The Role of Mycoplasma Infection, IL-1 Polymorphism, and $CD4^+CD25^+$ Regulatory T-Lymphocytes in Rheumatoid Arthritis

THESIS

Submitted in the Partial Fulfillment of the
MD Degree in Clinical and Chemical Pathology

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2012

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LIST OF ABBREVIATIONS

AcP	Accessory protein
ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
anti-CCP	Anti-cyclic citrullinated protein antibodies
APC	Antigen-presenting cell
aTreg	Adaptive regulatory T
BiP	Immunoglobulin heavy-chain binding protein
cDNA	Complementary DNA
COX	Cyclooxygenase
CSF	Colony-stimulating factor
CTLA	Cytotoxic T lymphocyte-associated antigen
DAF	Decay activating factor
DAS	Disease activity score
DC	Dendritic cell
EAM	Extra-articular manifestations
EBV	Epstein-Barr virus
FGF	Fibroblast Growth Factor
FoxP3	Forkhead box protein 3
GATA	A family of transcription factors that bind to DNA
gc	g-Chain
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glucose-6-phosphate isomerase
HLA	Human leukocyte antigen
hnRNP-A2	Heterogeneous nuclear ribonucleoprotein-A2
HSP	Heat shock proteins
IBD	Inflammatory bowel disease
ICAM-1	Intercellular Adhesion Molecule 1
ICE	IL-1β-converting enzyme
IgG	Immunoglobulin G
IL-1	Interleukin-1
IL-1F	IL-1 family
IL-1R	IL-1 receptor
IL-1Ra	IL-1 receptor antagonist
IRAK	IL-1 receptor-associated kinase

LFA-1	Lymphocyte function-associated antigen 1
MCP-1	macrophage chemoattractant protein-1
MHC	Major histocompatibility complex
MM	Molecular mimicry
MMPs	Matrix metalloproteinases
MS	Multiple sclerosis
NK	Natural killer
NKT	Natural killer T
nTreg	Naturally occurring regulatory T
OA	Osteoarthritis
PADI4	Peptidyl arginine deiminase type IV
PBS	Phosphate buffered saline
PDGF	Platelet-Derived Growth Factor
PTPN22	Protein Tyrosine Phosphatase
QKRAA	Glutamine-leucine-arginine-alanine-alanine
RA	Rheumatoid arthritis
RAI	Ritchie articular index
RANTES	Regulated upon Activation Normal T cell Expressed, Secreted
RF	Rheumatoid factor
ROG	Repressor of GATA
RUNX1	Runt-related transcription factor 1
SA	Superantigens
SLC22A4	Solute carrier family 22 member 4
SNPs	Single nucleotide polymorphisms
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Teff	Effector T cell
TF	Transcription factor
TGF-β	Transforming Growth Factor-ß
T_H	Helper T cell
TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor-α
Tr1	Type 1 regulatory T cell
Treg	Regulatory T cell
Vaa	Variable adherence-associated
VCAM-1	Vascular cell adhesion molecule1

INTRODUCTION

Rheumatoid arthritis (RA) is a debilitating autoimmune disease characterized by systemic inflammation of the joints and the ensuing destruction of cartilage and bone leading to progressive joint damage and disability **(Choy & Panayi, 2001)**. Its prevalence is 0.5–1% in Caucasians, being somewhat lower but still important in developing countries **(Mody & Cardiel, 2008)**. Although the exact mechanisms that are responsible for the disease remain unclear, it is accepted that different factors contribute to the etiology and pathogenesis of RA. Among these factors pro-inflammatory cytokines, regulatory T cells and also infectious agents like bacteria and viruses **(Bystry *et al.*, 2001; Roelofs *et al.*, 2005; Goronzy & Weyand, 2005)**.

IL-1 has been implicated in RA and the ability of IL-1 to drive inflammation and joint erosion and to inhibit tissue repair processes has been clearly established in invitro systems and animal models **(Dayer, 2003; Goldring, 2003)**. IL-1Ra prevents the interaction between IL-1 and its cell-surface receptors, thus acting as a naturally occurring inhibitor **(Arend, 2002)**.

