	COL9A1 (26-28)
Oestrogen α receptor (ER alpha) (28)	COL11A1 (27, 28)
Transforming growth factor β (TGF beta) (26)	COl1A1 (28)
Asporin (ASP) (28)	ANK (26)
Tissue inhibitor of metaloprotease 3 (TIMP3) (28)	Calponin homology domain containing protein 1 (LRCH1) (28)
Repetitions rich in leucine (28)	Metaloprotease ADAM12 (28)
Cartilage intermediate protein (CLIP) (28)	Tetranectin (TNA) (28)
Bone morphogenic protein 2 (BMP2) (27, 28)	

Extrinsic factors

Obesity is known to predispose individuals to OA, especially knee OA (25, 27). Obesity is a major source of mechanical load on joints. Evidence in human and animal studies found that joint loading causes changes to the structure, constituent and mechanical of the joint (6). There are reports that women who lost weight had reduced risk of development of knee OA (25).

Severe injury and/or surgery to the articular cartilage, meniscus and ligament increases the risk of the developing OA (25). It has been reported the higher prevalence of meniscal damage was higher in populations with radiographic knee OA (82%) than without OA (25%). Higher KL grading correlated with increase the prevalence of meniscal damage (12). Abnormal loading leads to recruitment of immune cells to the joint resulting in as increased risk of OA development.

Immunopathogenesis of osteoarthritis

Knee OA pathophysiology is caused by multiple factors in the joint (4). Multiple factors involved with OA such as cell/extra-cellular matrix (ECM), chondrocytes, enzymes (metalloproteinases (MMPs) and collagenases), and immune response (29, 30). This research focuses on immunopathogenic of OA.

Initial injury of the joint causes several genetic, metabolic factors and mechanical factors to release cartilage specific auto-antigens or damage-associated molecular patterns (DAMPs). The hypothesis is that specific auto-antigens of cartilage activates both innate and adaptive immune response resulting in infiltration of immune cells (T cell, B cell and macrophages), release of soluble proteins (cytokines and chemokines), activation of complement, and generation of immune complexes against cartilage components (7) (Figure 3). In addition, physical damage to the joint can cause chondrocytes to release catabolic enzymes and stop production of anabolic factors leading to cartilage damage and releasing of matrix components, causing inflammatory mechanisms (7). In a given immune response, both innate and adaptive immune response play a role.

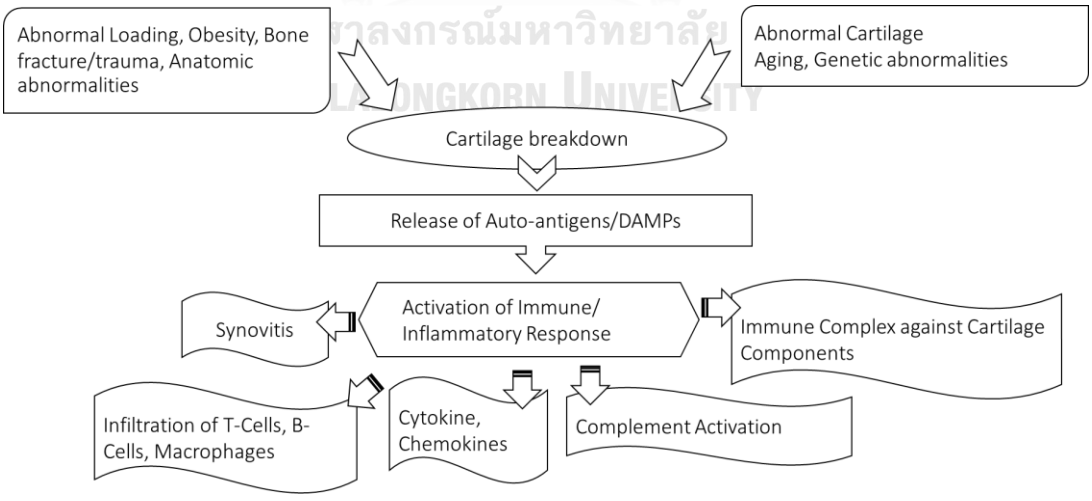


Figure 3 Immunopathogenesis of osteoarthritis (7)

Innate immunity in osteoarthritis

Innate immune responses in OA is activated by DAMPs, cartilage extracellular matrix molecules from injury and microdamage in the joints. In addition, these several stimuli can bind with pattern recognition receptors (PRRs) which further make activate monocytes (31).

DAMPs

DAMPs associated with OA are divided into 4 classes, these include (i) extracellular matrix (ECM) breakdown (biglycan, fibronectin and tenascin C), (ii) plasma proteins (α1-2 micro-globulin, fibrinogen, and vitamin D-binding protein), (iii) intracellular alarmins (HMGB1 and the S100 family of proteins) and (iv) microscopic crystals (basic calcium phosphate, calcium pyrophosphate dihydrate, and uric acid) (2). Plasma proteins induced cytokine and growth factor production via TLR 4 signaling of macrophage. In addition, intracellular alarmins also bind to TLR 4 of cartilage catabolism to increase catabolic mediator production consist of MMPs 1, 3, 9, 13 and IL-6. Microscopic crystals can bind TLR 2 of chondrocyte resulting to induced nitric oxide production (37). TLR activation leads to increased expression of pro-inflammatory cytokines via a number of transcription factors, such as activator protein 1 (AP1), cyclic AMP responsive element binding (CREB) protein, interferon regulatory factors (IRF) and NF-κB. Specifically, TLR-2 and TLR-4 are upregulated in the synovial tissue from patients with OA (15).

Macrophages

Macrophages can be activated many ways. The primary way is activation of PRRs, which activates a number of intracellular pathways, such as NF-κB. An alternate way, macrophages can be activated through inflammasome mediated pathway (31). In knee OA patients suggested that NLRP3 inflammasomes were activated by uric acid from synovial fluid in the pathogenesis of OA (32). Hyaluronan, a glycosaminoglycan from the

extracellular matrix after damage, also activates inflammasome pathways (33). Numerous growth factors, cytokines, chemokines and enzymes that are produced from macrophages can increase the formation of osteophyte, moderate cartilage breakdown and induce joint effusion in the synovium of OA joints (34).

Complement

The complement system contains more than 30 proteins that are involved in the pathogenesis of OA including serine proteases. Serine protease contributes to complement system associated with opsonization, chemotaxis, cell lysis, inhibitors and factor H (31). In transcriptome analysis from synovial membrane, levels of complement effectors transcripts (C5) were higher whereas complement inhibitor transcripts were lower in OA patients when compared with healthy individual (35). The formation of the membrane attack complex (MAC) is related with the level of inflammation in synovial membrane (31). DAMPs in OA joints can bind to complement and lead to complement activation (31).

Granulocytes

IL-6, IL-8 and metalloproteinase MMP8 -producing neutrophils increase cartilage breakdown and lead to necrosis of the adipose tissue (34). Moreover, eosinophils and basophils release histamine and enhances production of pro-inflammatory mediators and degradation of enzyme production in synovial fibroblasts and cartilage (36).

Adaptive immunity in osteoarthritis

The adaptive immune responses in OA include responses from T cells and B cells (7, 14, 37, 38). Evidences show that inflammatory responses in joint tissue exhibits characterization of a T cell immune response (8). Anti-specific antigen antibodies are also detected in OA patients (2, 8). However, the role of T cells and B immune response in the pathogenesis of OA still needs more investigation.

T cells in osteoarthritis

T cells may play an important role in the pathogenesis and progression of OA (8, 14). Mononuclear cell infiltration in the joint tissues contain primarily CD3+ T cells. CD4+ and CD8+ T cells present have a memory and activated phenotype (8). Activated phenotype represents early (CD69), intermediate (CD25, CD38), and late (CD45RO) activation antigens of CD3+ T cells were expressed in synovial membrane of OA patients (Figure4) (8). These cells can be activated by antigens leading to the release of cytokines in the local joint (21). In addition, they found CD4+ T cells at a higher level when compared with CD8+ T cells in peripheral blood, synovial tissue and IFP. In synovial tissue, CD4+:CD8+ T cells ratio were reported as 5:1 (10). Interestingly, peripheral blood CD8+ T cells was shown to be related with aging of knee OA patients, as higher CD8+ T cells correlated with older knee OA patients (13). Also, our group have previously shown that peripheral blood CD8+ T cells were in a higher activated state than CD4+ T cells in OA patients (10).

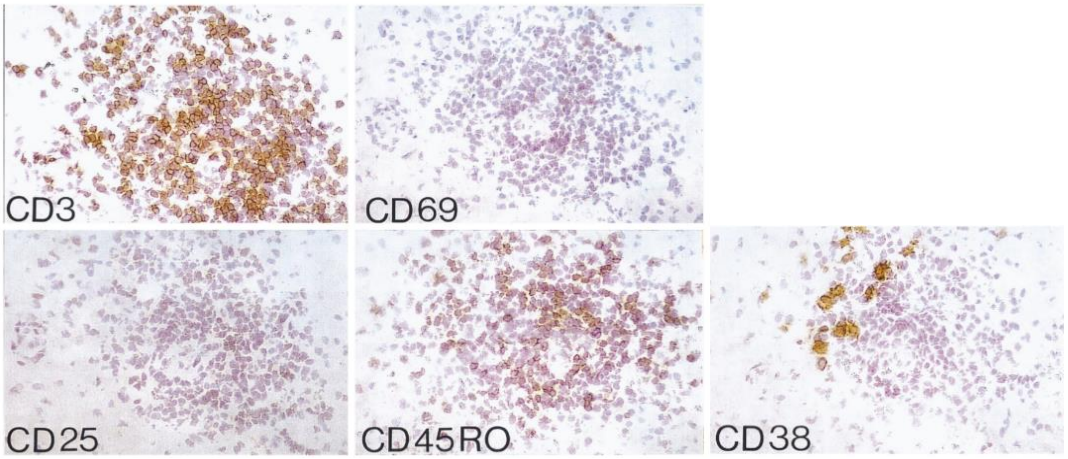


Figure 4 Immunohistochemistry of synovial membrane from OA patients expressing activation marker of T cells (CD69, CD25, CD45RO and CD38) (14)

B cells in osteoarthritis

Antibodies and immune complexes were detected at high levels in OA synovial fluid and joint tissues (2, 7). Cartilage or other cellular debris are able to bind to antibodies and lead to form immune complexes. These immune complexes may stimulate innate immune responses and inflammatory responses (2). A previous study found that antibody titers to cartilage proteoglycan aggrecan in serum of OA patients were higher when compared to healthy individuals (7). Anti–type II collagen antibodies were also found in OA cartilage (8). It is possible that cartilage proteoglycan aggrecan and type II collagen are self-antigen in OA. In addition, a potent chemo-attractor of B cell, CXCL13, was expressed in OA synovial membrane (8). The B cell repertoire in the synovium show an oligoclonal profile, suggesting an antigen-driven expansion (7).

Inflammation and osteoarthritis

Joint damage stimulates the immune system causing chronic, low-grade inflammation and development of clinical OA. Inflammation in joint OA has been demonstrated in many studies (2, 37). The inflammatory plasma proteins were found in both the blood and the synovial fluid of OA patients at a high level (2). Besides having a high level of plasma proteins, they also found complement components, chemokines and cytokines in synovial tissue and synovial fluid of OA patients (2). Joint inflammation of OA is a result of inflammatory mediators such as IL1-β, TNF, and nitric oxide (NO) -producing chondrocytes and synovial cells (2). Soluble inflammatory mediators, cytokines, are central to the inflammatory process (37). In addition, immune cells also infiltrate the joint tissues through blood vessels (38). These findings suggest that inflammation is related to immune cell infiltration and overexpression of inflammatory mediators in the joint tissue (2). Characteristic of joint inflammation of OA is associated with many pathologic changes, including increased severity of joint symptoms (morning stiffness, warmth, pain, and joint effusions), increased cartilage loss, decreased mobility, and elevated radiographic grading (2).

