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Exprese interleukinu 20 a jeho význam u revmatoidní artritidy

The expression of interleukin 20 and its role in rheumatoid arthritis

Diploma Thesis

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Declaration:

I declare that I worked and wrote this Diploma Thesis personally, only by using cited literatures, under the leadership of the Head of our Department.

Prague, 12.08.2013

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CONTENT

Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disease that is associated with formation of autoantibodies, activation of inflammatory cascade and up-regulation of several cytokines. These processes lead to persistent synovial inflammation, joint damage and systemic manifestations. The aim of this diploma thesis is to characterize the role of a novel cytokine interleukin-20 (IL-20) in the pathogenesis of RA and to investigate its involvement in different stages of the disease as a potential surrogate biomarker. In this work, several methods including Enzyme-Linked Immunosorbent Assay (ELISA), Immunohistochemistry and Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) have been employed.

We demonstrated increased expression of IL-20 in the synovial tissue of RA compared with control osteoarthritis (OA) patients. Along with the up-regulation at sites of inflammation, concentrations of IL-20 were higher in the synovial fluid compared with circulating levels of IL-20. Furthermore, serum and synovial fluid IL-20 levels significantly correlated with RA disease activity. Synthesis of IL-20 was significantly increased in peripheral blood mononuclear cells (PBMCs) and synovial fibroblasts upon stimulation with some TLR ligands and pro-inflammatory cytokines. Although not regulating PBMCs functions in vitro, IL-20 stimulated expression of IL-8 in RA synovial fibroblasts. Taken together, IL-20 represents significant cytokine involved in the pathology of RA, may reflect disease severity and potentially might be a good therapeutic target for RA.

Key words: rheumatoid arthritis, osteoarthritis, disease activity, cytokine, chemokine, interleukin-20, Interleukin-8, ELISA, PCR.

Abstrakt

Revmatoidní artritida (RA) je systémové autoimunitní onemocnění charakterizované tvorbou autoprotilátek a aktivací prozánětlivých cytokinů. Tento proces vede k chronickému zánětu v synoviální tkáni a následné kloubní destrukci. Cílem diplomové práce bylo charakterizovat roli interleukinu-20 (IL-20) jako potenciálního biomarkeru ve vztahu k patogenezi RA v jednotlivých stádiích onemocnění. Hladiny IL-20 a jeho exprese byla získána pomocí imunologických a molekulárně genetických metod jako je Enzyme-Linked Immunosorbent Assay (ELISA), Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) a imunohistochemickým barvením tkáně.

Prokázali jsme zvýšenou expresi IL-20 v synoviální tkáni u nemocných s RA oproti pacientům s osteoartrózou (OA). U pacientů s RA byla zjištěna rovněž vyšší hladina IL-20 v synoviální tekutině oproti hladinám v krevním séru. Hladina IL-20 korelovala s aktivitou onemocnění. Syntéza IL-20 u mononukleárních buněk (PBMC) a synoviálních fibroblastů byla signifikantně zvýšená po stimulaci TLR ligandy a prozánětlivými cytokiny. Po stimulaci IL-20 došlo u RA synoviálních fibroblastů ke zvýšené expresi IL-8.

Z výsledků pilotní studie vyplývá, že IL-20 se účastní patogeneze RA, jeho hladiny korelují s aktivitou onemocnění a potenciálně je možné ho využít k cílené terapii RA.

Klíčová slova: revmatoidní artritida, osteoartróza, aktivita onemocnění, cytokin, chemokin, interleukin-20, interleuzkin 8, ELISA, PCR.

Abbreviation

AA	Amyloidiosis
ACPA	Anti-Citullinated Protein Antibody
ACR	American College of Rheumatology
BMI	Body Mass Index
CRP	C - Reactive Protein
CLTA	Cytotoxic T-lymphocyte Antigen
DAS28 score	Disease Activity Score
DMARDs	Disease Modifying Antirheumatic Drugs
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DRP	Disease Repercussion Profile
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
ESR	Erythrocyte Sedimentation Rate
EULAR	European League Against Rheumatism
FCRL	Fc receptor- like protein
HDL-C	High Density Lipoprotein Cholesterol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human Leukocyte Antigen
HLA-DRB	Human Leukocyte Antigen complex on Chromosome 6

IgG	Immunoglobulin G
LDL-C	Low Density Lipoprotein Cholesterol
LPS	Lipopolysaccharides
MCP	Monocyte Chemotactic Protein
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
MRI	Magnetic resonance imaging
NK	Natural Killer (cells)
OA	Osteoarthritis
PBMC	Peripheral Blood Mononuclear Cell
PBS	Basic cell culture media contains phosphate and NaCl
Poly (I:C)	Polyinosinic: polycytidylic acid
PGE2	Prostaglandin E2
PTPN22	Protein Tyrosine Phosphate Non-receptor type 22
RA	Rheumatoid Arthritis
RANKL	Receptor Activator of Nuclear factor Kappa-β Ligand
RF	Rheumatoid Factor
S	Serum
SF	Synovial Fluid
SLC22A4	Solute Carrier Family 22, member 4
SLF	Synovial-Like Fibroblast
STAT	Signal Transducer and Activator of Transcription protein

TNF	Tumour Necrosis Factor
VDR	Vitamin D Receptor

1 INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease that is characterized by synovial hyperplasia, cartilage damage and bone erosions. The disease may be associated with disability, several organ involvements, and systemic complications with increased risk of cardiovascular comorbidities (KVIEN et al. 2009).

Pathogenesis of RA involves migration of several immune cells (e.g. macrophages, dendritic cells, lymphocytes, neutrophils) into the joint tissues and activation of resident cells such as synovial fibroblasts, chondrocytes, osteoblasts and osteoclasts (SCOTT et al. 2010). There are three different overlapping mechanisms involved in this process and consists of inflammation, modulated immune response and synovial hyperplasia. Synovial hyperplasia is induced by increased accumulation of immune cells within the synovial membrane that produce various cytokines and inflammatory molecules (MCINNES et al. 2007). All the cells act together in the inflamed synovial tissue via direct cell-cell contact or via soluble mediators, known as cytokines. Activated synovial fibroblasts are more resistant to apoptosis, undergo morphological differentiation, proliferate, and produce several matrix degrading enzymes leading to joint destruction (OSPELT et al. 2008). These changes are associated with inflammatory reaction that contributes to joint tenderness, pain, weakness and finally loss of function.

Systematic response to inflammation and cellular activation is mediated and determined by cell-cell contact or by soluble mediators - cytokines. Most of the cytokines are multifunctional. Cytokines are engaged in wide networks with synergistic and antagonistic interactions with both negative and positive effects on target cells (FEGHALI et al. 1997).

The purpose of this study was to explore the role of a novel cytokine interleukin (IL)-20 in the process of RA inflammation, joint destruction and to characterize its role as a potential biomarker reflecting disease severity.

2 AIMS OF THE THESIS

The aim of my diploma thesis was to examine the role of a novel cytokine IL-20 as biomarker in RA. In particular, we studied the difference in the levels of local as well as systemic amount of IL-20 between RA and control osteoarthritis (OA) patients. Furthermore, we studied the biologic effects of IL-20 on different resident tissue and systemic immune cells derived from synovial tissue and blood circulation, respectively.

3 LITERATURE REVIEW

3.1 Rheumatoid arthritis

RA is relatively common systemic autoimmune and longstanding disease affecting joints, which has a greater incidence in females (2-3:1), and sometimes is associated with systemic features and extra-articular manifestations. It is not known when RA first developed. There is controversy as to whether the disease is a modern illness (starting from 17th century) or whether the origin of the disease came from ancient. However, its name was first introduced in the 1850s and RA classification was first developed about 50 years ago (SCOTT et al., 2010).

The disease is characterized by symmetrical inflammatory polyarthritis that causes joint swelling, tenderness, pain, and morning stiffness particularly of small and middle-sized joints contributing generally to disability, increased risk of morbidity and premature mortality. On average, RA reduces life expectancy by seven to ten years, and creates the need for long-term medical treatment (FIRESTEIN et al. 2003, BEACKLUND et al. 2006).

During the process of RA, inflammatory cells invade the synovial membrane, which in turn results in cartilage damage and bone erosions leading to inability and functional loss of affected joints. However, the course of RA shows different profiles. There are cases ranging from mild to severe disease depending on the amount of inflammation and subsequent joint destruction. The heritability of RA is about 60%, which indicates that genetic factors (mostly HLA genes) account for a large proportion of the risk of the disease. Recent data indicate that, however, multiple genes and epigenetic factors, including interactions with environmental

factors may contribute to large heterogeneity of the broad spectrum of disease that is currently classified as RA (SCOTT et al., 2010 and MCINNES et al. 2011)

Recent studies have demonstrated that systemic autoimmunity characterized by presence of circulating autoantibodies including rheumatoid factors (RFs) and/or anti citrullinated peptide antibodies (ACPAs) usually precede the clinical onset of RA by many years (Figure 1) (KLARESKOG et al. 2009). One of the best established risk factors for RA is cigarette smoking that causes protein citrullination in lungs and breach of tolerance in genetically predispose individuals with subsequent synovial inflammation. RA is considered as a useful model for studying various inflammatory and immune mediated diseases. In addition to joint involvement, some other organs may be engaged such as the vascular system, lungs or eyes. The clinical picture of RA usually shows overlapping features with other rheumatic diseases and it may also appear more frequently with other autoimmune diseases including Hashimoto thyroiditis.

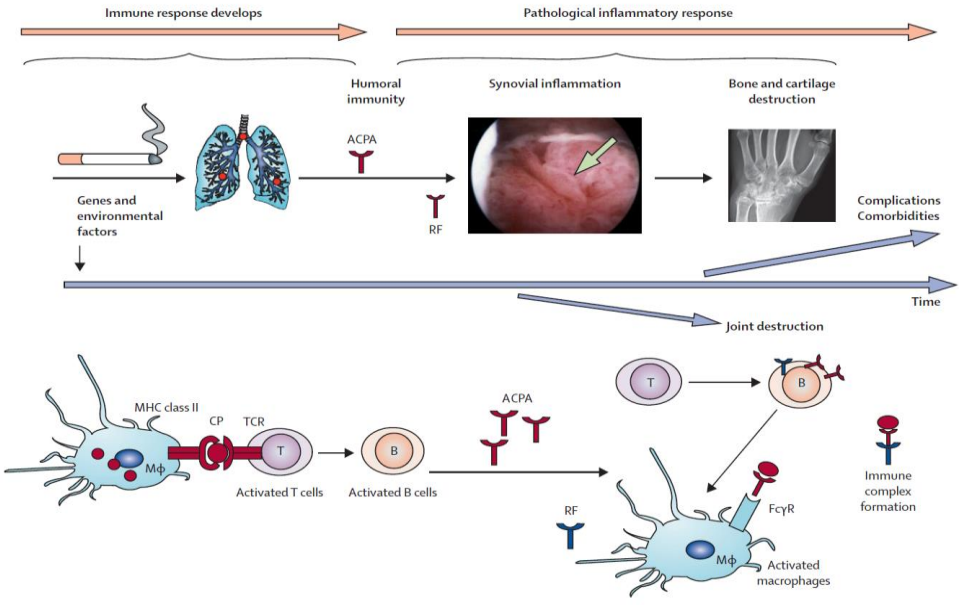

Figure 1 Hypothetical model for molecular pathogenesis of ACPA-positive rheumatoid arthritis (from Klareskog, et al. Lancet 2009).