Associations with polymorphisms or microsatellites have been identified for several cytokines, inflammatory mediators, and chemokines, among which interleukin-1β (IL-1β) and interleukin-1 receptor antagonist (IL-1Ra) **(Lee *et al.*, 2004; 2009)**. The relative contribution of each is still poorly defined, and variations in technique, stage of disease, and patient populations result in some disagreement among various reports **(Firestein, 2005)**.

Many evidences indicate that $CD4^+CD25^+$ T-regs play a key role in the maintenance of self-tolerance **(Lahl *et al.*, 2007; Sakaguchi *et al.*, 2006)**. However, the number of T-regs as defined by the number of $CD4^+CD25^{+/high}$ or $FoxP3^+$ T cells in the peripheral blood of RA patients appears to be inconclusive and contradictory across different studies **(Chavele & Ehrenstein, 2011)**. Also, the observed increase in T-reg cell numbers in the synovial fluid of RA patients is contradictory to the assumption that lower numbers of T-regs would be present in RA patients **(Cao *et al.*, 2003)**. This finding leads to the possibility that T-reg cells in RA patients may be non-functional and this hypothesis currently appears correct, as T-reg cells isolated from the synovial fluid of RA patients appear to be functionally inactive in regards to their ability to suppress pro-inflammatory cytokine production **(Ehrenstein *et al.*, 2004)**.

Mycoplasmas are a major cause of acute and chronic arthritis in animals and can induce arthritis in animal experimental models **(Rivera *et al.*, 2002)**.

Using PCR techniques, the presence of mycoplasmas was investigated in synovial fluids of patients with RA and other chronic arthritis. Schaeverbeke *et al.* **(1996)** showed that *M. fermentans*, but not *M. penetrans*, was detectable in 20% of these patients and other types of arthritis of unknown causes, but not in patients with reactive, post-traumatic or chronic juvenile arthritis.

AIM OF THE WORK

This study is planned to:

(1) Determine the association of IL-1β and IL-1Ra genes polymorphism to RA susceptibility and severity.

(2) Assess the role of $CD4^{+}CD25^{+}$ Regulatory T cells counts in RA.

(3) Assess the role of *Mycoplasma fermentans* in RA.

Chapter 1

RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is the most common systemic, autoimmune rheumatological disorder, characterized by chronic inflammation of multiple joints, synovial cell proliferation and the accumulation of T lymphocytes in synovial tissues, leading to the destruction and disability of joints **(Sharma *et al.*, 2004)**. Although RA is properly considered a disease of the joints, it can cause a variety of extra-articular manifestations. These manifestations clearly show that RA has features of a systemic disease capable of involving a variety of major organ systems. In some cases, rheumatoid factor (RF) production with the formation of immune complexes that fix complement contribute to these extra-articular findings **(Firestein, 2005)**.

Despite intensive work, causes of RA are still not completely known. Clues have been provided by detailed studies of immunogenetics of the class II major histocompatibility complex (MHC) loci and the usage of specific RF genes, but still what causes the disease is not known **(Orozco *et al.*, 2006)**. It is in the area of pathogenesis that most progress has been made since the early 1990s. The role of small-molecule mediators of inflammation, cytokines, growth factors, chemokines, adhesion molecules, and matrix metalloproteinases (MMPs) has been carefully defined. New appreciation of the pathogenic mechanisms evoked by these products has increased awareness that irreversible loss of articular cartilage begins relatively early in the course of RA; therapies to

suppress the synovitis must be effective early if joint destruction is to be avoided **(Firestein, 2005)**.

1.1. Pathogenesis of Rheumatoid Arthritis:

Although the etiology of RA remains a mystery, a variety of studies suggest that a blend of environmental and genetic factors is responsible. A contribution of either one is necessary but not sufficient for full expression of the disease. One or multiple genetic factors probably predispose an individual to developing RA. However, attempts to identify specific infectious agents as the etiology have generally met with disappointment. A guess, based on available data, is that several environmental stimuli, possibly viruses or retroviruses, infect an individual with the appropriate genetic background, and through some mechanism, the inflammatory response is focused in joints. After gaining a grip there, the synovitis persists—even in the absence of the offending agent—because of local autoimmunity and other influences that enable the disease to become self-perpetuating. However, the role of infectious agents in the occurrence of the disease remains unclear. Incident cases of RA do not report a history of increased number of infections, and cases of RA do not present any time or space clustering **(Alamanos & Drosos, 2005)**.