T cell-derived cytokines are important mediators of the outcome of immune responses (14). Several populations of T helper (Th) cells are distinguished into Th1 cells producing interleukin-2 (IL-2), tumour necrosis factor (TNF) and interferon-γ (IFN-γ), Th2 cells produce interleukin-4 (IL-4) and interleukin-5 (IL-5)) and Th17 cells produce interleukin-17 (IL-17) as well as regulatory T cells (Th3 and Tr1) (14). In normal conditions, the level of cartilage is stable as a result of balance between anabolic and catabolic cytokines, whereas the imbalance of anabolic and catabolic cytokines in OA contribute to the pathophysiology of the disease (15) (Figure 5). Many cytokines inhibit anabolic processes in OA progression (37). For example, IL-6 can activate inflammatory cells (B cells and T cells) and recruit these cells to sites of inflammation (39). IL-1β can inhibit cartilage ECM components production, including aggrecan and types II and IX collagen (37).

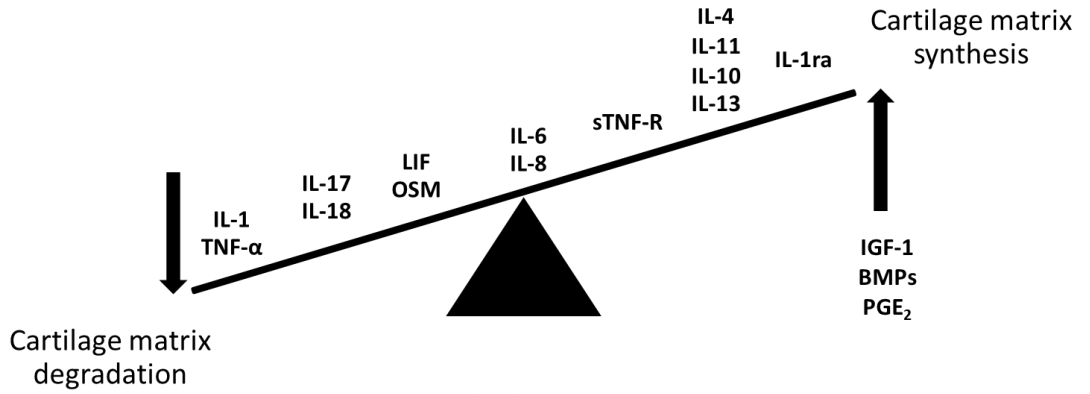


Figure 5 Cytokines association with cartilage homeostasis in osteoarthritis (37)

Several immune cells in the synovium can produce inflammatory cytokines and degradative enzymes, that reach the cartilage through the synovial fluid, in inflammatory diseases, of which includes rheumatoid arthritis (RA) and OA (40). In a previous study, they found IL-1β, TNF, IL-2 and IFN-γ in knee joints of synovial fluid samples (41). Other studies showed TNF and IL-6 were detectable in synovial fluid samples from 47 knee OA patients. KL grading had no correlation with TNF whereas there was a significant negative correlation with IL-6 (42). The examination of the levels of these cytokines in the synovial fluid from healthy knee is essential to understand more about OA.

Apart from cytokines from synovial fluid samples, cytokines from other tissues, including synovial membrane, synovial tissue, IFP, serum, plasma, articular cartilage and whole blood also have play a role in initiation and propagation of OA (7, 10, 34, 36, 40, 43-46). It has been reported that many cytokines, including IL-1β, TNF, IL-6, IFN-γ, TGF-β, IL-2, IL-4, IL-8, IL-10, and IL-18 are detected in OA patients (11, 15, 16, 40). IL-1β and TNF were the most detected in osteoarthritic inflammation. These two pro-inflammatory cytokines promote more catabolism and degradation of the cartilage in cartilage homeostasis process (7). In addition, IL-6 can be detected in early stage OA and is associated with cartilage loss. Therefore, IL-6 may be used as a diagnostic and prognostic biomarker in early stage OA (16). For this reason, these cytokines may serve as biomarkers for diagnostic and become targets for therapeutic agents of OA (47).

Rationale

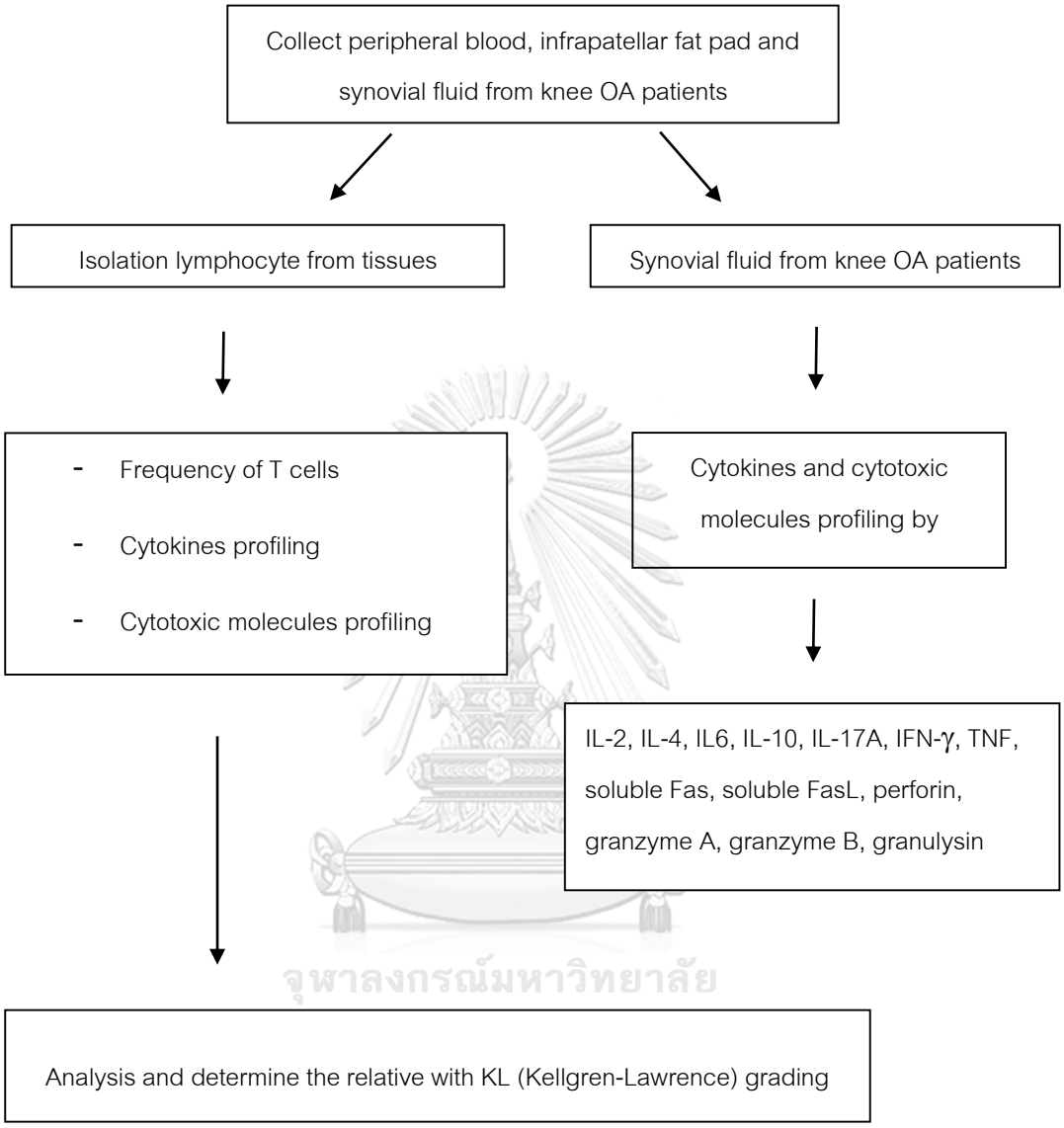
Knee OA is a chronic disease of the whole joint, which is associated with many joint tissues of the knee. Currently, many factors involved in the initiation, progression and pathogenesis in OA, including metabolic and mechanical factors. Innate and adaptive immune response are factors which play key roles in OA. There are studies regarding T cells and low-grade inflammation in OA. However, the mechanisms underlying the role of T cells in OA has not been much explored. Thus, in this thesis, our aim was to investigate the role of T cells in OA.

My research questions include

- What is the role of CD8+ T cells (and/or CD4+ T cells) in OA?
- What is the correlation between the frequency of CD4+ and CD8+ T cells, cytokines and cytotoxic granules with radiographic grading?
- Are there any predominant cytotoxic molecules found in synovial fluid of knee OA patients?

The goal of this project is to gain a more understanding of the role of cytokine and cytotoxic granule released from CD4+ and CD8+ T cells in peripheral blood and IFP and synovial fluid of OA patient. For methodology scheme is showed on the next page.

Methodology Scheme



CHAPTER III

MATERIALS AND METHODS

Materials tissue samples collection

Peripheral blood samples were collected from 15 healthy controls and 26 OA patients. IFP biopsies and synovial fluid samples were collected from 17 and 40 OA patients that underwent total knee arthroplasty (TKA) at King Chulalongkorn Memorial Hospital, respectively. Procedures were performed in accordance with the ethical standards of the responsible committee on human experimentation at the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB no. 574/57).

Demographic data collection

Demographic data of healthy individuals and knee OA patients were collected include age, weight, height and body mass index. A comparison of each characteristic between healthy individuals and knee OA patients was displayed in p-value.

Radiographic grading

All patients was reviewed preoperative standard plain radiography for radiographic grading according to the Kellgren-Lawrence (KL) grading system (grade 0: no radiographic features of OA are present; grade 1: doubtful joint space narrowing (JSN) and possible osteophytic lipping; grade 2: definite osteophytes and possible JSN on anteroposterior weight-bearing radiograph; grade 3: multiple osteophytes, definite JSN, sclerosis, and possible bony deformity; and grade 4: large osteophytes, marked JSN, severe sclerosis, and definite bony deformity) (42).

Isolation of PBMCs

PBMCs were isolated from whole blood using Ficoll-Paque density gradient centrifugation method. 10 ml of blood and 10 ml of RF 10 were layered over 15 ml of the Ficoll-Paque. Gradients were centrifuged at 600 × g for 30 min at 25 °C (Acceleration: Max, Deceleration: Off). The PBMC interface was carefully removed by pipetting and washed with RF 10 by centrifugation at 600 × g for 10 min at 4 °C. PBMCs were cryopreserved in liquid nitrogen in 90 % fetal bovine serum (FBS) / 10 % dimethyl sulfoxide (DMSO) and stored until experiments were performed.

Isolation of lymphocytes from infrapatellar fat pad

IFP from knee OA patients were cut into small pieces (~1-3 mm.) and stirred with digestion buffer for 90 min at 37 °C. Supernatant was centrifuged at 600 × g for 10 min at 4 °C. Lymphocytes were washed with RF 10 by centrifugation at 600 × g for 10 min at 4 °C. Cells were cryopreserved in liquid nitrogen in 90 % FBS / 10% DMSO and stored until experiments were performed.

Synovial fluid collection

Synovial fluid from knee OA patients were centrifuged at 600 × g for 30 min at 4 °C and supernatants were collected to remove any cells. Supernatants were cryopreserved in -80 °C and stored until experiments were performed.

Cell surface staining and intracellular cytokine staining

10^5 cells of PBMCs or 10^5 isolated lymphocytes were incubated in RF 10 with or without 6 µL of anti-CD3/CD28 bead for 1 hour at 37 ºC. After that brefeldin A (BFA) (final concentration is 20 µg/ml) was added and incubated at 37 ºC overnight. Cells were labeled with monoclonal antibodies for 30 minutes at 4 ºC in the dark with the following antibodies: antihuman-CD3-phycoerythrin-cyanine 7 (PE-Cy7), antihuman-CD4-phycoerythrin-cyanine 5 (PE-Cy5), and antihuman-CD8-PerCP-cyanine 5.5

(PerCp/Cy5.5). The plate was centrifuged at 600 × g for 5 minutes at 4 °C and remove the supernatant. Then, cells were fixed with 1% formaldehyde in PBS and incubated for 1 hour at 4 ºC in the dark. Cells were subsequently labeled with monoclonal antibodies for 1 hour at 4 ºC in the dark with the following antibodies: antihuman-interleukin-17 (IL-17)-phycoerythrin (PE), antihuman-interleukin-1β (IL1β)-fluorescein isothiocyanate (FITC), antihuman-interleukin-6 (IL6)-allophycocyanin (APC), antihuman-IFN-γ-allophycocyanin-cyanine 7 (APC-Cy7), and antihuman-tumor necrosis factor (TNF)-Alexa Fluor 700. Cells were washed with FACS buffer (10% FBS in PBS) and centrifuged at 600 × g for 5 minutes at 4 °C. After washing the cells twice with FACS buffer, cells were acquired on the BDLSRII flow cytometer. Analysis was performed using the Flowjo software (Treestar, USA). All antibodies were purchased from Bio-legend, CA, USA. Except for antihuman-IL-17-PE was purchased from R&D Systems™, USA.