RA is a disorder with multifactorial causes; diverse genetic and environmental factors, different prevalence among ethnic groups, and variety of clinical, radiological and laboratory features of this disease. Most studied factors include auto-antibodies, cytokines, hormones and genetic molecules that are suggested to influence RA development. (HYRICH et al. 2005, AGGRAWAL et al. 2009)

Treatment with synthetic disease modifying anti-rheumatic drugs (DMARD), mainly methotrexate, and applying biological agents such as tumour necrosis factor (TNF) inhibitors and other new biological agents with different mode of action have contributed to dramatic revolution in the development of RA management (VIEIRA et al. 2011, SENOLT et al. 2009). However it should be noted that using glucocorticoids and synthetic DMARDs is applied without complete understanding of the mechanism of their action (VIEIRA et al.2011).

3.1.1 Clinical symptoms

The onset of RA and the course of the disease may be extremely variable. The main features of RA are defined as joint pain, tenderness, swelling and morning stiffness that can lead to subsequent development of joint deformities (Figure 2). These are the common physical symptoms and signs that define RA as a chronic inflammatory disorder. The chronicity of the disease may be finally followed by severe disability, increased morbidity and premature mortality.

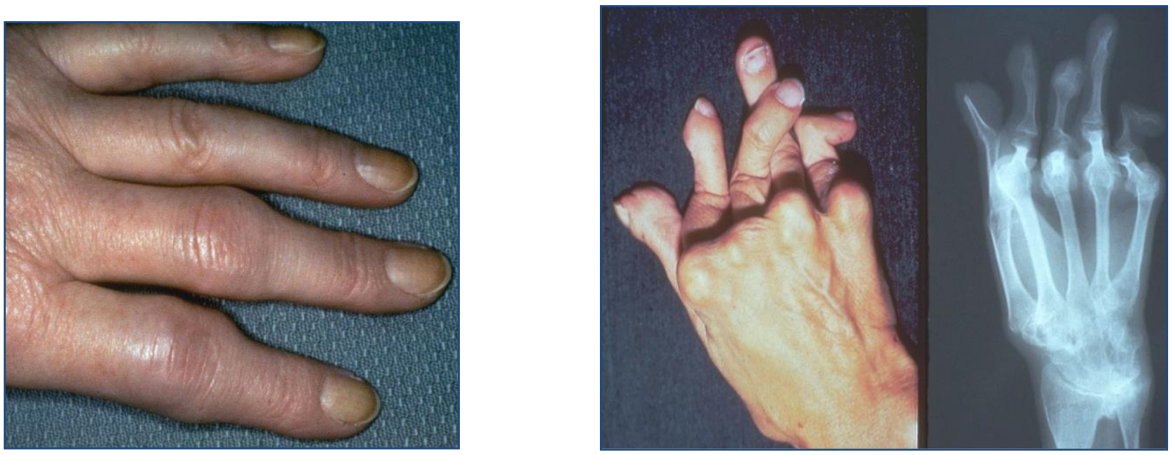

Figure 2 The hand joints in early and longstanding rheumatoid arthritis, including radiographic findings.

The clinical presentation of RA does not show similar patterns in affected patients. Most common finding is long-lasting symmetric arthritis accompanied by tenderness, pain and swelling of the hand and feet joints, wrists and ankles, sometimes also knees, elbows and shoulder joints. RA is often associated with systematic features such as fatigue, fever, and in severe cases also anorexia and weight loss. The onset of the disease is variable, ranging from one single affected joint (monoarthritis) to progression to severely polyarticular involvement. Subcutaneous granulomatous lesions, called rheumatoid nodules, are late symptoms presented in few patients. Parenchymal lung disease, ocular involvement, including secondary Sjögren's syndrome, cutaneous vasculitis and pericartidis represent further extra-articular manifestations of RA (STANICH et al. 2009, MCINNES et al. 2011).The main characteristics of the disease are given in table 1 (ASSIL et al. 2008).

Characteristic of rheumatoid arthritis	
Clinical	Female more than male (2 to 3 times more), morning stiffness (more than 1hour), fatigue, fever.
Laboratory	Rheumatoid factor (RF), anti citrullinated peptide antibodies (ACPAs), elevated inflammatory markers including erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP).
Radiographic	Erosions, periarticular osteopenia, joint space loss.
Musculoskeletal	Symmetric, polyarticular involvement, most commonly affects metacarpophalangeal, proximal interphalangeal joints, wrists, metatarsophalangeal joints. Sometimes cervical spine, spares thoracolumbar spine.
Extra-articular	Cardiovascular disease (atherosclerosis), rheumatoid nodules, ocular involvement (episcleritis), pulmonary fibrosis and nodules, renal manifestations (AA amyloidiosis), sicca symptoms (dry eyes and mouth), hematologic (anaemia of chronic diseases and Felty's syndrome), osteoporosis.

Table 1 Characteristic of rheumatoid arthritis (ASSIL 2008)

Although conventional radiographic examination is still considered as a gold standard, it is very often normal within the early phases of the disease. In some cases, when detection of arthritis is difficult to interpret, ultrasonography of the joints may be helpful to identify synovial inflammation. Magnetic resonance imaging (MRI) may be an important tool for early diagnosis of RA, since bone erosions are observed with MRI earlier than with conventional radiography. Despite being somewhat restricted in a routine clinical practice,

MRI is mostly used in research as an experimental tool in clinical trials evaluating efficacy of novel drugs.

Doctor-based examination of joint inflammation, but also patient reported outcomes, extra-articular manifestations, laboratory measures, mostly acute phase reactants and autoantibodies, represent the most common assessments in RA (Table 2).

Panel: Assessments in rheumatoid arthritis

Disease activity

Core assessments

- Joint counts (tender and swollen joint counts)
- Global assessment (doctor and patient) and pain score
- Laboratory (erythrocyte sedimentation rate and C-reactive protein)
- Disability (eg, health assessment questionnaire)

Additional assessment

- Fatigue
- Radiological damage

Combined status indices

- Disease activity score
- Simple disease activity score
- Clinical disease activity score

Change in status (trials only)

- ACR20, ACR50, and ACR70 responders

Extra-articular disease

- Nodules
- Pulmonary
  - Pulmonary nodules
  - Pleural effusion
  - Fibrosing alveolitis
- Ocular
  - Keratoconjunctivitis sicca
  - Episcleritis
  - Scleritis
- Vasculitis
  - Nail fold
  - Systemic
- Cardiac
  - Pericarditis
  - Pericardial effusion
  - Valvular heart disease
  - Conduction defects
- Neurological
  - Nerve entrapment
  - Cervical myelopathy
  - Peripheral neuropathy
  - Mononeuritis multiplex
- Cutaneous
  - Palmar erythema
  - Pyoderma gangrenosum
  - Vasculitic rashes
  - Leg ulceration
- Amyloidosis

Comorbidities*

Cardiovascular

- Myocardial infarction
- Heart failure
- Stroke
- Peripheral vascular disease
- Hypertension

Cancer

- Lymphoma and lymphoproliferative diseases
- Lung cancer
- Skin cancer

Infection

- General
- Bacterial

Other

- Depression
- Gastrointestinal disease
- Osteoporosis
- Psoriasis
- Renal disease

ACR20, ACR50, and ACR70=20%, 50%, and 70% improvements in five of the seven measures of American College of Rheumatology criteria. *Some comorbidities are mainly associated with rheumatoid arthritis (eg, cardiovascular), some with treatment (eg, gastrointestinal disease), and some with both disease and treatment (eg, infection).

Table 2 Clinical assessments in rheumatoid arthritis (Scott L. D. et al.2010)

3.1.2 Epidemiology

According to population-based studies, about 0.5-1% of the European and North American adult populations suffer from RA. The disease is 2 to 3 times more frequent in females compared to males. Incidence of RA ranges from 5 to 50 per 100 000 adults in developed countries and increases with age. The most often onset of RA is in the age ranging between 40 and 70 years, but it can develop either earlier or later.

3.1.3 Etiology and pathogenesis

The initial recognition that mechanisms of autoimmunity underlie RA pathogenesis came from the discovery of autoantibodies targeting the Fc fragment of human IgG (so-called "Rheumatoid Factors") that are elevated in the blood of affected patients. RFs have, however, lower specificity and can be found also during infectious diseases, in some tumours and also in up to 15-20% of elderly (KVIEN et al. 2009).

The exact cause of RA is poorly known; however, detail understanding the pathogenesis of the disease is increasing within the last years (MCINNES et al. 2011). The clinical process of arthritis starts as the inflammation within synovial membrane at the joints, however, the initial process of RA probably starts with a breach of tolerance to self-protein(s) occurring in secondary lymph nodes or bone marrow. In healthy joints, the thin synovial membrane is composed of two to three layers of cells mostly composed from fibroblast-like synoviocytes and macrophage-like synoviocytes. Altered apoptosis and increased cell proliferation in this layer results in synovial hyperplasia (over 10 cell lines within the lining layer) that along with leukocytes infiltration contribute to characteristic feature of RA (STANDICH et al. 2009). These processes finally contribute to the activation of resident joint tissue cells (fibroblasts

and chondrocytes) that increasingly produce pro-inflammatory mediators and matrix degrading enzymes resulting into the cartilage and bone damage.

3.1.4 Individual risk factors of RA development

Persistent synovial inflammation associated with joint damage in RA represents inflammatory pathway that is based on interaction among several genetics, environmental and immunological factors (MCINNES et al 2011). Most data have studied association of ACPA positive RA phenotype with environmental risk factors. The gene-environment interaction is of special interest and point to the shared epitope of HLA gene and also some non-genetic factors (LIAO et al. 2009).

Genetic factors

The comparison of monozygotic and dizygotic twins was the basis of evaluation of the familial risk for RA which is influenced by inheritable genetic and environmental factors. Estimated risk for the development of RA among monozygotic twins is about 15-30% and among dizygotic twins about 5%. Moreover, there is evidence that about 50% of risk of RA development is related to genetic factors. The most important genetic factor in RA is the human leucocyte antigen (HLA) class II with a common motive of amino acid sequences within the position 70-74 of the DRβ1 chain that is known as shared epitope. Shared epitope is thought to allow presentation of modified (e.g. citrullinated) antigens and further loss of tolerance and antibody formation against these antigens. Presence of HLA-DRB1 alleles significantly correlate with RA susceptibility and also with severity of the disease.

Whole genome-wide association studies identified several risk loci that are linked to RA development, e.g. C1854T single nucleotide polymorphism (SNP) in the PTPN22 gene and

A49G SNP in the CLTA4 gene. Products of these genes regulate T-cell activation (BALDING et al. 2006, CHRISTENSEN et al. 2007, BIJLSMA et al. 2009). Many other genes have been studied, including PADI4, SLC22A4 and FCRL3 and have been shown to have different potential in disease outbreak. Based on diverse genetic background, genetic research supports the idea that RA is a group of overlapping syndromes with similar phenotype.

In the recent years, other important factors such as epigenetic changes (e.g. DNA methylation, histone acetylation, sumoylation or altered microRNA expression) were identified to be associated with an intrinsic activation and aggressive cell behaviour in RA (KLEIN et al 2012).

Environmental factors

The main and well-investigated environmental risk factor for the development of RA is cigarette smoking (Figure 3). There is a positive association between cigarette smoking and the RF and ACPA positive RA (KLARESKOG et al 2006). Moreover, the doses of cigarettes per day and the years of smoking have been found to have the greatest impact with the fact that smoking and HLA-DRB1 alleles synergistically increase the risk of having ACPA. Furthermore, not only active, but also previous cigarette smoking represents an independent risk factor for the development of RA and its severity (BAKA et al 2009). A mechanistic link has been proposed for carrying specific HLA-DRB1 alleles encoding the shared epitope and smoking, which results into ACPA positive RA (KLARESKOG et al 2006). An increasing rate of lung cancer observed in RA patients may thus be explained by the association between RA and smoking (MCINNES 2011).

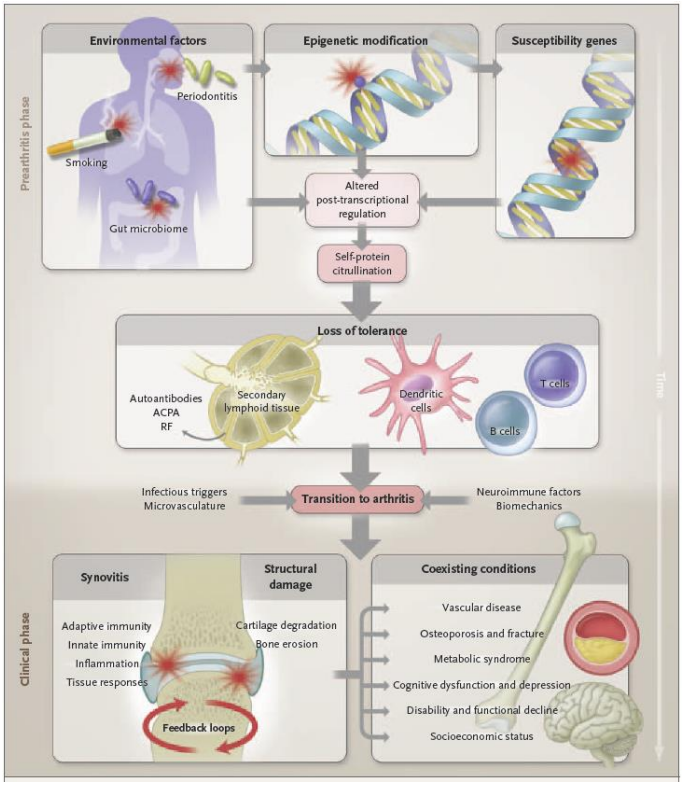

Figure 3 Environmental-gene interactions that are suggested to play a significant role in rheumatoid arthritis development (McInnes 2011).

The association between RA risk and other factors such as diet, alcohol and caffeine intake and body mass index are weak or not proven. According to a Danish study comparing RA patients who drink alcohol and those who do not drink revealed that alcohol may decrease the risk of RA, especially in ACPA positive subgroup (LIAO et al. 2009). This was supported by meta-analysis of prospective studies demonstrating that low to moderate alcohol consumption inversely associates with the development of RA (JIN et al. 2013).

Vitamin D, an essential hormone for bone and mineral homeostasis, has been hypothesized to have a role in RA development. It is involved in both innate and adaptive immune systems via vitamin D receptor (VDR) as a suppressor in pro-inflammatory reactions. There is some evidence that vitamin D deficiency play a role in the development of some autoimmune diseases, including RA (SONG et al 2012).

Although no direct evidence has been found to show the role of infectious agents, there are some data showing that RA is associated with periodontal disease (Porphyromonas gingivalis) or presence of some gastrointestinal microbes. Due to greater risk of RA in women, sexual hormones or other reproductive factors can play some role in the development of RA.

3.1.5 Classification and diagnosis of rheumatoid arthritis

The diagnosis of RA is based on the novel American college of Rheumatology (ACR) and European league against rheumatism (EULAR) classification criteria enabling early diagnosis and institution of therapy to struggle against progression to devastating disease (ALETAHA et al. 2010). The criteria can be applied for patient with at least one joint with definite clinical synovitis (joint swelling) and consist of four main domains (Table 3): 1) joint involvement, 2) serology (RF and ACPA), 3) symptom duration and 4) acute phase reactants (CRP and ESR). Achievement of a total score of 6 or greater enables classifying as having RA.

JOINT DISTRIBUTION (0-5)	
1 large joint	0
2-10 large joints	1
1-3 small joints (large joints not counted)	2
4-10 small joints (large joints not counted)	3
>10 joints (at least one small joint)	5
SEROLOGY (0-3)	
Negative RF AND negative ACPA	0
Low positive RF OR low positive ACPA	2
High positive RF OR high positive ACPA	3
SYMPTOM DURATION (0-1)	
<6 weeks	0
≥6 weeks	1
ACUTE PHASE REACTANTS (0-1)	
Normal CRP AND normal ESR	0
Abnormal CRP OR abnormal ESR	1

≥6 = definite RA

Table 3 American college of Rheumatology (ACR) and European league against rheumatism (EULAR) classification criteria for rheumatoid arthritis (Aletaha, et al. 2010)

3.2 Cytokines

Cytokines represent low molecular weight peptides that are produced by various cells connected to the immune system, including lymphocytes, macrophages, monocytes, dendritic cells, mast cells or blood vessel endothelial cells, but also by synovial fibroblasts and chondrocytes (Figure 4). Pleiotropic roles and redundancy define the multiple functions of each cytokine in the human body. Depending on the target cell (tissue); different cytokines can show similar biologic functions and vice versa (CARP et al. 2004).

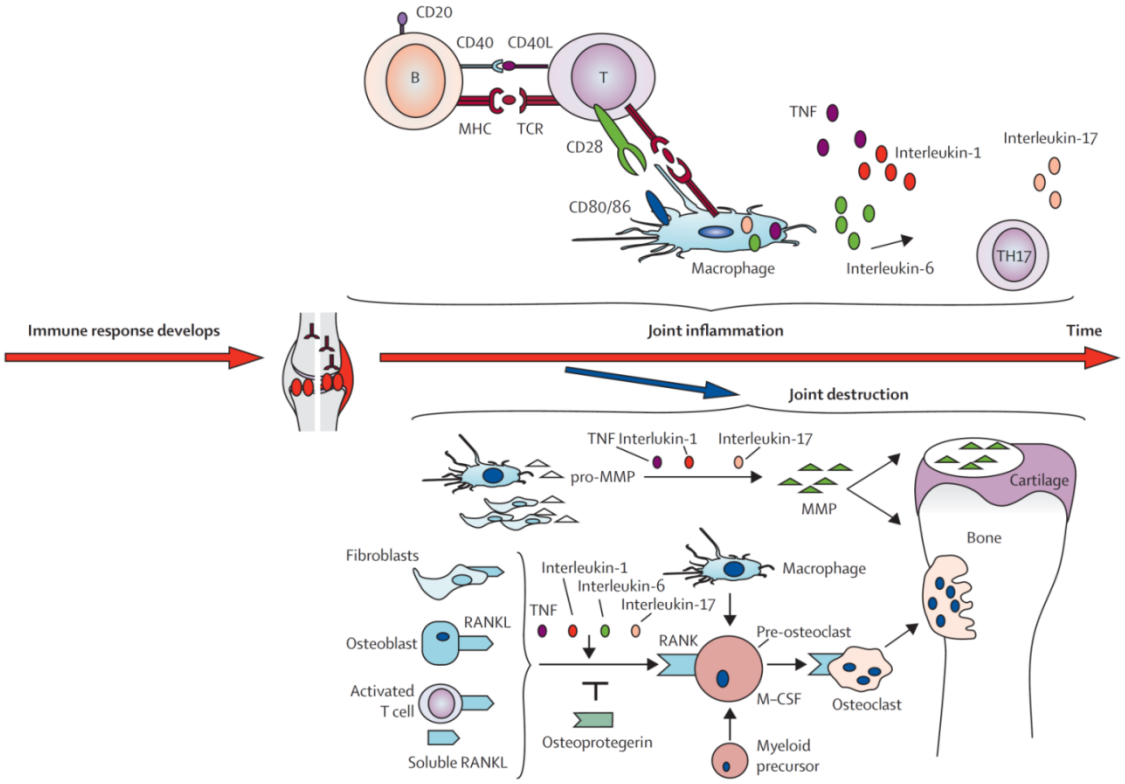

Figure 4 The interplay among immune cells and cytokines in rheumatoid arthritis (from Klareskog, et al. Lancet 2009).

Increased production of cytokines is the hallmark of RA (MCINNES 2011). Furthermore, there is a positive correlation between disease activity and the number of macrophages and lymphocytes along with elevated amount of IL-6 and TNF-α in the RA synovial tissue.

Cytokines as soluble mediators play a significant role in the process of inflammation, particularly by responding to tissue injury. Inflammatory cytokines can be divided into two groups according to the state of inflammation, i.e. acute and chronic (Figure 5), but several cytokines belongs to both groups (FEGHALI et al.1997). Inflammatory cytokines such as IL-1β or TNFα can significantly perpetuate systemic inflammation and can contribute to the damage of several tissues in patients with RA, mainly to joints, particularly by inducing the release of extracellular matrix degrading enzymes (KANEKO et al. 2001).

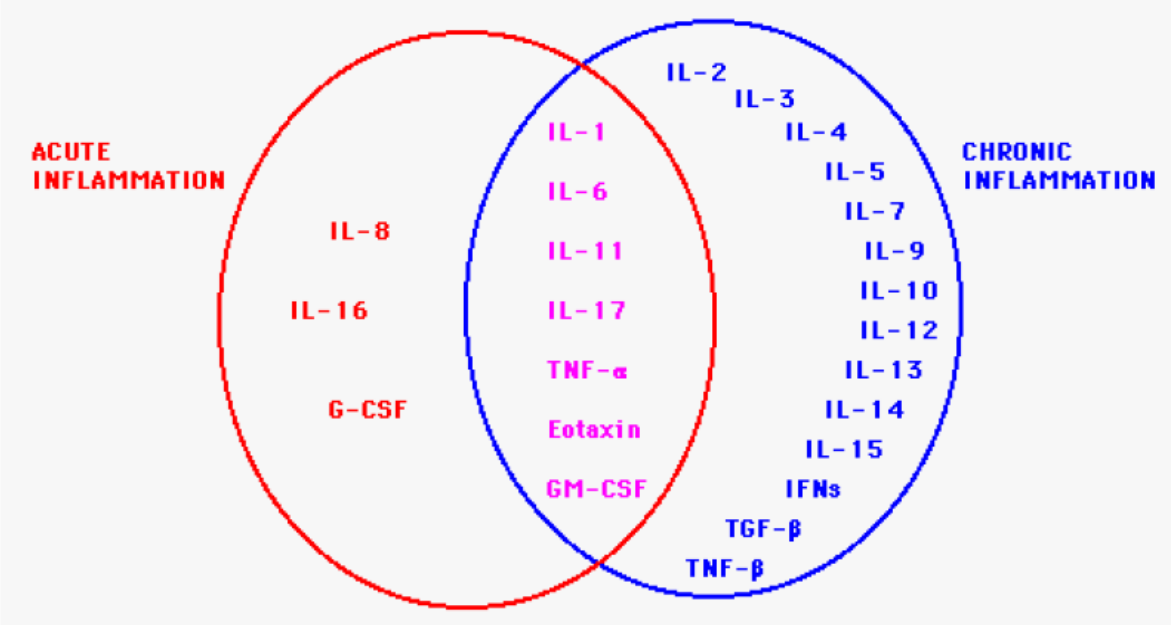

Figure 5 Cytokines involved in acute and chronic inflammation (from FEGHALI et al.1997)

Other important roles of cytokines are involved in differentiation, maturation and activation of the cells with important effects on immune-inflammatory responses (Figure 6). Several cytokines have been demonstrated to be involved in the pathogenesis of RA. Based on these data, better understanding to the pathogenesis of RA contributed significantly to the development of novel biological therapies for RA, mostly monoclonal antibodies targeting pro-inflammatory cytokines or their receptors (SENOLT et al. 2009). Using biological

therapies, final effects of particular cytokines such as tissue destruction can be abrogated and joint structures can be thus preserved (BRENNAN et al. 2008, SENOLT, et al. 2009).