Degranulation assay

10^5 cells of PBMCs or 10^5 isolated lymphocytes were incubated with RF 10 and antihuman-CD107a-PE-Cy7 with or without 6 µL of anti-CD3/CD28 bead for 1 hour at 37 ºC. After that BFA (final concentration is 20 µg/ml) and monensin (final concentration is 20 µg/ml) were added and incubated at 37 ºC overnight. Cells were labeled with monoclonal antibodies for 30 minutes at 4 ºC in the dark with the following antibodies: antihuman-CD3-APC, antihuman-CD4-APC-Cy7 and antihuman-CD8-Alexa Fluor 700. The plate was centrifuged at 600 × g for 5 minutes at 4 °C and remove the supernatant. Then, cells were fixed with 1% formaldehyde in PBS and incubated for 1 hour at 4 ºC in the dark. Cells were labeled with monoclonal antibodies for 1 hour at 4 ºC in the dark with the following antibodies: antihuman-granzymeB-FITC, antihuman-perforin-PerCp/Cy5.5, and antihuman-granulysin-PE. Cells were washed with FACS buffer and centrifuged at 600 × g for 5 minutes at 4 °C. After washing the cells twice with FACS buffer. Cells were acquired on the BDLSRII flow cytometer. Analysis was performed using the FlowJo software (Treestar, USA). All antibodies were purchased from Biolegend, CA, USA.

Except for antihuman-CD107a-PE-Cy7, antihuman-granzymeB-FITC and antihuman-perforin-PerCp/Cy5.5 were purchased from Becton Dickinson (BD), NJ, USA.

Cytometric bead array (Human CD8/NK Panel)

Synovial fluid samples were diluted 2-fold with assay buffer before being tested (50 µL of synovial fluid sample: 50 µL of assay buffer). Standard were prepared based on the manufacture's procedure (LEGEND plex™ Human CD8/NK Panel). Standard cocktail or synovial fluid was added into 96-well V bottom microplate and incubated with hyaluronidase (final concentration is 20 U/ml), 25 µL of mixed beads and 25 µL of detection antibodies. Then, the plate was oscillated at 90 × g for 2 hours at room temperature in the dark and 25 µL of Streptavidin-PE (SA-PE) was added into each well for another 30 minutes. The plate was centrifuged at 600 × g for 5 minutes at 4 °C and remove the supernatant. The remaining bead were washed with 200 µL of 1X wash buffer. The plate was centrifuge at 600 x g for 5 minutes at 4 °C and supernatant were removed. After washing the remaining bead twice, 200 µL of 1X wash buffer were added to each well. Samples were acquired on a FACS Calibur™ flow cytometer. Analysis was performed using the LEGEND plex™ data analysis software.

Statistical analysis

All data were analyzed using the Statistical Package for Social Sciences (SPSS 15.0, SPSS Inc, Chicago, IL, USA) or Graph Pad InStat version 5.0 software (San Diego, CA, USA). The results were presented as mean ± S.D. Independent sample t-tests was used to compare the value between two groups (between healthy control and knee OA patients and peripheral blood and IPFPs of knee OA patients). Statistical significance was defined as $P \leq 0.05$ (*), $P \leq 0.01$ (**) or $P \leq 0.001$ (***). Analysis of the radiographic grading (the calculation: clinical grading (the Kellgren-Lawrence (KL) grading system) and cytometric bead array using the LEGENDplex™ Data Analysis software.

CHAPTER IV

RESULTS

PART 1
(SOLUBLE MEDIATORS)

Demographic characteristics data of healthy individuals and OA patients

We collected demographic characteristics of healthy individuals (n=15) and knee OA patients (n=31). A comparison of each characteristic between knee OA patients and healthy individuals is shown in Table 2. Our sample population was collected from healthy individuals with low risk in OA. The mean values of age and body mass index (BMI) was significantly lower in healthy individuals than knee OA patients. The mean age of healthy individuals was 29.21 (± 4.46) years and OA patients was 68.88 (± 7.91) years. The mean BMI of healthy individuals was 22.73 (± 3.76) kg/m^2 and OA patients was 26.35 (± 4.76) kg/m^2.

Table 2 Comparison of demographic characteristics between healthy individuals and OA patients.

	Healthy (n=15) mean values (SD)	OA Patient (n=31) mean values (SD)	p-value
Age (years)	29.21 (4.46)	68.88 (7.91)	<0.001
Weight (kilograms)	60.93 (12.31)	61.81 (11.26)	0.81
Height (centimetres)	1.63 (0.07)	1.54 (0.06)	<0.001
Body mass index (kg/m^2)	22.73 (3.76)	26.35 (4.76)	0.016

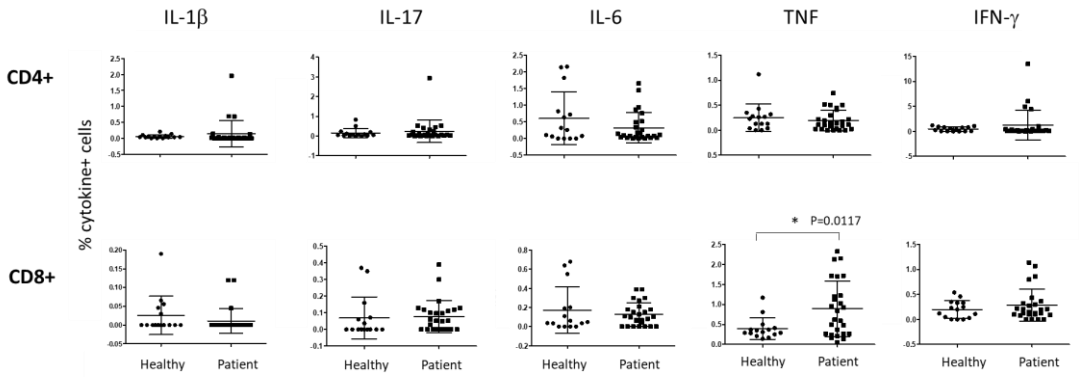
CD4+ and CD8+ T cell cytokine and cytotoxic granule production from peripheral blood of healthy individuals and knee OA patients

The systemic inflammation may be involved with local inflammation in OA joints (2). We investigated the role of T cells in OA patients evaluation of cytokine and cytotoxic granule production from peripheral blood T cells of OA patients and compared the levels with healthy individuals.

We performed cytokine and cytotoxic granule analyses by flow cytometer on peripheral blood of 15 healthy individuals and 26 knee OA patients. We stimulated the PBMCs with anti-CD3/anti-CD28 beads. We evaluated the secretion of cytokine production by intracellular cytokine staining that included IL-1β, IL-17, IL-6, TNF and IFN-γ and cytotoxic granule production by degranulation assays that included perforin, granzyme B and granulysin. Moreover, we also evaluated the degranulation level by evaluated the degranulation marker (LAMP1 or CD107a).

Unstimulated TNF-producing CD8+ T cells from OA patients were significantly higher than healthy individuals (p=0.0117) (Figure 6). Unstimulated cytokines-producing CD4+ and CD8+ T cell (IL-1β, IL-17, IL-6, and IFN-γ) and TNF-producing CD4+ T cell were not significantly different (Figure 6). Furthermore, IL-6-producing CD4+ T cells and IFN-γ-producing both CD4+ and CD8+ T cells from OA patients when stimulated with anti-CD3/anti-CD28 beads was significantly decreased when compared to healthy individuals (IL-6+CD4+ T cell, p=0.0253; IFN-γ+CD4+ T cell, p=0.0002; IFN-γ+CD8+ T cell, p=0.0019) (Figure 6). In peripheral blood of OA patient, the ability of T cells to secret some cytokines (TNF) was increased whereas the ability of T cells after stimulation to secrete some cytokine (IL-6 and IFN-γ) was decreased. However, limitation of this comparison includes non-age matched between healthy individuals and knee OA patients since age is one of the risk factor of OA. Old age can lead to T cell dysfunction (48). Thereby, the comparison with age matched has a better conclusion.

Unstimulated



Stimulated with anti-CD3/CD28 bead

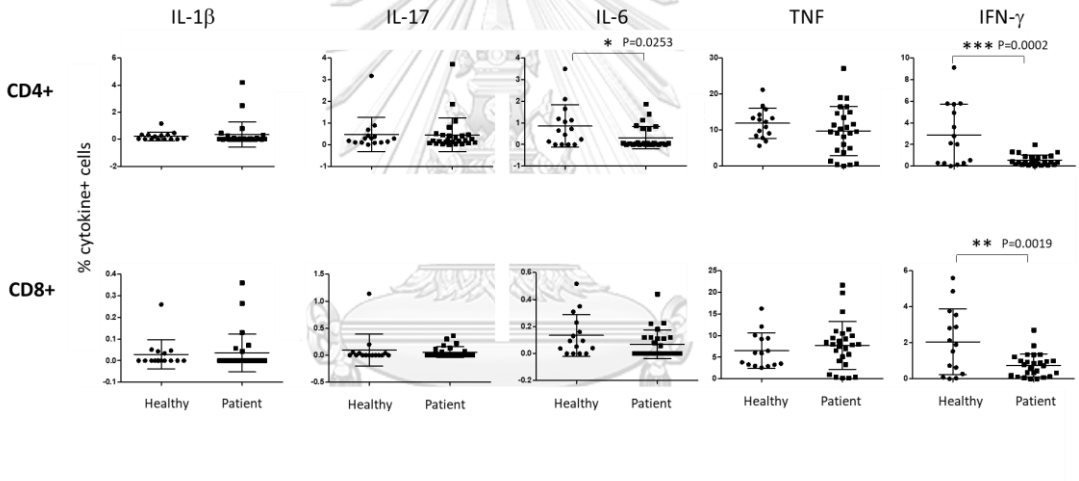


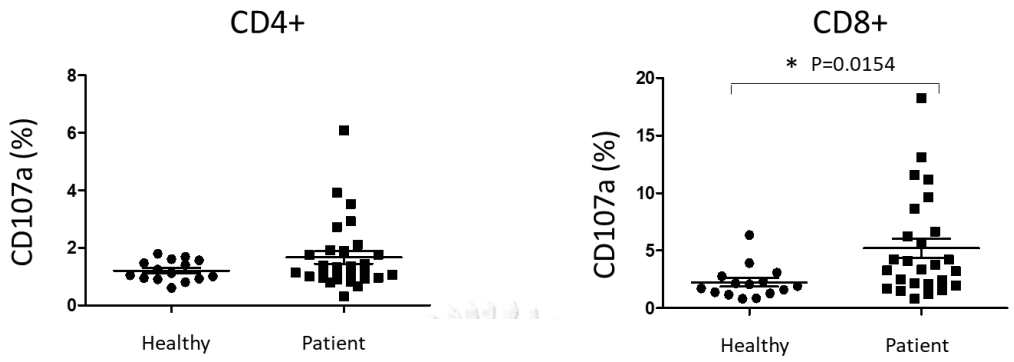
Figure 6 Cytokine-producing CD4+ and CD8+ T cells from peripheral blood of healthy individuals and knee OA patients. The frequency of cytokine-producing CD4+ and CD8+ T cells (IL-1β, IL-17, IL-6, TNF and IFN-γ) in unstimulated (above) and stimulated with anti-CD3/anti-CD28 beads (below) from peripheral blood from healthy individuals and knee OA patients. (healthy individuals; N=15, knee OA patients; N=26)

Degranulation levels in CD4+ T cell was slightly higher in knee OA patients when compared to healthy individual, despite not being significant (figure 7A). In CD8+ T cells, degranulation levels from knee OA patients were higher ($p=0.0154$) than healthy individuals (Figure 7A). Levels of degranulation after anti-CD3/CD28 bead stimulation of CD8+ T cells were comparable. CD8+ T cells in peripheral blood of knee OA patients had higher levels of degranulation than healthy individuals, referring to a more activated state of CD8+ T cells in knee OA patients (49).