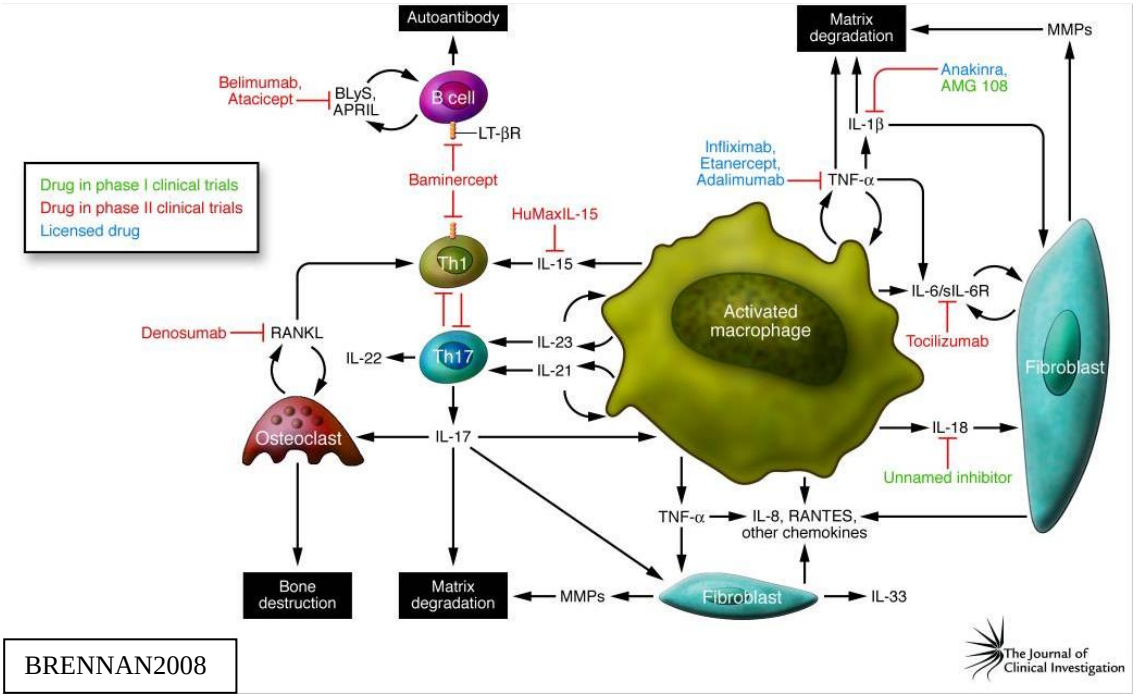

Figure 6 Cellular interactions and cytokines playing significant role in the pathogenesis of rheumatoid arthritis. Cytokines and some immune cells are depicted as potential targets for novel therapies of inflammatory and immune-mediated diseases.

Recent investigation and identification of novel cytokines and inflammatory molecules being involved in the pathogenesis of RA have been facilitated by new technologies. The novel potential targets are first explored in experimental studies (in vitro and ex vivo) in the diseased tissues from pathogenic sites such as synovial membrane, synovial fluid, tissue (cell) supernatants, but also from patients' blood circulation.

3.2.1 The most important cytokines in rheumatoid arthritis

Interleukin-1

IL-1 has been firstly discovered in 1977 as a protein that induces fever and was initially called human leukocytic pyrogen (DINARELLO et al. 1977). IL-1 causes fever by increasing the level of prostaglandin E_2 (PGE_2) (FEGHALI et al. 1997). The cytokine is made up of two major molecules IL-1α and IL-1β (with 20-25% of homology in amino acids), that are coded by two different genes on the human chromosome 2 with the size range of 22-31 kDa. It is mostly produced by mononuclear cells, fibroblasts, keratinocytes and the T and B lymphocytes. Inhibition of IL-1 was originally developed for the treatment of RA, but is has shown limited efficacy in this disease in humans.

Interleukin-6

IL-6 is a glycoprotein with 21 to 28 kDa size, and its gene is on the chromosome 7. It is a multifunctional, pleiotropic cytokine that is involved in regulation of immune responses, acute-phase responses and inflammation. This cytokine acts also as a growth factor contributing to the maturation of B cells and is involved in T cell differentiation and activation. IL-6 is produced by monocytes and macrophages, dendritic cells, endothelial cells and fibroblasts in response to different stimuli during systemic inflammation (NEURATH et al. 2011). IL-6 is up-regulated in different autoimmune and chronic inflammatory diseases. The treatment targeting IL-6 signalling represents promising novel approach for therapy of these diseases and has been already shown to be extremely effective in patients with RA.

Tumour necrosis factor

TNF-α was first described in 1975 as a factor inducing necrosis of tumours, however further studies demonstrated it can also induce signs and symptoms of shock and multiorgan damage

via pro-inflammatory effects on vascular endothelium. There are two cytokines - TNF-α and TNF-β with 17 and 25 kDa sizes that are found on MHC region of chromosome 6. TNF is produced by macrophages and monocytes, some T cells, mast cells, NK cells and fibroblasts. TNF-α play a key role in RA and represent one of the major cytokines from the complex cytokine network in RA. Inhibition of TNF-α represents very effective strategy in the treatment of patients with RA and with insufficient response to traditional DMARDs.

3.2.2 Novel cytokine in rheumatoid arthritis – interleukin 20

IL-20 belongs to IL-10 family and represents newly described cytokine known from 2001 as an important factor promoting hyper-proliferation of keratinocytes (RICH et al. 2001). IL-20 is located on chromosome 1q32 locus and has been demonstrated to regulate angiogenesis, to promote chemotaxis, proliferation of keratinocytes and inflammation (WEGENKA et al 2010). *In vitro*, IL-20 stimulates production of IL-6, IL-8, monocyte chemoattractant protein (MCP-1), and receptor activator of NFκB ligand (RANKL) in synovial fibroblasts (HSU et al. 2006). Based on these preclinical data IL-20 was characterized as a pro-inflammatory cytokine, which may play a significant role in inflammatory and autoimmune diseases, including RA (Figure 7). IL-20 and its receptors are up-regulated in synovial membranes of patients with RA in contrast to little or no expression in patients with osteoarthritis or healthy individuals. The main sources of IL-20 are activated monocytes, keratinocytes and dendritic cells.

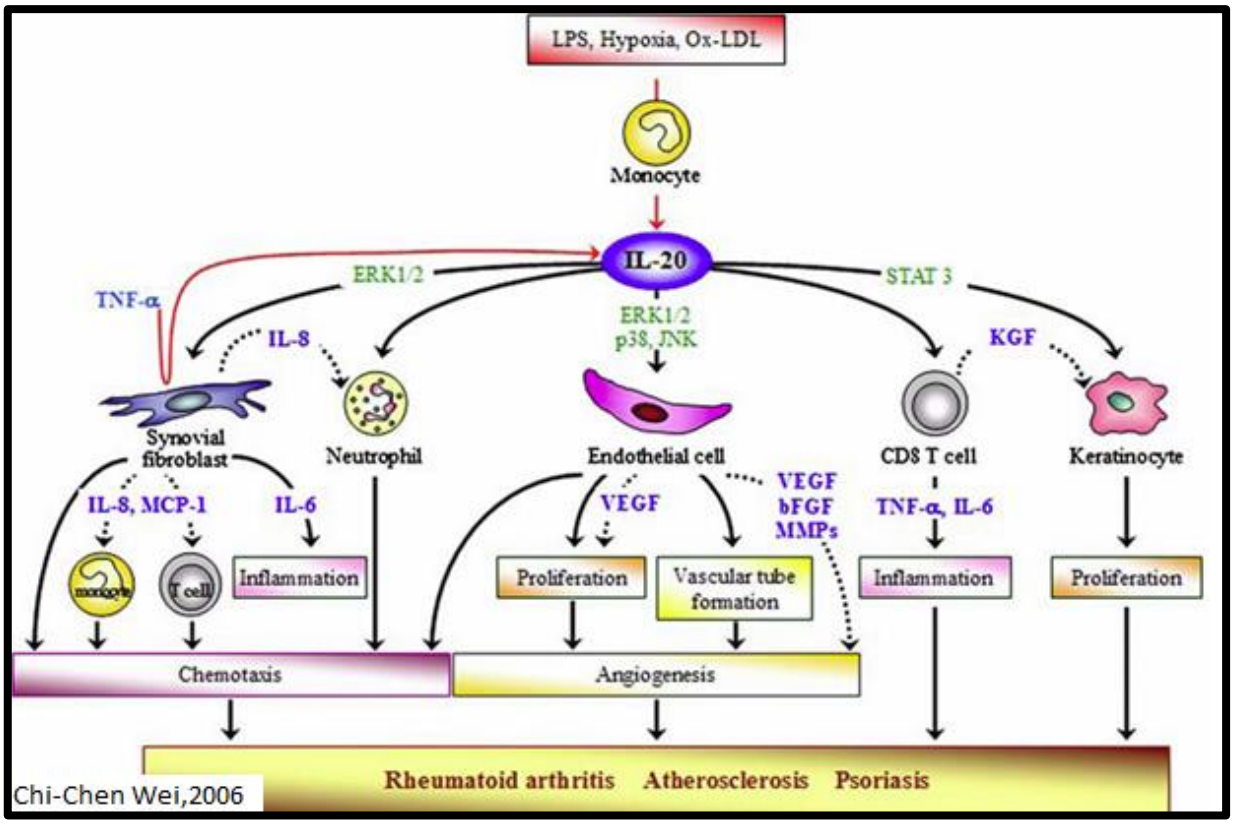

Figure 7 The role of interleukin-20 in activation of immune response and inflammation (from Chi-Chen Wei. 2006).

IL-20 exerts its effects through either of two heterodimeric receptors, IL-20R1/IL-20R2 (type I), and IL22R1/IL-20R2 (type 2) which have been demonstrated to be expressed on monocytes, granulocytes, endothelial cells and keratinocytes (FICKENSCHER et al. 2002), and both lining layer macrophage-like and fibroblast-like synoviocytes (HSU et al. 2006).

Moreover, novel recombinant human monoclonal antibody, which targets and neutralizes IL-20 was safe, well tolerated and effective in reducing disease activity of patients with active RA (ŠENOLT et al. 2012).

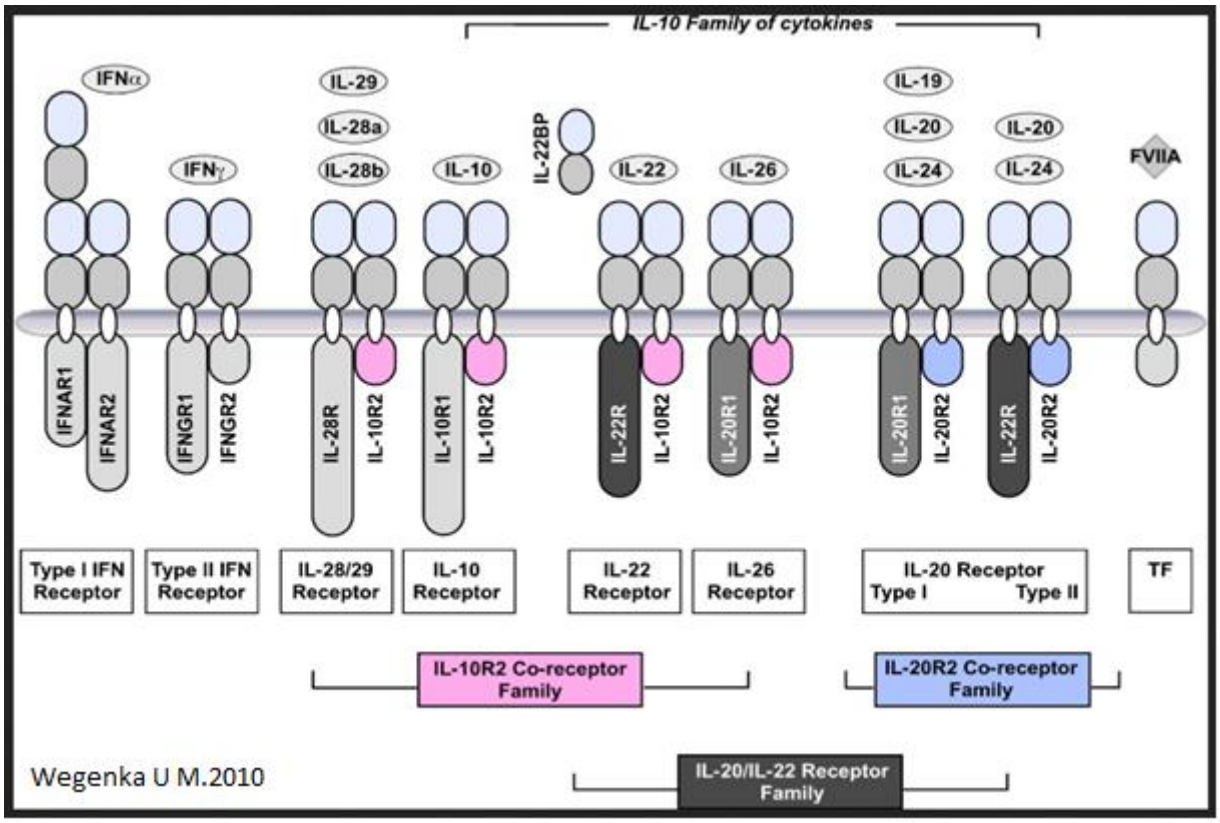

Figure 8 The receptor complexes of interleukin-20 which are common between different members of IL-10 family.