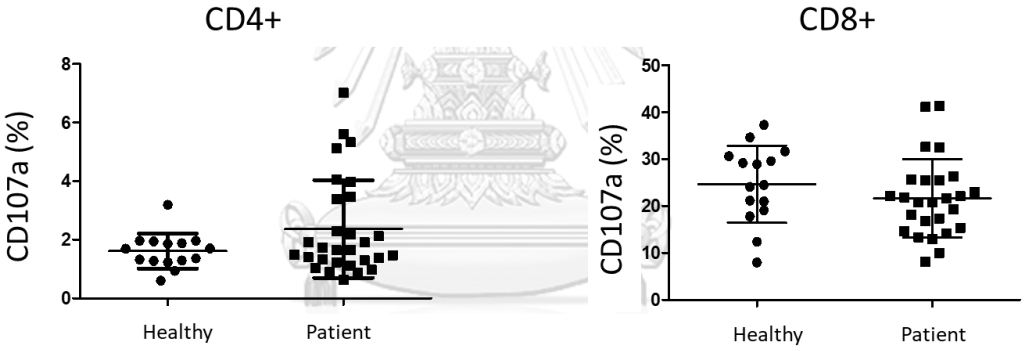
Cytotoxic granule production in unstimulated condition showed that perforin levels in CD8+ T cells from knee OA patients were lower ($p=0.0186$) than healthy individual. Other cytotoxic granules in both CD4+ and CD8+ T cells did not differ significantly (Figure 7B). When stimulated with anti-CD3/anti-CD28 beads results show that CD4+ T cells was produced granzyme B lower ($p=0.0096$) in knee OA patients when compared to healthy individuals. (Figure 7B). In peripheral blood of OA patient, the ability of T cells to secrete some cytotoxic granule (perforin) was decreased. Similarly, the ability of T cells after stimulation to secrete some cytotoxic granule (granzyme B) also was decreased. Similarly, Old age effects T cell function (48) and age-matched healthy group may be more comparable.

A

Unstimulated

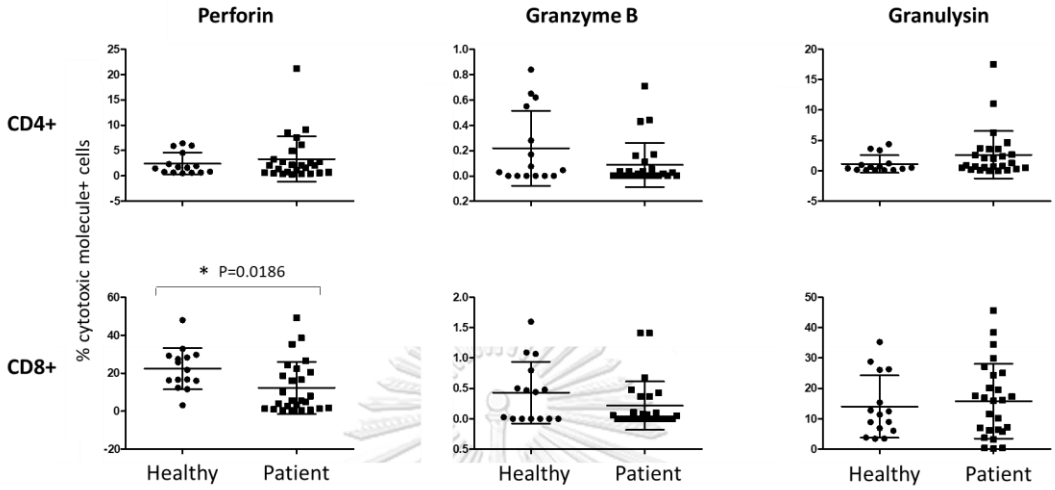


Stimulated with anti-CD3/CD28 bead



B

Unstimulated



Stimulated with anti-CD3/CD28 bead

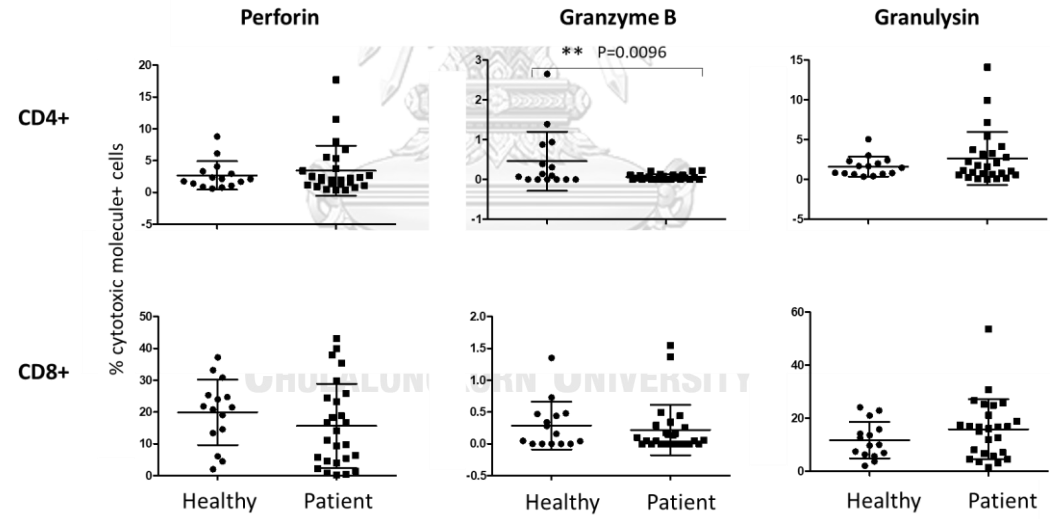


Figure 7 Degranulation levels and cytotoxic granules production from peripheral blood CD4+ and CD8+ T cells in healthy individuals and knee OA patients. Peripheral blood T cells from healthy individuals and knee OA patients were isolated in unstimulated and stimulated (anti-CD3/anti-CD28 beads). Degranulation assays were performed on these cells to determine the level of (A) CD107a cell surface expression and (B) cytotoxic granules (perforin, granzyme B and granulysin) in both CD4+ and CD8+ T cell subpopulation. (healthy individuals; N=15, knee OA patients; N=26)

A comparison of CD4+ and CD8+ T cell cytokine and cytotoxic granule production between peripheral blood and IFP of knee OA patients

Since local inflammation plays a role in OA (2). We compared cytokine and cytotoxic granule production, from T cell between peripheral blood and IFP of knee OA patients.

When we compared the levels of cytokines in peripheral blood and IFP of knee OA patients in CD4+ T cells, results indicated that IL-1β, IL-17, IL-6 and TNF -producing CD4+ T cell in IFP were higher than in peripheral blood (IL-1β, p=0.0228; IL-17, p=0.0154; IL-6, p<0.000; TNF, p=0.0006). IFN-γ producing CD4+ T cells did not differ significantly between these tissues (Figure 8). The level of cytokines in peripheral blood and IFP of knee OA patients in CD8+ T cells showed that four cytokines include IL-1β, IL-6, TNF and IFN-γ producing CD8+ T cells from IFP were higher than peripheral blood (IL-1β, p=0.0229; IL-6, p=0.0012; TNF, p=0.0003; IFN-γ, p=0.003). However, IL-17 producing CD8+ T cells did not differ significantly (Figure 8). From these findings, IFP T cells are capable of secreting cytokines at higher levels than peripheral blood from both CD4+ and CD8+ T cells, especially pro-inflammatory cytokines. In addition, we also found increases in IL-17 and IFNγ production.

Next, we also compared the cytotoxicity function of T cells by examining the degranulation level and cytotoxic granule production in peripheral blood and IFP of knee OA patients. The degranulation marker (CD107a) in CD4+ T cell was significantly higher in IFP than peripheral blood of knee OA patients (p<0.0001). However, CD107a in CD8+ T cells did not differ significantly between peripheral blood and IFP of knee OA patients (Figure 9A). These results suggest that CD4+ T cells may have cytotoxic effects due to its increased level of degranulation in IFPs. Perforin- and granulysin- producing CD4+ T cells were increased in IFP of knee OA patients when compared with peripheral blood of knee OA patients (perforin, p=0.0379; granulysin, p=0.0059) (Figure 9B). In addition, perforin- and granzyme B- producing CD8+ T cells also were at higher levels in IFP than peripheral blood of knee OA patients (perforin, p=0.018; granzyme B, p=0.0112) (Figure 9B). These results suggest that cytotoxicity may contribute to the pathogenesis of knee OA, however

the cytotoxicity function of CD4+ and CD8+ T cells in knee local inflammation may be not the same mechanism due to presence of different cytotoxic molecules.

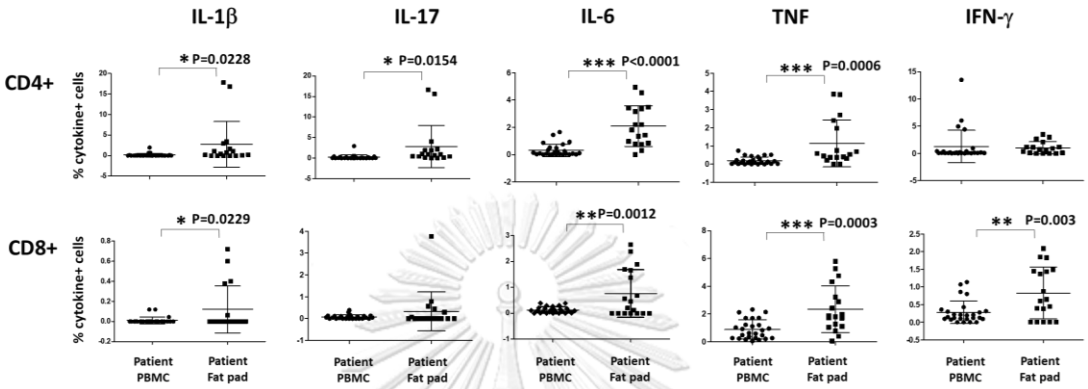


Figure 8 A comparison of cytokine levels of CD4+ and CD8+ T cells isolated from peripheral blood and IFP of knee OA patients. T cells were isolated from peripheral blood and IFP of knee OA patients. Intracellular cytokine staining was performed for detect of IL-1β, IL-17, IL-6, TNF and IFN-γ from CD4+ and CD8+ T cell. Each dot represents one individual. (N=26; peripheral blood, N=17; IFP)

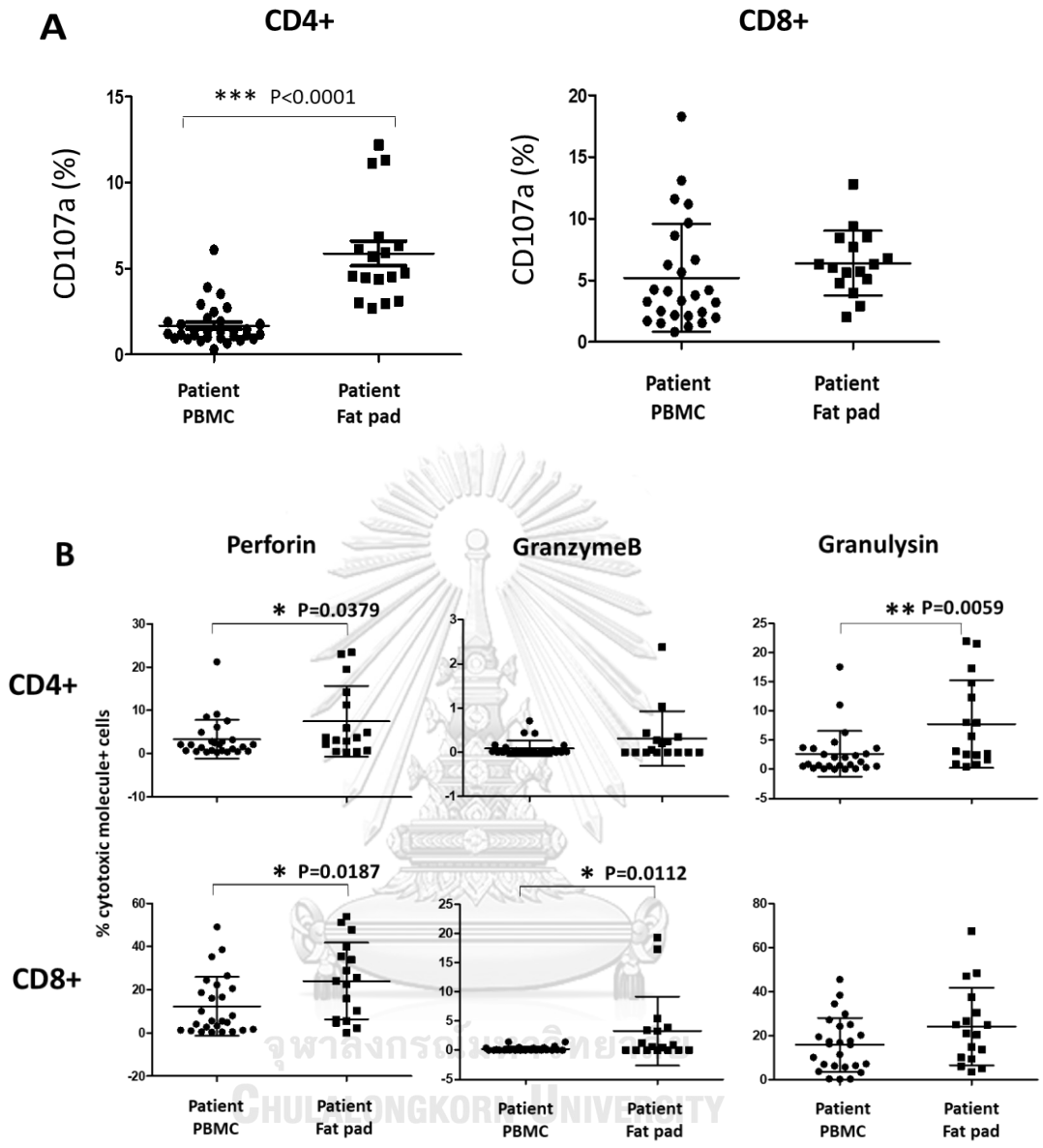


Figure 9 A comparison of degranulation levels and cytotoxic granules production of CD4+ and CD8+ T cells isolated from peripheral blood and IFP of knee OA patients. Degranulation assays were performed degranulation levels and cytotoxic granules. Degranulation levels based on cell surface expression of (A) CD107a of CD4+ and CD8+ T cells and (B) cytotoxic granules production include perforin, granzyme B and granulysin of CD4+ and CD8+ T cells were isolated from peripheral blood and IFP of knee OA patients. Y-axis represents percentage of CD107a cell surface expression or cytotoxic granules-producing cells. Each dot represents one individual. (N=26; peripheral blood, N=17; IFP)

Polyfunctional cytokines and cytotoxic granules production and with or without degranulation marker (CD107a) by T cells

The release of soluble molecules by T cell is associated with functional activity *in vivo*. The ability of T cells to produce multiple soluble molecules is usually beneficial in immune response (49). The functional responses of T cells have been associated with measures of these functions include releasing of one or more soluble molecules. However, to test whether T cells in IFP of OA patients had this phenotype, we studied the functional responses of T cells by measuring cytokines and cytotoxic granules in OA. We evaluated polyfunctional secretion of cytokine (IL-1β, IL-17, IL-6, TNF and IFN-γ) and cytotoxic granule (perforin, granzyme B and granulysin) production of both CD4+ and CD8+ T cells.

The dominant population of T cells from IFPs were those that released one cytokine. However, we detected CD4+ T cells that released two cytokines occur that is IL-1β/IFN-γ and IL-1β/IL-6. T cells that secreted multiple cytokines (three, four and five cytokines) were not detected (Figure 10A). For cytotoxic granule production, the majority of IFP CD4+ and CD8+ T cells were those that produced one cytotoxic granule, followed by two and three cytotoxic granules, respectively, regardless of degranulation status (LAMP1+/- or CD107a+/-) (Figure 10B). These findings show that T cell clones from IFP are more likely to release multiple cytotoxic molecules, but not multiple cytokines.

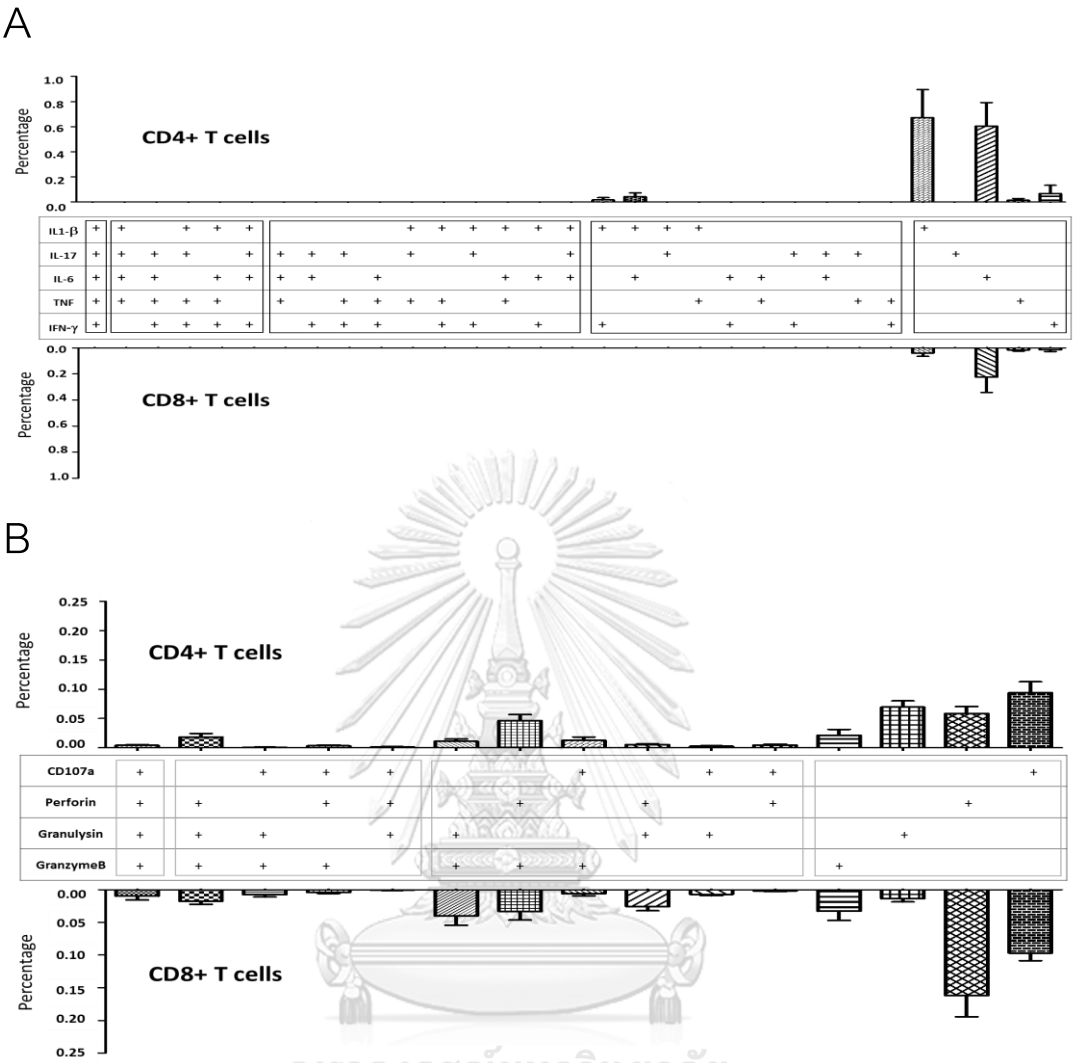
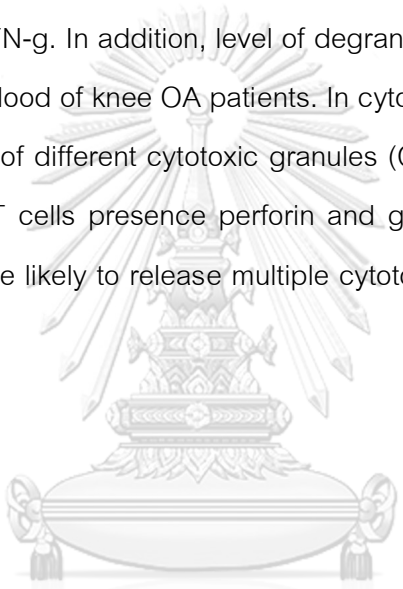


Figure 10 Polyfunctional cytokine and cytotoxic granule production by CD4+ and CD8+ T cells from IFP of knee OA patients. T cells were isolated from IFP of knee OA patients (N=17) and intracellular cytokine staining was performed on these cells. Polyfunctional (A) cytokine (IL-1β, IL-17, IL-6, TNF and IFN-γ) and (B) cytotoxic granule production (perforin, granzyme B and granulysin) and with or without degranulation marker (CD107a) by CD4+ and CD8+ T cells. X-axis represents the panel of cytokines or cytotoxic granules/degranulation expressed in a single cell. Y-axis represents the percentage of cells that are present in the total population of T cells. Bars represent mean values with error bars.

In this chapter, peripheral blood T cells able to release some cytokine (TNF) was higher whereas peripheral blood T cells after stimulated release some cytokine (IL-6 and IFN-g) was lower in knee OA patients than healthy individuals. Level of degranulation in only peripheral blood CD8+ T cells was higher than healthy individuals. Peripheral blood T cells from OA patients secreted some cytotoxic granule (perforin and granzyme B) was decreased in unstimulated and stimulated condition than healthy individuals. Comparison of IFP and peripheral blood, demonstrated the increase of pro-inflammatory cytokines (IL-1b, IL-6, TNF) from IFP T cells. It is interesting that only CD8+ T cells in the IFP had significant increase of IFN-g. In addition, level of degranulation of IFP CD4+ T cells was higher than peripheral blood of knee OA patients. In cytotoxicity function, IFP CD4+ and CD8+ T cells presence of different cytotoxic granules (CD4+ T cells presence perforin and granulysin/ CD8+ T cells presence perforin and granzyme B). In addition, T cell clones from IFP are more likely to release multiple cytotoxic molecules, but not multiple cytokines.



PART 2
(CORRELATION WITH RADIOGRAPHIC GRADING)

Correlation between the frequency of CD107a of CD3+, CD4+ and CD8+ T cells, cytotoxic granules and cytokines of knee OA patients with radiographic grading

Immune responses affect with disease severity (19). T cells are one of the mediators that are important in immune responses. We determined the correlation between the frequency of CD107a+ T cells, cytokine and cytotoxic granule -producing T cells with radiographic grading.

We isolated CD4+ and CD8+ T cells from peripheral blood (n=26) and IFP (n=15) of knee OA patients. We performed cytokines and cytotoxic granules production by Flowcytometry (BDLSRII). We evaluated the secretion of cytokines production by intracellular cytokine staining include IL-1β, IL-17, IL-6, TNF and IFN-γ and cytotoxic granules production by degranulation assays include perforin, granzyme B and granulysin. We also evaluated the degranulation levels by checking the degranulation marker (LAMP1 or CD107a). Then, we correlated between CD107a of CD3+, CD4+ and CD8+ T cells, cytotoxic granules- and cytokines- producing T cell with radiographic grading using Kellgren-Lawrence (KL) classification.

A correlation between cytokines producing CD4+ and CD8+ T cell and KL grading in peripheral blood and IFP. Our sample population were obtained from OA patients in grade 3 and grade 4 undergoing total knee arthroplasty. There were no patients for KL grades 0, 1 and 2. We found that cytokine production levels, IL-1β, IL-17, IL-6, TNF and IFN-γ, secreted from both CD4+ and CD8+ T cells isolated from peripheral blood and IFP of knee OA patients were not significantly different between knee OA patients grade 3 and grade 4 (Figure 11A and 11B).