3.3 Cell cultures

3.3.1 Basic guidelines for working with cell and tissue cultures

The most important point during the work in a laboratory of cell and tissue cultures is the hygienic instructions to protect all the laboratory facilities and materials from potential contamination.

3.3.2 Work environment

It should be very calm, clean and with low personnel traffic. The laboratory should be used only for work in this field; it means culturing and its related affairs. The facilities and

materials have to be placed correctly to give suitable accessibility to everything that is necessary during the work with cell and tissue samples.

Personal hygiene

Every person who is working in the laboratory has to put on clean working clothes, wash their hands before and after work and use hygienic gloves. Individuals suffering from certain infectious diseases are not allowed to work in the laboratory until recovery.

Materials and equipment

Every laboratory is equipped with the necessary instruments and materials that are used daily and should be kept clean and disinfected. Routinely, it is necessary to disinfect the places including chairs, tables and instruments such as refrigerators, freezers, incubators, centrifuges and so on. The incubators have to disinfect before and after usage.

Work technique

There are disposable materials including pipettes, which should be used according to the instructions. The chemical and biological materials have to be labelled in detail so that all personnel are able to find and use them easily. The date of first use is written on the label. If anything is added to the stock material or if it is diluted, it should be reported. Every person has his or her own materials in the cases to decrease the risk of contamination.

Work in laminar box

Work with the laminar box has its own principles. The UV light should be switched on before and after every time using the box for 20 minutes. It is necessary to turn the ventilation system on for a minimum of 15 minutes before starting work in the box. In addition, suitable disinfectant (70% alcohol, 70% benzinalcohol, incidur) should be used to disinfect all the empty spaces in the box and everything used in the box. Once work is completed the laminar box should be cleaned again and everything is returned to its rightful place.

3.3.3 Contamination

Contamination can happen at any time with only a small mistake. All of these: bacteria, moulds, yeasts or mycoplasma can cause microbial contamination in the laboratory. Routine aseptic instructions should be kept to prevent any contamination, and if it happens, it has to be removed as soon as possible. Contaminated cultures are discarded.

3.3.4 Preparing water, solutions and media for tissue cultures

Water

The used water in preparing media or dilution of biochemical materials should be selected and provided as appropriate. Water without organic and an-organic substances is suitable but should be autoclaved for 30 minutes in 120°C.

Preparing solutions

There are few solutions that are used in the cell and tissue cultures continuously.

Phosphate buffered saline (PBS)

PBS solution is used in different steps of cell and tissue cultures. Before using trypsin, PBS is added to the cell culture to rinse grown cells. When preparing the PBS, one must consider the pH value (between 7.3 to 7.5), and the final solution needs to be autoclaved for 30 minutes in 120°C and 1 atm.

Trypsin

One important solution in cell passage is Trypsin, which is applied to separate the cell masses from each other and also from the solid substrate. The employee can order and use sterile one with or without EDTA (EthylenDiaminTetraAcid) in the appropriate concentration (Trypsin-EDTA, Gibco, distributor KRD, Prague). The solutions are prepared in lower amounts, 10ml to 15 ml, and stored in – 20°C.

Media

Different media are applied for culturing the cells and tissues from storing ones in the freezer.

Culturing media

In cell and tissue cultures, DHLBECCO'S MEM NUT MIX F-12(Gibco, distributor KRD, Prague) medium is used in the laboratory. Preparing the medium depends on the amount of fetal bovine serum (FCB and BOFES, which must be inactivated in 56°C for 30 minutes and shaken every 10 minutes) according to the cell growth conditions. For example, the medium used for Fibroblasts have 10% FCS, HeLa cells (transformed cancer cells) and only needs 5% FCS. Antibiotics are also added to the medium (to keep the cell lines out of any bacterial contamination), L-glutimin (essential aminoacid), HEPES (buffer in stabilizing pH in the solution), and fungicide (to protect the cells against moulds).

Freezing media

The suitable media used for storing the cell lines is a mix of FCS and DMSO.

3.3.5 Culture of the adhering cells

Some cells do not grow if there is not a solid substrate to adhere. The substrate can be glass or plastic material. The cells grow in a thermostat incubator at 37°C and in 5% CO2. As they start to grow, they will cover all the surface of the substrate (90-100% confluence), and the grown cells must then be changed and divided in different substrates (passage). Depending on the cell types, this is done 1 to 2 times per week. The passage process is done under the laminar box in aseptic conditions. Some necessary solutions need to have the 37°C temperature before adding to medium.

Passage process starts with the aspiration of the old medium to waste container, and then one must rinse with PBS and apply Trypsin. The Flask should be kept in incubator for 10 minutes.

In these 10 minutes, the cells will disengage, as observable under a microscope. By adding the medium, the effect of Trypsin is blocked. The cells are divided into the new flasks according to necessity. The specification of the cells, the date and passage number are written on every flask and they are then returned to the incubator.

3.3.6 Long-time cells storing, freezing

The cell lines are stored in liquid Nitrogen. The cells which are in logarithmic or top phase are suitable for storing. The medium changes a day before freezing. The cells should be checked to ensure that they are vital and not contaminated.

The cells are aspirated to falcon tubes and rotated in centrifuge 10 000 rpm for 15 minutes. The supernatant is poured and quickly added to the related medium for freezing. The cells are chilled in cryo-tubes (Nunc. Schöeller, Prague) and must be labelled in detail including the kind, number, and the passage number of cell and the date of storage. Every tube can be used for 1 to 1.5ml media. At first the tubes are put in Cryo 1°C freezing container (NALGENE®), then in – 20°C for 20 minutes. The ideal condition for freezing is when the temperature comes down slowly. Finally the cell tubes are transferred to -80°C. If the cells are to be stored for a long time, the tubes must be moved often.

3.3.7 Defrosting the cells

A lukewarm bath is first prepared with 20-30°C water, and then the chilled tubes are transferred quickly into it. After melting, the cells are dispersed in the separate flasks, and the medium is added so as to be ten times more than the volume of cell solution. The medium is changed the next day.

4 MATERIALS AND METHODS

4.1.1 Serum and synovial fluid

Blood contains the fluid and cells which are circulated in the vessels. The blood elements are suspended in a liquid part named plasma. Serum and plasma are individually derived from whole blood. Serum, in contrast to plasma, is lacking fibrinogen and other clotting factors (JUNQUEIRA et al. 1998). Synovial fluid represents an ultrafiltration of serum, and under the normal conditions, it is viscose, colourless and transparent. Hyaluronic acid and other proteins acting as lubricants in the synovial fluid are produced by synovial lining layer. Synovial fluid also transfers nutrients to the joint cartilage surfaces.Fasting blood samples were collected from the participants when they underwent therapeutic knee arthrocentesis or not more than 5 days post-arthrocentesis, or during regular visits at the Institute of Rheumatology in Prague. Disease activity of RA was based on the Disease Activity Score of 28 joints (DAS28) using the number of the tender and swollen joints. Paired samples were then centrifuged, and both the serum and synovial fluid were stored at -80°C. Before analysis, the synovial fluids were incubated with Hylase Dessau for 30 min at 37°C.

4.1.2 Synovial fibroblasts and tissue cultures

Synovial tissue samples were obtained at the time of arthroscopy or open joint surgery (1st Orthopaedic Clinic, 1st Faculty of Medicine, Prague, Czech Republic) from patients with RA and OA. The samples were cut to small pieces to be cultured in the flasks with suitable medium. The particular medium was chosen according to the recommendation of provided companies included in the protocol. Synovial fibroblasts were grown in culture flasks in DMEM F-12 medium (Lonza, Verviers, Belgium) supplemented with 10% FBS and 1% Pen-Strep (Lonza, Verviers, Belgium) at 37°C with 5% CO2. Cells were harvested after four to six

passages. Before stimulatory experiments, fibroblasts were washed by PBS, trypsinized in 0,5% Trypsin (Lonza, Verviers, Belgium) and seeded at 1 x 10^5 cells per 1ml DMEM cultivation medium in the 35mm diameter culture plates.

4.1.3 Peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMCs) have a round nuclei and represent immune cells derived from blood such as monocytes, macrophages and lymphocytes. Human PBMCs were isolated from peripheral blood of patients with RA and OA by Ficoll-Pague density gradient centrifugation (Figure 9). PBMCs were quantified by CASY® Model DT cell counter (Roche Innovatis, Basel, Switzerland) and seeded at 1 x 10^6 cells per 1ml of RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% FBS (Lonza, Verviers, Belgium) and 1% Pen-Strep (Lonza, Verviers, Belgium) in 24mm diameter culture plates.

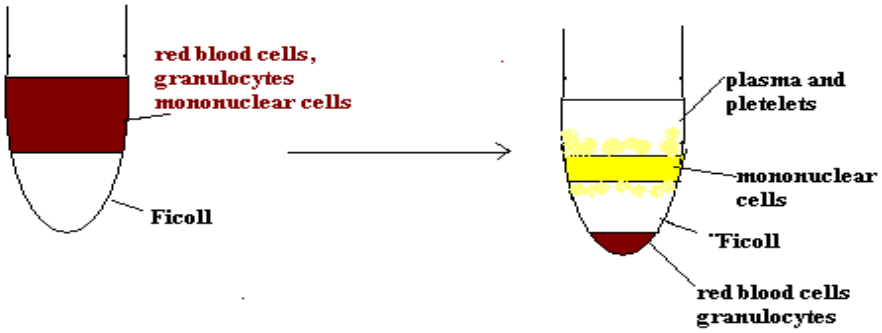

Figure 9 Peripheral blood mononuclear cells isolation from whole blood by Ficoll- Paque.