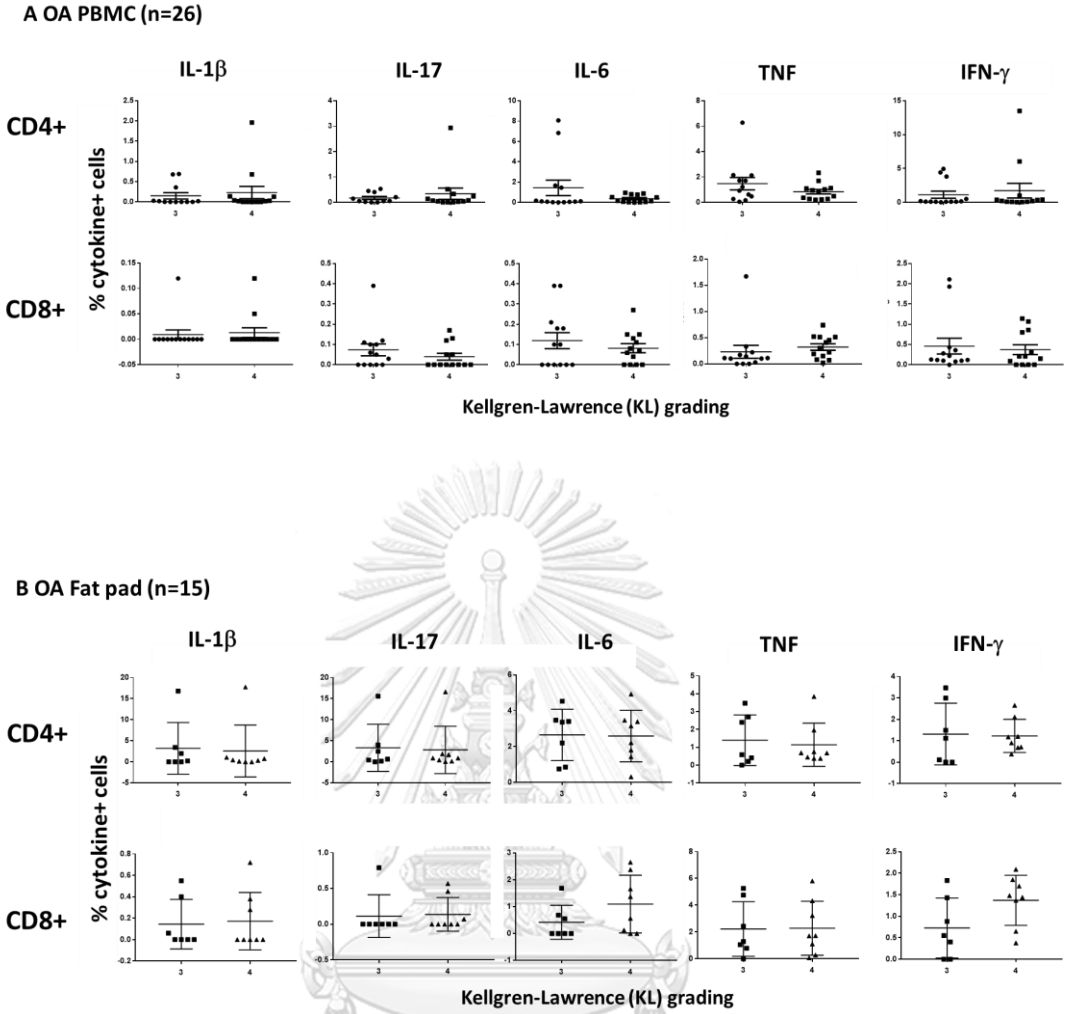
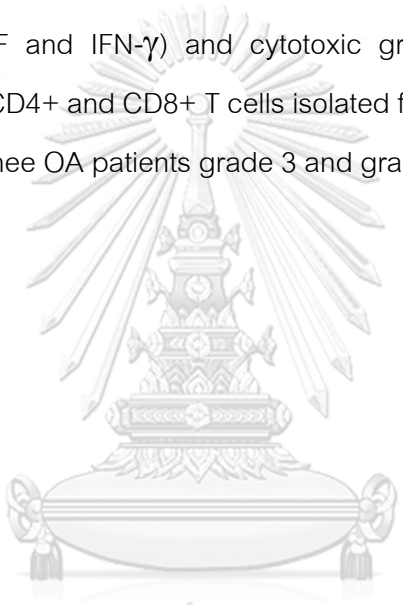


Figure 11 A correlation of cytokine production of CD4+ and CD8+ T cells isolated from peripheral blood and IFP of knee OA patients with KL grading. T cells were isolated from peripheral blood and IFP of knee OA patients. The correlation between cytokine-producing CD4+ and CD8+ T cells (IL-1β, IL-17, IL-6, TNF and IFN-γ) from (A) peripheral blood and (B) IFP of knee OA patients with KL grading. (peripheral blood (OA PBMC); N=26, IFP (OA Fat pad); N=15)

In addition, we correlated the frequency of degranulation levels (CD107a) of CD3+, CD4+ and CD8+ T cells with KL grading in peripheral blood and IFP. The mean frequency of CD107a expression on CD3+, CD4+ and CD8+ T cells were similar between KL grade 3 and grade 4 both in peripheral blood and IFP (Figure 12A and 12B). When correlate between cytotoxic granules (perforin, granzyme B, granulysin) and KL grading in peripheral blood and IFP indicated that it also was not significantly different between grade 3 and grade 4 both in CD4+ and CD8+ T cells (Figure 13A and 13B).

In conclusion, we found no correlation of the frequency of CD107a+, cytokines (IL-1β, IL-17, IL-6, TNF and IFN-γ) and cytotoxic granules (perforin, granzyme B, granulysin) -producing CD4+ and CD8+ T cells isolated from peripheral blood and IFP of knee OA patients with knee OA patients grade 3 and grade 4.



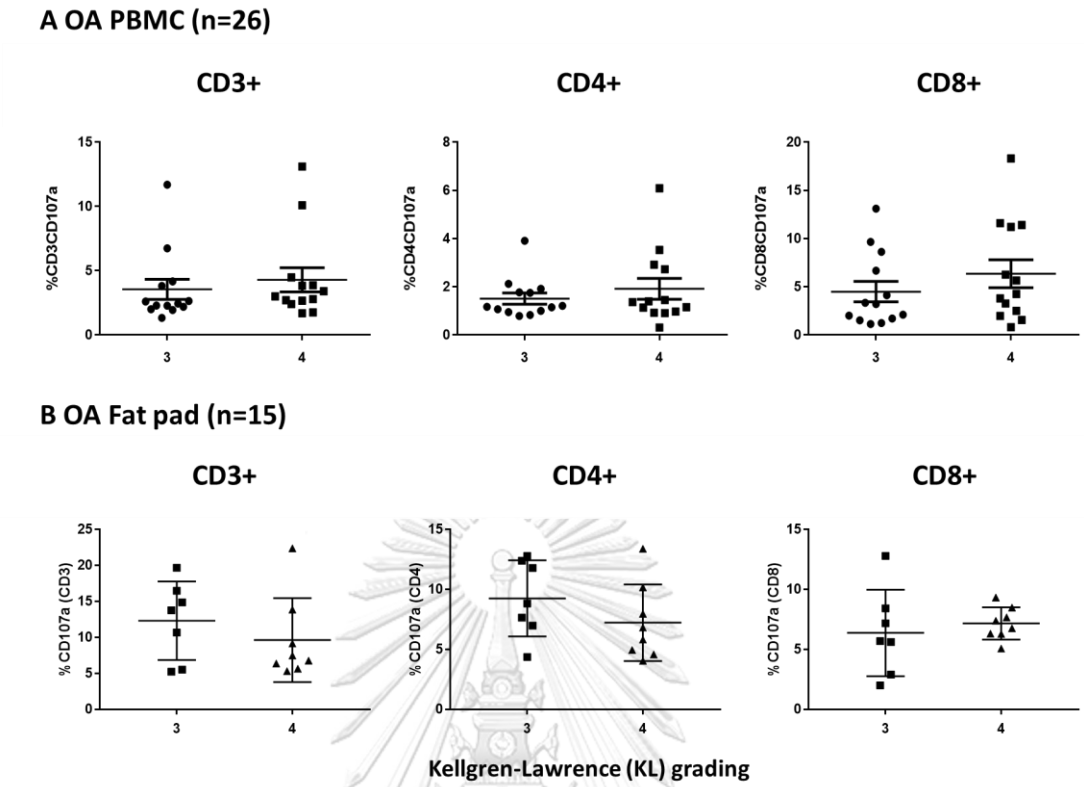


Figure 12 A correlation of the frequency of CD107a of CD3+, CD4+ and CD8+ T cells isolated from peripheral blood and IFP of knee OA patients with KL grading. T cells were isolated from peripheral blood and IFP of knee OA patients. The correlation of the frequency of CD107a of CD3+, CD4+ and CD8+ T cells from (A) peripheral blood and (B) IFP of knee OA patients with KL grading. Each dot represents one individual. (peripheral blood (OA PBMC); N=26, IFP (OA Fat pad); N=15)

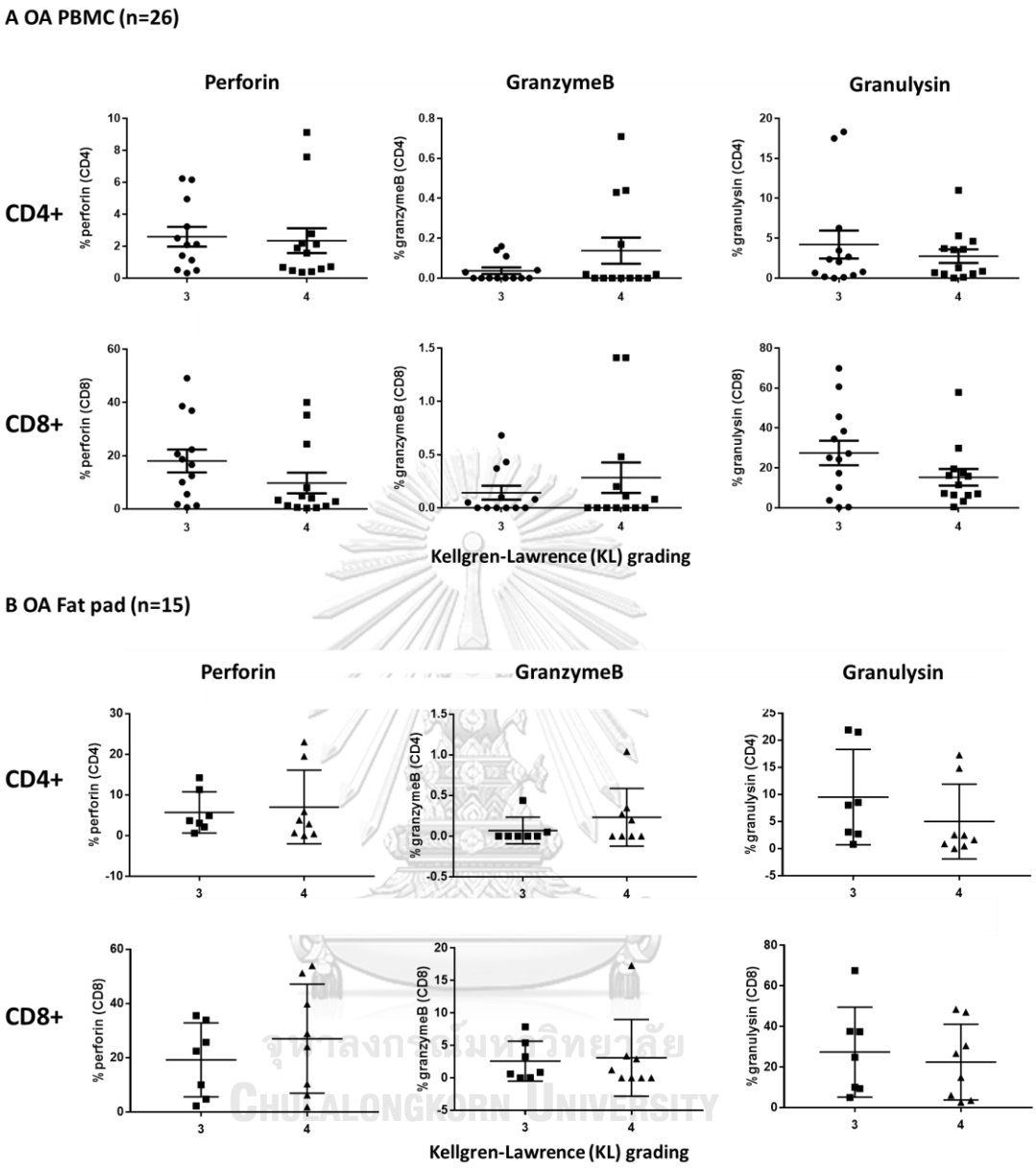


Figure 13 A correlation of the cytotoxic granule production of CD4+ and CD8+ T cells isolated from peripheral blood and IFP of knee OA patients with KL grading. T cells were isolated from peripheral blood and IFP of knee OA patients. The correlation of cytotoxic granules producing CD4+ and CD8+ T cells (perforin, granzymeB, granulysin) from (A) peripheral blood and (B) IFP of knee OA patients with KL grading. Each dot represents one individual. (peripheral blood (OA PBMC); N=26, IFP (OA Fat pad); N=15)

PART 3
(SOLUBLE PROTEINS FROM SYNOVIAL FLUID)

Soluble proteins from synovial fluid of knee OA patients

Soluble protein can infiltrate the synovial fluid (40). These soluble molecules are secreted by immune cells which affects local inflammation in knee OA (11). Due to the synovial fluid being a soluble matrix infiltrating the knee cavity and our previous findings that show cytotoxic granules and cytokines from T cells are present higher in IFPs than peripheral blood, we studied the soluble proteins from synovial fluid of knee OA patients.

We performed a cytometric bead array (LEGEND plex™ Human CD8/NK Panel) on synovial fluid of 40 knee OA patients. We detected for the presence of 13 soluble proteins, including IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF, soluble Fas (sFas), soluble FasL (sFasL), granzyme A, granzyme B, perforin and granulysin. To digest extracellular matrix that may interfere with the assay sensitivity, we needed to treat synovial fluids with hyaluronidase. We tested hyaluronidase treatment for any enzymatic effect on our soluble proteins of interest. Standard cocktail was divided into two conditions (test with or without hyaluronidase) at 1:4, 1:16 and 1:64 dilution. Detection of soluble proteins did not differ between the two conditions at tested dilutions (1:4, 1:16 and 1:64 dilution) (Figure 14).

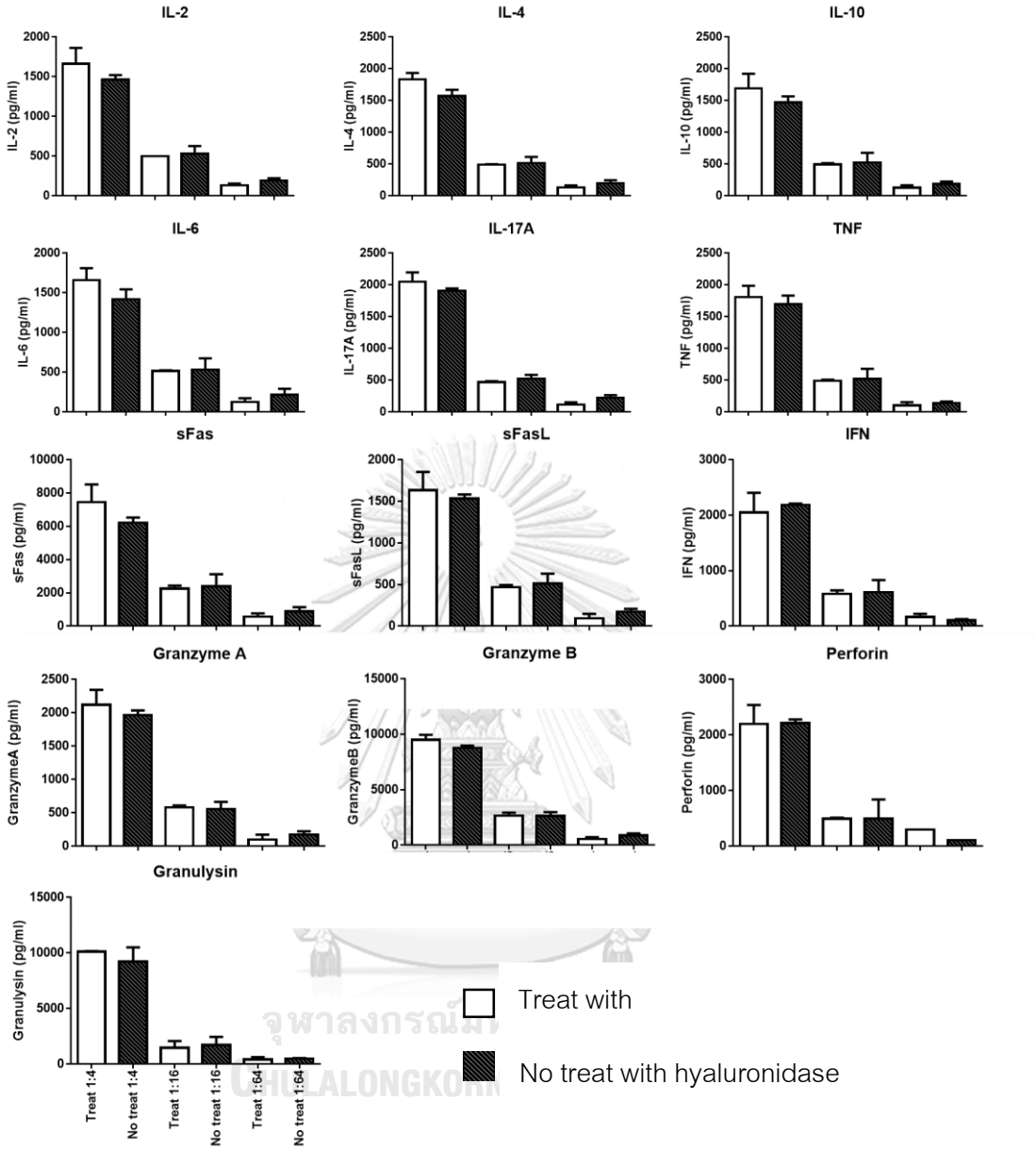


Figure 14 Soluble proteins from standard cocktail. Graphs of 13 soluble proteins (IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF, sFas, sFasL, granzyme A, granzyme B, perforin and granulysin) to test with or without hyaluronidase dilution at 1:4, 1:16 and 1:64 from standard cocktail (duplicate). X-axis represents treating with or without hyaluronidase. Y-axis represents the percentage of each soluble protein (pg/ml). Bars represent mean values with error bars.

Mean values (±SD) of each analyte levels from synovial fluid are show in Table3. Concentrations of soluble proteins were different in each soluble protein. IL-6, sFas, granzyme A, perforin and granulysin, were highly detectable (90-97.5%) (n=40). IL-2, IL-10, IL17A, TNF, IFN-γ and granzyme B were intermediately detectable (7.5-67.5%) whereas IL-4 and sFasL were not detected (0%) in synovial fluid (n=40) (Table 3) (Figure 15). Among the highly detectable mediators, a comparison of soluble proteins revealed sFas with the highest concentration (835.91 ± 370.86) followed by granzyme B (272.63 ± 216.13), granulysin (210.77 ± 177.46), IL-6 (130.91 ± 239.01) and perforin (102.98 ± 86.83), respectively (Figure 16).

In conclusion, we detected a predominant of IL-6, sFas, granzyme A, perforin and granulysin from synovial fluid of knee OA patients suggesting cytokine and cytotoxic molecules may be as mediators that cause inflammation in local site of knee OA.

Table 3 Soluble proteins from synovial fluid of OA patients

Cytokines (pg/ml)	Synovial fluid of OA (n=40) (Mean ± SD)	Detectable (%)
IL-2	12.23 ± 6.23	25
IL-4	0 ± 0	0
IL-10	3.392 ± 1.80	60
IL-6	130.91 ± 239.01	92.5
IL-17A	10.92 ± 10.34	67.5
TNF	14.97 ± 27.63	37.5
sFas	835.91 ± 370.86	97.5
sFasL	0 ± 0	0
IFN-γ	6.50 ± 4.51	50
Granzyme A	23.05 ± 18.00	92.5
Granzyme B	272.63 ± 216.13	7.5
Perforin	102.98 ± 86.83	90
Granulysin	210.77 ± 177.46	95

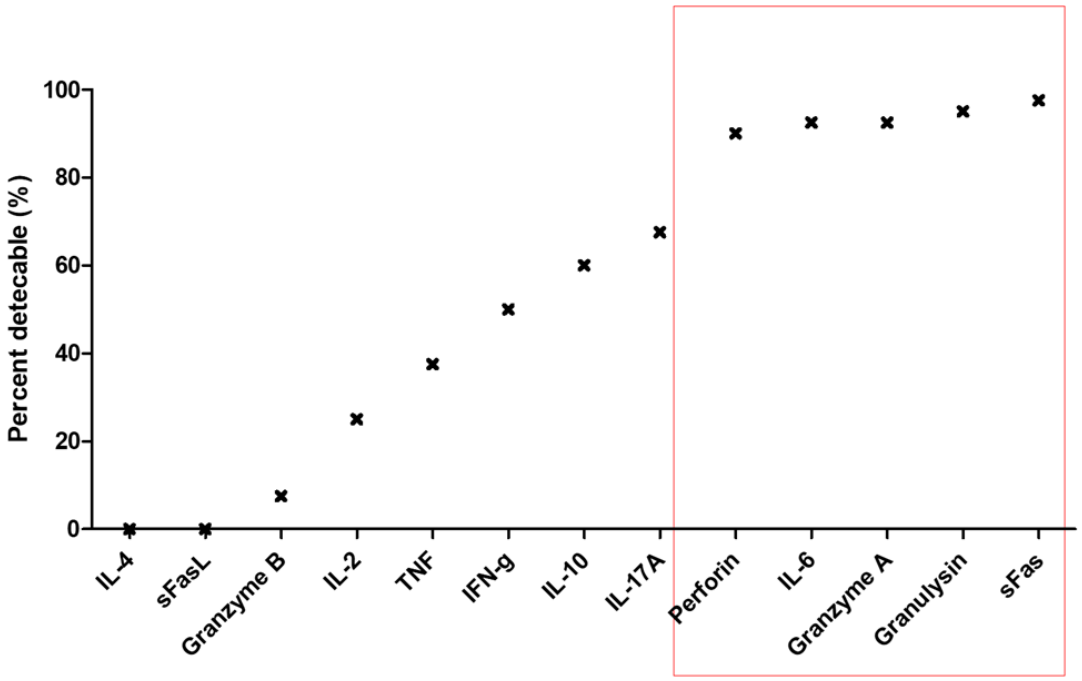


Figure 15 Soluble proteins from synovial fluid of OA patients. This graph represents percentage of detectable of 13 soluble proteins (IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF, sFas, sFasL, granzyme A, granzyme B, perforin and granulysin) from synovial fluid of OA patients which detected. X-axis represents the percentage of detectable of each soluble protein (%). Y-axis represents the panel of soluble proteins.