4.1.4 Stimulatory experiments

Synovial fibroblasts and PBMCs isolated from RA patients' synovial tissue and whole blood, respectively, were calculated to be sufficient number for the experiments (PBMC: 10^6

cells/well and fibroblast: 10^5 cells/well). The cells were treated with IL-20 (20 and 200 ng/ml), LPS (100 ng/ml), IL-1β (1 ng/ml), TNFα (10 ng/ml) and Poly I:C (20μg/ml) for 6 and 24 hours at 37°C with 5% CO_2. After 6 hours, harvested cells were centrifuged and pellets were lysed by RLT Buffer (Qiagen, Hilden, Germany) and stored at -80°C until RNA isolation. After 24 hours of stimulation, the cells were also centrifuged and supernatants were stored at -80°C until protein analysis. All stimulatory experiments were done in duplicates. Starving medium (DMEM F-12 medium, Lonza, Verviers, Belgium) was used while stimulating adhered fibroblasts of floating PBMCs.

4.1.5 Demographic data of patients

The synovial fluid and the serum samples were collected from all the patients and stored at -80°C until analysis. Disease activity was based on the Disease Activity Score of 28 joints (DAS28) using the number of the tender joints. The body mass index (BMI) was calculated as weight (kg) / height2 (m2). Table 1 lists the patient details and their disease characteristics. The patients with RA had higher CRP levels compared to the patients with OA, while the patients with OA had higher BMI. There is a table including a summary of demographic data of the patients those were used in this experiment. These data are indicated in the appendix 9.1.

4.1.6 Interleukin 20 (IL-20)

The human recombinant IL-20 (R&D systems, E. coli-derived, Leu25-Glu176, with an N-terminal Met, Cat.No.1102-IL-025) was provided commercially and concentrations used in our experiments were used based on previous data.

4.1.7 RNA isolation

Total RNA from the cells treated with IL-20 or other stimulatory molecules was extracted using the MagNA Pure Compact RNA Isolation kit for MagNA Pure Compact Instrument (Roche Diagnostics GmbH, Germany). Exactly after RNA isolation (Figure 10), the RNA concentrations were measured by biophotometer (Eppendorf) according to the protocol :

1-Add 5 µl of isolated RNA ti 65µl of distilled water (dH2O).

2- Measure the blank at first step.

3- Measure of all samples one by one (The suitable concentration of RNA is 10µg/ml, and a purity between 1.7-2.1 is acceptable).

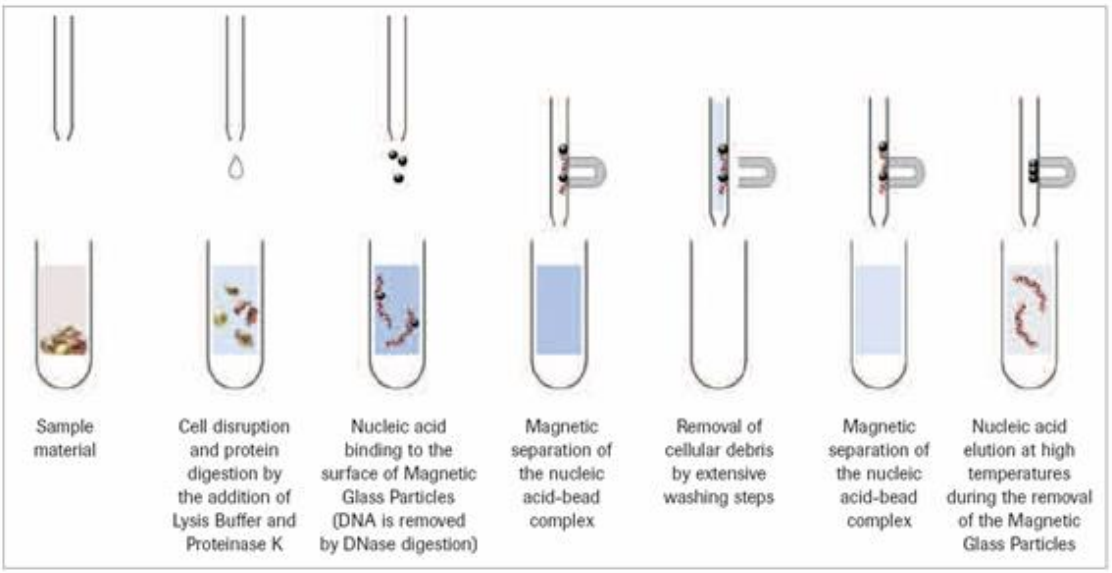

Figure 10 The process of RNA isolation by MagNA Pure Compact Instrument.

4.1.8 Reverse transcription and quantitative Real Time PCR

Complementary DNA was obtained by reverse transcription using High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Forster City, CA). PCR was performed using TaqMan® Universal PCR Master Mix (Applied Biosystem, Foster City, CA) according to a standard protocol. Pre-developed primers (Applied Biosystem, Foster City, CA) were used to

detect different chemokines, cytokines and matrix degrading enzymes. Real-time PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystem, Foster City, CA). 18S rRNA was used as an internal control gene. Data were analysed using comparative threshold cycles (Ct) method for relative quantification (also referred to as the $2^{-\Delta\Delta CT}$ method). All these steps were done in a place that was RNase free and on the ice bath.

The work protocol for reverse transcription:

1- Write on the microtubes and leave them on ice in the rack.
2- Transcriptase is the inhibitor brings out of freezer before adding to the tubes.
3- 1µl of primers do pipetteto every microtubes.
4- Add11µl of homogenized RNA to related named microtubes.
5- Centrifuge at 3000 rounds per minute, then vortex and again centrifuge at the same speed for a few seconds.
6- At the same time prepare the master mix and include4µl of buffer 5x conc.,1µl inhibitor and 2µl dNTPs.
7- Add 7µl of master mix to every sample tube.
8- Activate RNase during the 5 minutes incubation of the samples in 37°C.
9- Add 1µl of reverse transcriptase to the samples.
10- Centrifugeat 3000 rounds per minute , vortexand again centrifuge.
11- Transfer the microtubes, including samples, to thermocycler.
12- Check the saved related program and start the process.
13- Store the samples in -80°C.

Work protocol for RT-PCR:

1- Prepare the probes which have to be traced, premix and cDNA samples.

2- Add 80µl of dH2O (DNase and RNase free, Water PCR-Grade, Bioline) to every 20µl samples of cDNA
3- Prepare the premix including the Master mix, dH2O and probes.
4- Total volume reaction is 25µl that contains 3 µl of cDNA sample and 23 µl of premix (12.5µl Master mix, TaqMan Universal PCR Master Mix (Roche), 1.25µl of probes and finally 8.25µl of dH2O)
5- Prepare the plate; be careful not to touch the plate.
6- Pipette 22µl of premix to every well of plate and add 3µl of the sample to relate well.
7- Wrap by adhesive covers.
8- Shake the plate and centrifuge for a few seconds.
9- Put the plate on the RT-PCR machine and start the related saved program.

4.1.9 Immunohistochemistry

Indirect method of immunohistochemical staining uses primary and secondary antibodies. The fact that several secondary antibodies can react with the same primary antibody makes this method more sensitive (KARLSSON 2009). The synovial tissue samples for immunohistochemistry were obtained from wrist and elbow joints of RA (n=5) patients and from hip and knee joint of OA patients (n=7).

Five micrometre (5µm) thick formaldehyde fixed paraffin sections were deparaffinised and rehydrated using Xylen (PETNA, Chrudim, Czech Rep.). Endogenous peroxidase activity was inhibited by the addition of 3% hydrogen peroxide in methanol for 30 minutes followed by 15 minutes of rinsing in tap water. Non-specific binding activity was avoided by pre-treatment of the sections with 1% normal bovine serum for 2 hours. Immunohistochemical labelling was

performed after antigen retrieval in 0.2 mol/l citrate buffer (pH 6.0) for 40 minutes at 91^{o} C. Sections were incubated in the TBS (Tris Buffered Saline) buffer and incubated with antibodies in different dilutions for 1 hour and rinsed again in TBS buffer. Antigen-antibody complexes were visualized by Histofine detection system (Nichirei Biosciences Inc.), by using 3, 3´-diaminobensidine as chromogene. Sections were slightly counterstained with Harris´ haematoxylin.

Work protocol:

1- Fix the tissue sample and cover with paraffin.

2- Cut the samples into very thin slices.

3- Remove the paraffin by putting the slices in a hot water bath.

4- Capture 3 slices (one of them is control sample) on a clean slide.

5- Deparaffinise slides:

A. Add Xylol I 10 min.

B. Add Xylol II 10 min.

C. Add ethanol absolute I 3-5 min.

D. Add ethanol absolute II 3-5 min.

E. Add ethanol 96% 3-5 min.

F. Add ethanol 80% 3-5 min.

G. Add distilled water 5 min.

6- Pre-treat with Trypsin for 30 minutes.

7- Wash the well in ddH2O for 5 minutes.

8- Wash 2X in buffer1/Tween (PBS) for 5 minutes.

9- Block unspecific binding with 2-5% serum (horse, or goat) in buffer 1 30-40min.

10- Title the slides (do not wash).

11- Incubate primary AB and negative control diluted in buffer 1 at RT for 1 hour (or at 4°C over night).

12- Wash 2X in buffer1/Tween for 5 minutes.

13- Incubate secondary biotinylated AB diluted in buffer 1for 30 minutes.

14- Prepare ABC reagent for alkaline Phosphate in buffer 1

15- Wash in buffer1/ Tween.

16- Incubate with ABC reagent for AP.

17- Wash with TBS.

18- Wash with buffer2.

19- Develop Fast Red, Fast Blue Substrate or any other substrate for alkaline phosphate.

20- Stop colour reaction with ddH2O and rinse well.

21- Cover slides with aqueous mounting medium.

The expression of IL-20 (biosystems, Catalog #1102-IL-025), IL-R20Rα (Human IL-20 R alpha MAb (Clone 173707), Mouse IgG1, applied biosystems, Catalog #MAB11761) and IL-20Rβ (Human IL-20 R beta Affinity Purified Polyclonal Ab, applied biosystems, Catalog #AF1788) were analysed in RA and in OA synovial tissue samples. The slides were incubated with primary monoclonal anti-human IL-20 (50 µg/ml and 25µg/ml), IL-20Rα (25µg/ml) and with human IL20-Rβ Antibody (20µg/ml) (R&D systems, Minneapolis, MN) diluted in REALTM Antibody diluent (DAKO, Glostrup, Denmark) for 1 hour at room temperature in Magnetic Immuno Staining Tray (CellPath, Newtown, UK). Isotype IgG (Dako, Cytomation) diluted 1:200 was used as a negative control. The EnVision Dual Link System-HRP (DAKO,

Glostrup, Denmark) was used to visualize sections incubated with the primary antibody; in addition Liguid DAB Sustrate (DAKO, Glostrup, Denmark) was used as a chromogen. The slides were counterstained with Mayer's Hematoxylin Histological Staining Reagent (DAKO, Glostrup, Denmark) for 1 minute, dehydrate and fixed in resin solution Entellan (MERCK, Darmstadt, Germany). All the sections were analysed semi-quantitatively using a Nikon Eclipse E600 microscope. The intensity of IL-20 expression was scored on a four-point scale: 0 represented negative staining intensity, and scores of 1–3 represented weak, moderate and strong staining intensity, respectively.

4.1.10 Enzyme-linked Immunosorbent Assay (ELISA)

Enzymed- Linked Immunosorbent Assay (ELISA) methods are immunoassay techniques used for detection and quantification of a substance based on immunological reaction. An antibody can be bound to an antigen in physiological conditions including salt concentration, pH and temperature and by a covalent bond. ELISA is a qualified method in detecting a specific protein in a complex mixture. Like other Bioassay categories, it is done on a 96-well microplate. There are different types of ELISA, but the basic elements are the same in all of them and are included in capturing antigens in the sample by its fixation in a microplate directly or indirectly, thus blocking unnecessary molecules in the sample by covering all the free sites on the wells. The incubation of the antibody-antigen complex is a part of probing and detection, and finally detection of the signal and measuring it (SUTLA et al. 1986, KEMENY 1991, FOSSCECO et al.1999).