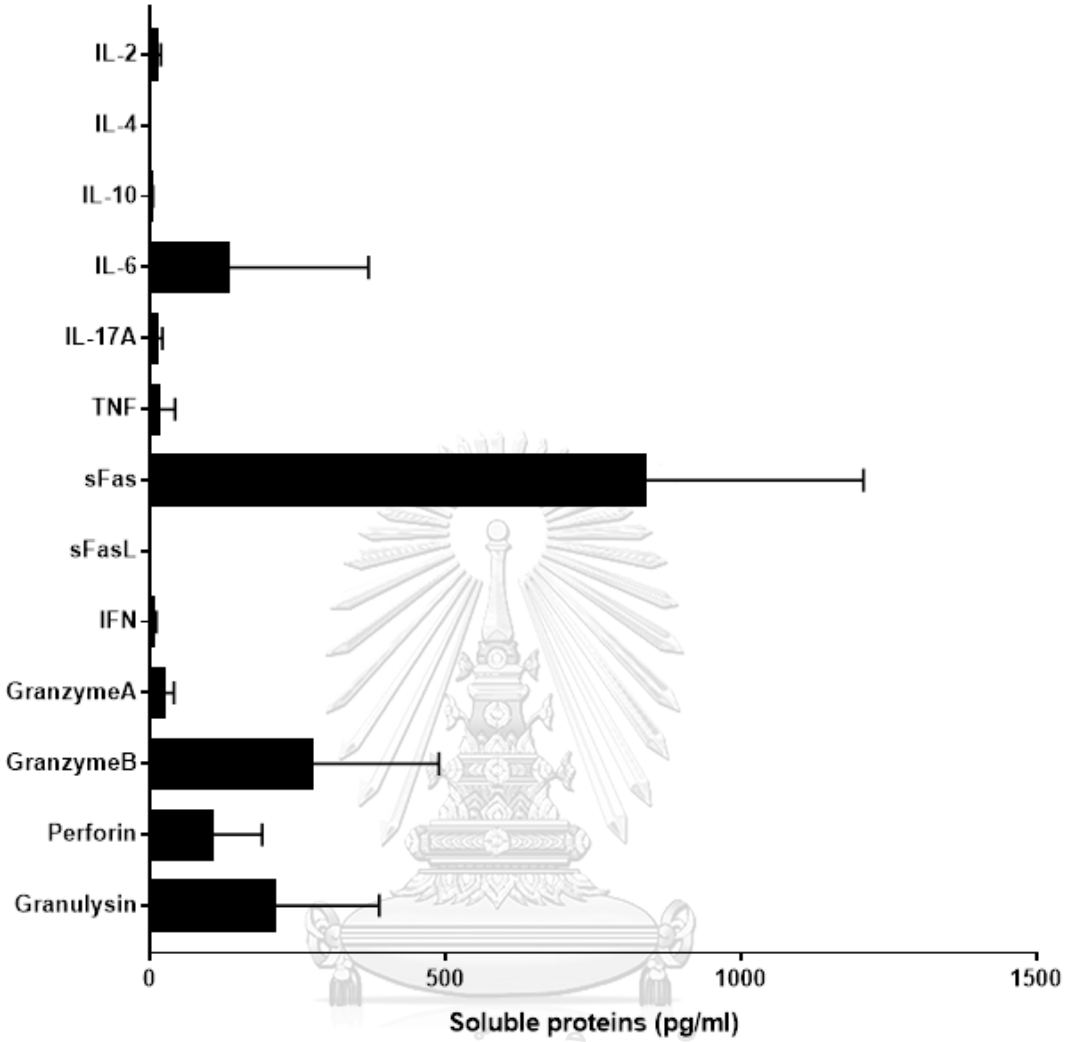


Figure 16 Soluble proteins from synovial fluid of OA patients. This graph represents 13 soluble proteins (IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF, sFas, sFasL, granzyme A, granzyme B, perforin and granulysin) from synovial fluid of OA patients which detected. X-axis represents the percentage of each soluble protein (pg/ml). Y-axis represents the panel of soluble proteins. Bars represent mean values with error bars.

CHAPTER V

DISCUSSION

We studied T cells in knee OA patients and investigated the soluble molecules that are released from T cells in peripheral blood, IFP and soluble mediators in the synovial fluid. In addition, we correlated these soluble molecules and degranulation level of T cells with severity grading. Here, we summarize our key findings of the study.

Immune response of T cells in OA is evident and characterized by the presence of T cell infiltrated in synovial membrane, IFP, subcutaneous adipose tissue (ScAT), and synovial tissue (10, 21, 34, 38). The IFP is a main adipose tissue within the knee joint capable of T cell infiltration and also a major source of several mediators (cytokines and adipokines) (10, 22, 38).

Several mediators are involved in OA pathogenesis, such as IL-1β, TNF, IL-6, IFN-γ, TGF-β, IL-2, IL-4, IL-8, IL-10, and IL-18 (11). In our study, we only investigated cytokines that were present in large amounts from these studies (7, 11, 15, 38, 40, 42, 46). Specifically, we evaluated the production of TNF, IL-6, IFN-γ, IL-17 and IL-1β in peripheral blood and IFP T cells. Our results show higher levels of TNF-producing CD8+ T cells in peripheral blood of knee OA patients than healthy individuals. When compared the level of cytokines in peripheral blood and IFP in knee OA patients, IL-1β, IL-6 and TNF -producing CD4+ and CD8+, IL-17-producing CD4+ and IFN-γ-producing T cells from IFP were present at higher levels than peripheral blood of knee OA patients. Likewise, the previous studies shown that IFN-γ, TNF and IL-6 are produced from both CD4+ and CD8+ T cells that stimulated with PMA/ionomycin in IFP of OA patients (38). In contrast, in our study, we stimulated T cells via T cell receptor-mediated T cell activation and compared cytokines-producing T cells between IFPs and peripheral blood of OA patients. We also determined IL-1β- and IL-17-producing T cells in our study.

In addition, a comparison of the cytotoxicity function of T cell found that T cells from knee OA patients had higher levels of degranulation than healthy individuals,

especially CD8+ T cells. This finding reflects an “activated” state of CD8+ T cells as degranulation are usually due to responses of T cell activation (50). Cytotoxicity function of T cell is the expression and regulated of secreted the cytotoxic molecule including perforin, granzyme B and granulysin. A preliminary previous study reported that granulysin-producing T cell from peripheral blood of OA patients counts about 30% and it was approximately twice higher, when compared with healthy individuals (51). In the IFP of knee OA patients, there were higher levels of perforin- and granulysin-producing T cells in the CD4+ T cell population. Interestingly, in the CD8+ T cell population higher levels of perforin- and granzyme B-producing T cells was observed instead, when compared to peripheral blood. Granule exocytosis by cytotoxic T lymphocytes can be mediated via perforin and granzymes; or perforin and granulysin (50). Despite the findings showing similar cytotoxicity potential in both IFP CD4+ and CD8+ T cells, however, the mechanisms may differ. Altogether, these results confirm the detection of certain cytokines and cytotoxic molecules similar to many previous studies (11, 16, 52). We describe that these cytokines and cytotoxic molecules detected are produced from T cells.

Furthermore, in inflammatory diseases such as rheumatoid arthritis or OA, the immune cell in synovial tissue produced several soluble mediators which reach the cartilage through the synovial fluid (40). Based on early findings that suggests the importance of CD8+ T cells in OA (10), we focused to determine the levels of soluble mediators that were related to cytotoxicity. In the synovial fluid, we were able to detect IL-6, sFas, granzyme A, perforin and granulysin in more than 90% of all specimen and IL-2, IL-4, IL-10, IL-17A, TNF, sFasL, IFN-γ and granzyme B less than 90%. It has been reported about cytokines in synovial fluid, they found a large amount of IL-6, IFN-γ and IL-2 (16). In these study, sFas was found at the highest levels in synovial fluid and even higher than IL-6. IL-6 has been detected in synovial fluid, serum, IFP, ScAT, cartilage tissue and synovial tissue and also plays a major pro-inflammatory role in OA (11, 16, 38, 40, 42, 46). However, sFas was also detected both in the serum and synovial fluid of patients with rheumatoid arthritis and OA (55). They found that the concentration of sFas from serum

was significant higher than from synovial fluid of OA patients (serum; 1488±192 pg/ml and synovial fluid; 884±478 pg/ml) (55). One of cytotoxicity of CTLs is the Fas-Fas ligand system. The Fas antigen (CD95) is a molecule that transmits apoptotic signals to several types of cells and perhaps the presence of sFas can cause chondrocytes, which are the cells of the cartilage, cell death via Fas-dependent apoptosis. sFas have been reported in many conditions, including supernatant of activated lymphocytes (55). In the local site of knee OA, activated lymphocytes have been identified in knee tissues (10) suggesting that sFas is probably released from activated infiltrating lymphocytes around periarticular tissues. Studying the soluble mediator profile in synovial fluid which local joint may be beneficial in targeted therapy for treatment of knee OA.

Overall, we found that IL-6, perforin and granulysin were soluble mediators present at high levels synovial fluid of OA patients. In conclusion, IL-6, perforin and granulysin; and potentially granzyme A and sFas may be interesting candidates as prognostic markers of knee OA. However, these aspects still require further investigation, confirmation and validation in serum patient samples.

Moreover, we determined the correlation of mediators and radiographic grading using KL grading system. We observed no correlation between CD107a, cytokines/cytotoxic granules and grade 3, grade 4. This could be due to the lack of patients with KL grades 0, 1 and 2 in our study. Similar to previous study of Vangsness C T et al, they reported none significant differences of cytokines level, IL-1a, IL-1b, IL-6, IL-8, IL-17, IL-4 IL-10, IL-13, IL-2, IL-5, IL-7, IL-12p70, IL-15, IFN-γ, when using KL grading system. However, contrary to international cartilage repair society (ICRS) grading system, they found significant differences in IL-2 and IL-5 between advanced arthritis and little or no arthritis (53). Unfortunately, IL-2 and IL-5 were not included our study. the different found between using the KL system and ICRS categorization is that the ICRS categorization is based on direct visualization of the joint surface during arthroscopy whereas the KL categorization is on the basis of radiographic findings and may not refract the true pathology of the discussion (53).

APPENDICES

APPENDIX A

REAGENTS PREPARATION

Phosphate Buffer Saline (PBS)

Stock solution (10xPBS)

NaCl	80	g.
KCl	2	g.
Na_2HPO_4	11.5	g.
KH_2PO_4	2	g.
ddH_2O	1000	ml.

Working solution (1xPBS)

PBS 10X	100	ml.
ddH_2O	900	ml.

Supplemental Complement (SC)

RPMI	320	ml.
Non-essential Amino Acid Solution	100	ml.
Penicillin Streptomycin Solution (Gibco)	80	ml.
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	11.9	g.
L-glutamine	100	ml./ 3 g.
b-mercaptoethanol	35	ul.

Filtered by 0.2 um and aliquot to 30 ml., store at -20 °C

Fetal bovine Serum (FBS)

Heat-inactivated at 56 °C for 30 min, store at -20 °C

RF 10 Medium

10% FBS	60	ml.
SC	30	ml.
RPMI	500	ml.

Digestion Buffer for Synovial Tissue and IFP

PBS	47.25	ml.
FBS	2.5	ml.
Collagenase type IV	50	ul.
DNase	5	ul.

Freezing Reagent

FBS	30	ml.
Dimethyl sulfoxide (DMSO)	3.3	ml.

FACS Buffer

PBS	100	ml.
FBS	2	ml.

Fixation Buffer

PBS	100	ml.
FBS	2	ml.
Formaldehyde	2.8	ml.

APPENDIX B

CHEMICAL AND REAGENTS

Phosphate Buffer Saline (PBS)	THAILAND
Roswell Park Memorial Institute (RPMI) medium (Gibco)	USA
Ficoll (GE Healthcare)	SWEDEN
Collagenase type IV	THAILAND
Hyaluronidase	THAILAND
DNase	THAILAND
Fetal bovine serum	THAILAND
Dimethyl sulfoxide (DMSO) (AMRESCO)	USA
Anti-CD3 antibody, PE-Cy7 label (clone: UCHT1)	USA
Anti-CD4 antibody, PE-Cy5 label (clone: RPA-T4)	USA
Anti-CD8 antibody, PerCp/Cy5.5 label (clone: SK1)	USA
Anti-CD107a antibody, PE-Cy7 label (clone: H4A3)	USA
Anti-interleukin-17 (IL-17) antibody, PE label (clone: SCPL1362)	USA
Anti-interleukin-1β (IL1β) antibody, FITC label (clone: H1b-98)	USA
Anti-interleukin-6 (IL6) antibody, APC label (clone: MQ2-13A5)	USA
Anti-IFN-γ antibody, APC-Cy7 label (clone: 4S.B3)	USA
Anti-tumor necrosis factor (TNF) antibody, AF700 label (clone: Mab11)	USA
Anti-perforin antibody, PerCP-cy5.5 label (clone: dG9)	USA
Anti-granzymeB antibody, FITC label (clone: GB11)	USA
Anti-granulysin antibody, PE label (clone: DH2)	USA
Brefeldin A (BFA)	USA
Monensin	USA
LEGEND plex™ kit (BioLegend)	CALIFORNIA

APPENDIX C

MATERIALS & EQUIPMENT

Flow cytometer (BDLSRII)

Hemacytometer

Falcon Tube 15 and 50 ml

Disposable plastic transfer pipettes

Glass pipettes 2, 5, 10, and 25 inch

Polystyrene U-Bottom plate 96 well

Polystyrene V-Bottom plate 96 well

Autopipette 1000, 200, 50 and 10 ul

Multichannel pipette 20-200 ul

Vortex mixer

Water bath

96 U-bottom plates

96 V-bottom plates

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