The levels of IL-20 were measured in the cell culture supernatants and in synovial fluid (n=20 OA, and n=17 RA) and serum (n=20 OA and n=17 RA) using an ELISA kit according to the

manufacturer's protocol (PeproTech, NJ). The analysis was performed using a Sunrise ELISA reader (Tecan, Salzburg, Austria) at wavelength of 405 nm. The sensitivity of IL-20 was 16 pg/ml. The IL-20 ELISA Kit was used for measuring the quantity of IL-20 in the serum and synovial fluids of patients with RA and OA. The results were analysed by Kim32 program software.

5 RESULTS

5.1 Increased expression of IL-20 in RA synovial tissue

The staining intensity of IL-20 was significantly enhanced in RA compared with OA synovial tissue (Figure 11). Furthermore, the expression of IL-20 was significantly greater in the lining as well as in the inflammatory infiltrates of the affected sublining layer and vessels of RA compared with OA synovial tissues (Table 4). RA synovial membranes derived from the knee or hip joints of patients with RA and OA synovial membranes revealed little or no expression of the cytokine in contrast to RA synovial membrane from affected wrist or elbow joints.

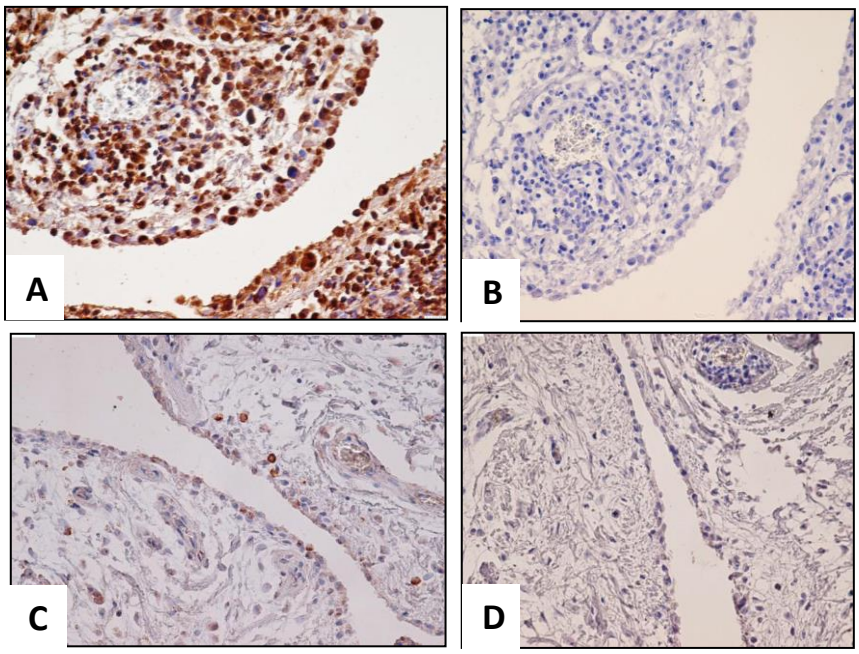

Figure 11 Expression of IL-20 in rheumatoid arthritis (A) and osteoarthritis (C) synovial tissue. Strong staining for IL-20 was observed in synovialocytes of the lining layer, mononuclear cells within the inflammatory infiltrates as well as in vessels and capillaries of the RA synovial tissue. Mouse IgG was used as an isotype control (B, D). The original magnification is 40 x.

.

OA samples	Lining layer	Sublining layer	Vessels
OA279	1	0-1	0
OA293	1	0	1
OA316	1	1	0
OA320	1	0	0
OA326	0-1	0-1	0
OA330	1	0	0
OA336	1	0-1	0
Mean (SD)	0.9 (0.2)	0.4 (0.4)	0.1 (0.4)
RA samples			
RA337	1-2	2	0
RA363	3	2-3	2
RA368	2-3	1-2	1-2
RA395	3	3	2
RA410	3	2	1-2
Mean (SD)	2.4 (0.8)	2.2 (0.5)	1.4 (0.8)
p	0.0004	< 0.0001	0.002

Table 4 Expression of IL-20 in rheumatoid arthritis (RA) and osteoarthritis (OA) patient synovial tissue samples scored using a semi-quantitative four-point scale analysis: 0 represented negative staining intensity, and scores of 1–3 represented weak, moderate and strong staining intensity, respectively.

5.2 The levels of IL-20 in serum and synovial fluid are elevated and correlate with disease activity in patients with RA

The synovial fluid levels (38,87 vs. 17,0; p=0,0051) were significantly higher in the RA than in the OA patients. In patients with RA, the median levels of IL20 were significantly higher in the synovial fluid compared with serum counterparts (38.87 (6,2 - 207,2) vs. 0.38 (0,1 - 195) pg/ml; p=0.005). There were no significant differences between serum and synovial fluid IL-20 levels in OA patients and in IL-20 serum levels between RA and OA patients (Figure 12).

Importantly, the IL-20 serum levels significantly correlated with clinical and laboratory markers of RA, such as DAS28 ($r=0.7102$, $p<0.001$) and CRP levels ($r=0.449$, $p<0.01$).

These data were comparable when synovial fluid IL-20 levels were calculated. The detailed table has been shown in appendix 9.2.

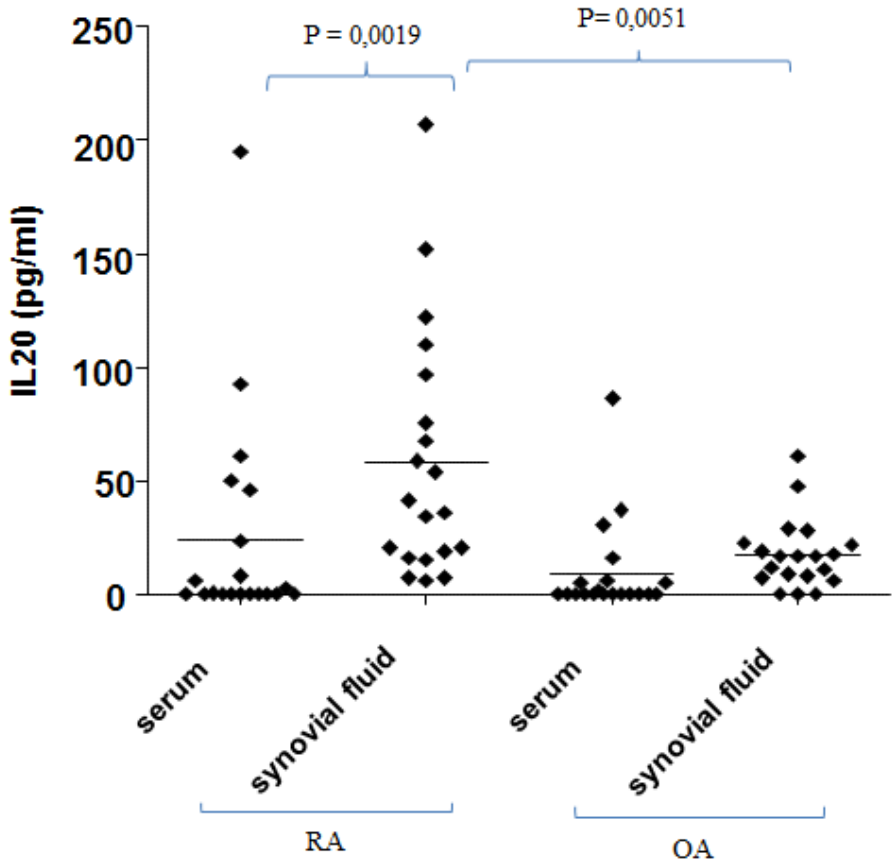

Figure 12 The median levels of IL-20 in the serum and synovial fluid of RA and OA patients

5.3 IL-20 is up-regulated upon pro-inflammatory stimuli in mononuclear cells and synovial fibroblasts

To evaluate whether pro-inflammatory stimuli can increase the production of IL-20, we measured IL-20 in the cell culture supernatants after 24 hours of stimulation with different molecules using ELISA (n=2 experiments of ten blood samples of RA Patients). As

demonstrated in figure 13, secretion of IL-20 from PMBCs was induced particularly by Poly I:C (TLR-3 ligand) ($p<0.0001$), and in a lesser extend also by LPS (TLR-4 ligand), IL-1 and TNFα compared with un-stimulated cells.

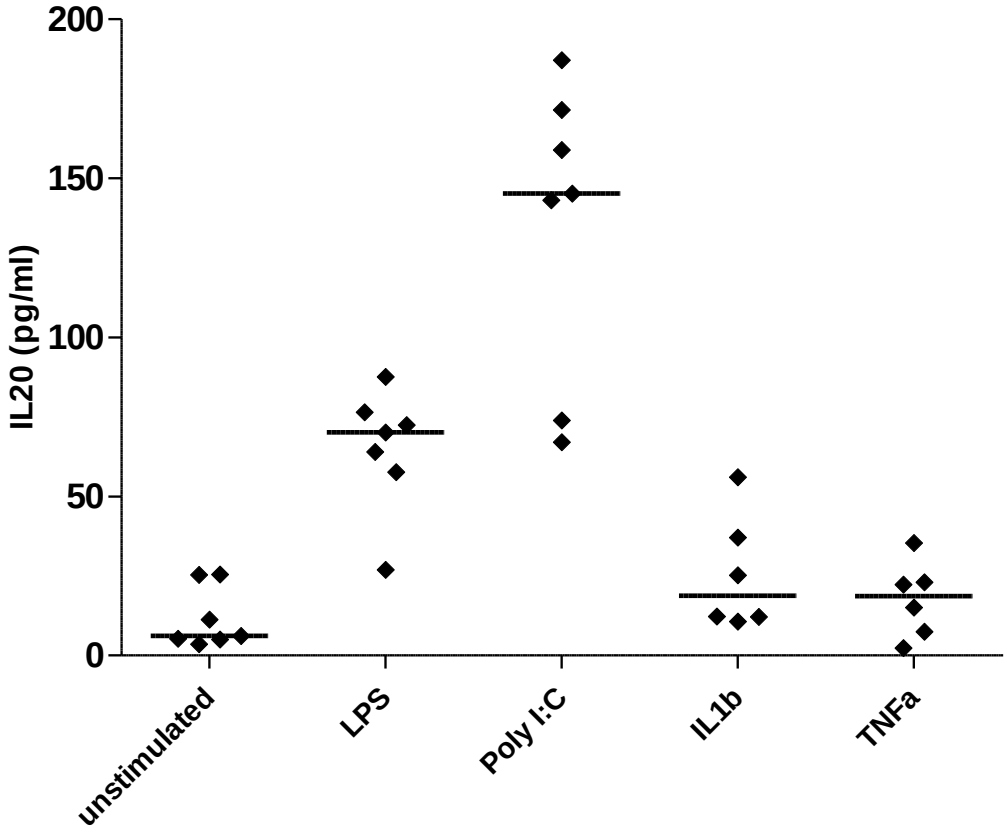

Figure 13 The median levels of IL-20 secreted from RA PMBCs incubated for 24 hours with different pro-inflammatory ligands including Poly(I:C), LPS, IL1β and TNFα.

In contrast to circulating PMBC, synovial fibroblasts stimulated with abovementioned pro-inflammatory mediators did not show such an effect upon IL-20 secretion from these cells (Figure 14).

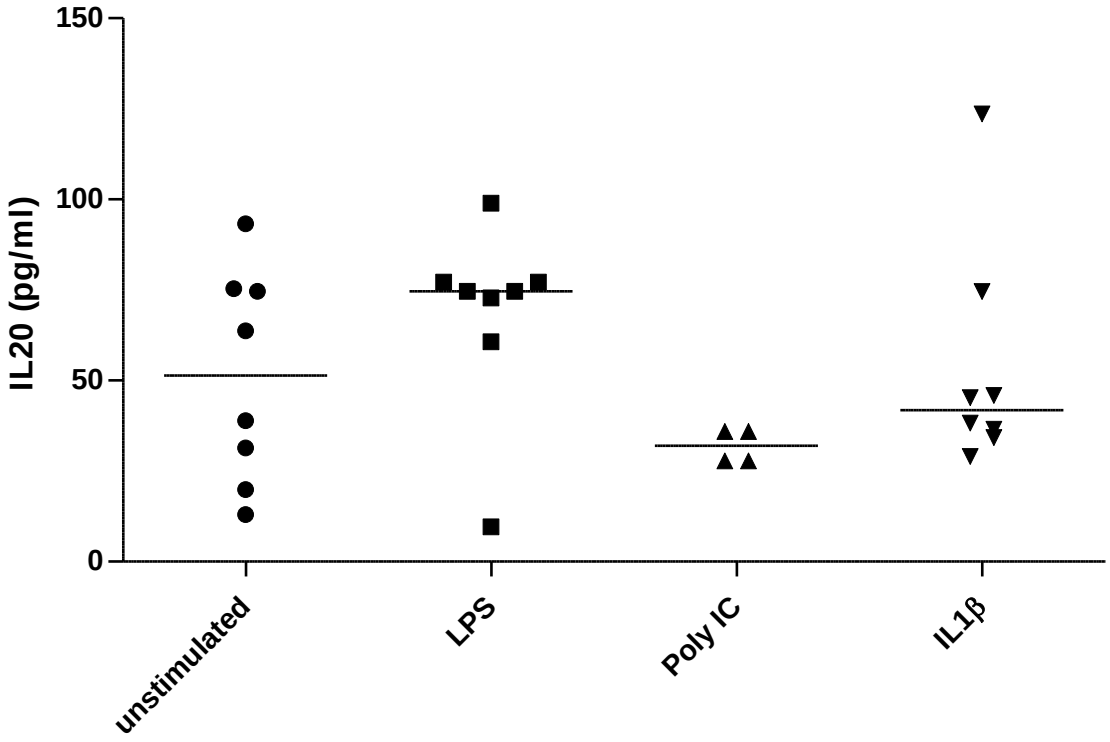

Figure 14 The median levels of IL-20 secreted from RA synovial fibroblasts incubated for 24 hours with different pro-inflammatory ligands including Poly(I:C), LPS, IL1β and TNFα.

5.4 Stimulation of synovial fibroblast with recombinant IL-20

To evaluate the pro-inflammatory effects of IL-20, we stimulated RA synovial fibroblasts (n=8 experiments) and PBMCs (n=2 experiments) with the IL-20 for 6 hours and analysed the expression of selected pro-inflammatory genes. Although, PBMCs treated with IL-20 did not regulate expression of any of the selected cytokine or chemokine, RA (but not OA) synovial fibroblasts significantly up-regulated the expression of chemokine IL-8 (Figure 15). In addition, IL-20 exerted stronger effect on RA than OA synovial fibroblasts. A detailed results of RT-PCR is shown in appendix 9.3.

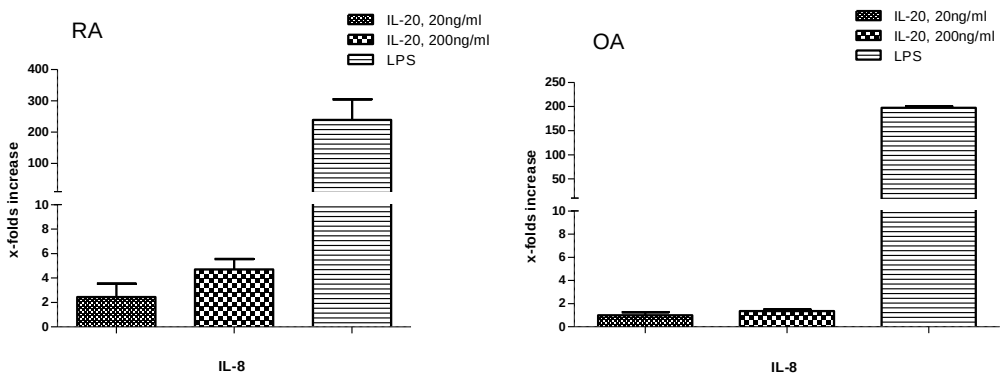

Figure 15 The expression of IL-8 mRNA in RA (A) and OA(B) synovial Fibroblast upon treatment with IL-20 for 6 hours.

6 DISCUSSION

In this study we demonstrated elevated amount of IL-20 at sites of local inflammation in RA and association between IL-20 levels and RA disease activity. Here we show increased expression of IL-20 in RA compared with OA synovial tissue and elevated concentrations of IL-20 in RA compared with OA synovial fluid. Based on our results, we suggest that several pro-inflammatory stimuli, e.g. TLR ligands and cytokines, can induce secretion of IL-20 out of some immune as well as resident tissue cells. However, pro-inflammatory effects of IL-20 were barely confirmed.

In agreement with previous report (HSU et al. 2006) we confirmed that IL-20 expression is enhanced in synovial tissue obtained from RA patients. Furthermore, we showed that the cytokine was localized in both lining as well as sublining layers, and in a lesser extend also around vessels. There was negligible expression of IL-20 in control OA synovial membrane. Since RA is a highly inflammatory disease, we speculated that the cytokine may be induced by some pro-inflammatory ligands, and found that both cell types - synovial fibroblasts and mononuclear cells produce IL-20. In addition, mostly TLR-3 ligand (Poly I:C), but to a lesser extend also other inflammatory molecules (LPS, TNFα, IL-1), are responsible for increased production of IL-20 from the immune cells. Our results thus support the fact that IL-20 up-regulation is induced by inflammatory milieu of the disease.

We have demonstrated that IL-20 is significantly elevated in RA synovial fluid, but not in the circulation in contrast to OA counterparts. These data also confirm previous results of Hsu et al. In addition to that, we found significant association of IL-20 levels and RA disease activity, which has not been reported so far. Therefore, we speculate that the increased

expression of IL-20 at sites of inflammation is responsible for greater secretion of the cytokine to the synovial fluid, which can thereafter reflect severity of the disease. Furthermore, some reports demonstrated that IL-20 induces osteoclast activity via the RANKL/RANK pathway in mice (HSU et al. 2011) and thus, it can be speculated that IL-20 may contribute to more erosive form of the disease in humans.

IL-20 is thought to play a role as a pro-inflammatory cytokine in the pathology of RA. It has been shown that IL-20 regulates chemokine expression in synovial tissue, promotes neutrophil chemotaxis, RA synovial fibroblasts migration, and endothelial cell proliferation (HSU et al. 2006). In contrast to these results, we were unable to show such strong pro-inflammatory effects of IL-20, at least on human cells in vitro. We confirmed only up-regulation of IL-8, but not other cytokines, chemokines or matrix degrading enzymes in synovial fibroblasts stimulated with IL-20. While there are reports supporting IL-20 as a novel target for treating some inflammatory diseases and cancer (HSU, et al. 2012), further studies are necessary to elucidate the biological role and potential link among IL-20, inflammatory and immune mediated diseases such as RA.

7 CONCLUSION

In summary, our results confirm significant increase of IL-20 at the sites of local inflammation in RA. Since the involvement of IL-20 in RA is not yet clearly elucidated, it can be speculated that pro-inflammatory milieu of the disease is responsible for the higher amount of the cytokine in RA, which can in turn regulate expression of some chemokines in synovial tissue. Furthermore, based on our results, IL-20 can be suggested as a potential biomarker of RA severity. Taken together, IL-20 is an important cytokine involved in the pathophysiology of RA, may reflect disease activity and potentially might be a good therapeutic target for RA and other immune mediated diseases.

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9 APPENDIX

9.1 A summary of demographic data

Studied samples	RA (n=20)		OA (n=20)	
Gender (F/M)	15/5(75 to 25%)		11/9(55 to 45%)	
	Mean SD	**Median**	**Mean SD**	**Median**
Age (years)	61,8 ± 15,17	63	62,65 ± 9,896	59,5
BMI (kg/m^2)	26,59 ± 4,371	26,3	28,69 ± 0,7889	28,61
CRP (mg/l)	17,38 ± 15,99	14,59	1,876 ± 1,101	1,870
FW	25,46 ± 20,20	16	9,611 ± 5,992	8,50
TC (mmol/l)	5,477 ± 1,034	5,270	5,763 ± 1,267	5,690
HDL (mmol/l)	1,531± 0,3549	1,545	1,609 ± 0,3012	1,550
LDL (mmol/l)	3,273 ± 0,9887	3,295	3,417 ± 1,265	3,280
TG (mmol/l)	1,305 ± 0,6351	1,27	1,872 ± 0,8772	1,440
Drugs (DMARDs/GC)	15/10		0/2	
Biological therapy	4		0	
DAS28	4,239 ± 1,080	3,950	-	
anti CCP positivity, n (%)	10 (50%)		0	
RF IgG positivity, n (%)	5 (25%)		1 (5%)	

9.2 Correlation with the disease activity and levels of IL20 in OA and RA sera and synovial fluids.

Levels of IL20	Correlation with
RA serum	DAS28; P 0,0096; r=0,7102 CRP; P 0,0017; r=0,4491
RA synovial fluid	DAS28; P 0,0221; r=0,6499 CRP; P 0,0007; r=0,7058 anti CCP; P 0,0034; r=0,6227 IgM; P 0,02; r=0,5154
OA serum	TC; P 0,0347; r=0,4865
OA synovial fluid	-

9.3 Detailed table of RT-PCR results

RA Fibroblast stimulation		**18s**	**IL-8**	**dCt**	**ddCt**	**X-Fold**
1st experiment	Control	14,17846	33,18035	19		
	trail IL20 - 20 ng/ml	14,16216	31,34488	17,18	1,82	3,53
2nd experiment	Control	14,15092	31,97898	17,83		
	trail IL20 - 20 ng/ml	14,21509	31,60691	17,39	0,44	1,35
1st experiment	Control	14,17846	33,18035	19		
	trail IL20 - 200 ng/ml	14,16001	30,68698	16,53	2,47	5,56
2nd experiment	Control	14,15092	31,97898	17,83		
	trail IL20 - 200 ng/ml	14,19291	30,07743	15,88	1,94	3,85
1st experiment	Control	14,17846	33,18035	19		
	trail LPS - 100ng/ml	14,2712	25,0209	10,75	8,25	304,9
2nd experiment	Control	14,15092	31,97898	17,83		
	trail LPS - 100ng/ml	14,21047	24,60613	10,4	7,43	172,73

OA Fibroblast stimulation		18s	IL-8	dCt	ddCt	X-Fold
1st experiment	Control	14,33333	31,12321	16,79		
	trail IL20 - 20 ng/ml	14,49509	31,72285	17,23	-0,44	0,74
2nd experiment	Control	14,29309	31,38844	17,1		
	trail IL20 - 20 ng/ml	14,7828	31,55711	16,77	0,32	1,25
1st experiment	Control	14,33333	31,12321	16,79		
	trail IL20 - 200 ng/ml	14,71849	31,24908	16,53	0,26	1,2
2nd experiment	Control	14,29309	31,38844	17,1		
	trail IL20 - 200 ng/ml	14,82008	31,3182	16,5	0,6	1,51
1st experiment	Control	14,33333	31,12321	16,79		
	trail LPS - 100ng/ml	14,41647	23,60291	9,19	7,6	194,47
2nd experiment	Control	14,29309	31,38844	17,1		
	trail LPS - 100ng/ml	14,31524	23,76451	9,45	7,65	200,31