1.1.1. Genetic factors

1.1.1.1. The role of HLA-DR:

The structure of class II surface molecules on antigen-presenting cells is of great importance in the susceptibility and severity of RA and accounts for about 40 percent of the genetic influence. Initiation of certain T cell immune responses is

dependent, in part, on the presence or absence of particular MHC (in this case DR) allelic products. The role of the MHC in shaping T cell receptor (TCR) gene usage has been confirmed by intense studies that characterized specific T cell repertoires in the joint or blood of RA patients, and hence a bias in the usage of TCR genes has been assumed to be an evidence of ongoing T cell activation **(Goldrath & Bevan, 1999)**.

The genetic link between HLA-DR and RA (described in the 1970s) revealed that HLA-DR4 occurred in 70 percent of RA patients, compared to about 30 percent of controls, giving a relative risk of having RA to those with HLA-DR4 of approximately 4 to 5. Careful study of the MHC using complementary DNA (cDNA) probes directed against specific α- and β-chains of the DR loci have revealed "susceptibility cassettes," or shared epitopes on the β-chains of DR that predispose to the development of RA. The susceptibility to RA is associated with the third hypervariable region of DRβ-chains, from amino acids 70 through 74 **(Nepom *et al.*, 1989 ; Orozco *et al.*, 2006)**. The susceptibility epitope is glutamine-leucine-arginine-alanine-alanine (QKRAA), a sequence found in DR4 and DR14 (in which RA is more prevalent), in addition to some DR1β-chains. Individuals with DRβ-chains exhibiting other substitutions in this region have no increased susceptibility to RA and up to 96 percent of patients with RA exhibit the appropriate HLA-DR locus, in some populations **(Weyand *et al.*, 1992)**.

The QKRAA epitope might also predict the severity of established RA **(Calin *et al.*, 1989)**. Data from one study suggest a

"dose response" effect of the HLA genes and imply that severity, rather than susceptibility, is the major contribution of HLA-DR to the disease. However, this notion is not universally true, and the interpretation depends greatly on the inclusion of patients with transient, self-limited arthritis and varies with race and ethnic background **(Thomson *et al.*, 1993)**.

However, the dose effect of the QKRAA epitope argues against a role for binding of a specific rheumatoid antigen, because DR surface density usually does not alter T cell responses. Also other genes must be involved, because many healthy individuals carry the QKRAA motif and do not develop RA; and the association between the shared epitope and RA might have little to do with antigen recognition and might function by shaping the T cell repertoire in the thymus. A reinterpretation of the shared-epitope hypothesis suggests that disease-associated alleles are not involved with presentation of organ-specific autoantigens but rather fail to provide protection against escape from tolerance **(Zanelli *et al.*, 2000)**.

1.1.1.2. HLA-DQ associations:

Additional associations with HLA molecules implicate the DQ locus as a key MHC protein that can display arthritogenic peptides, although certain HLA-DR genes are actually protective **(Zanelli *et al.*, 1995)**. Perhaps the DQ/DR haplotype is responsible for RA predisposition in humans and the polymorphism of the HLA-DR4 allele determines the degree of protection. This observation is supported by the fact that HLA-DQB1*0302 and HLA-DQB1*0301 are in linkage disequilibrium with most DR4 haplotypes, and the majority of HLA-DR4+ RA patients express one of these alleles

(Doherty *et al.*, 1992). However, a recent study failed to confirm a specific association between HLA-DQ and disease susceptibility in a cohort of Dutch patients **(de Vries *et al.*, 2000)**.

1.1.1.3. Role of other genes:

Although HLA-DRB1 is the main RA gene, it accounts for only part of the familial risk for RA. HLA-DRB1 alleles are neither necessary nor sufficient to cause the development of RA in a given individual. Several genome scans conducted in populations from France, Japan, North America and UK have confirmed the role of the HLA region and suggested several other susceptibility loci. Association studies support a role for several genes, including Tumor Necrosis Factor-α (TNFR2), peptidyl arginine deiminase type IV (PADI4), Solute carrier family 22 member 4 (SLC22A4), Runt-related transcription factor 1 (RUNX1), and Protein Tyrosine Phosphatase (PTPN22) **(Dieudé & Cornélis, 2005)**.

1.1.1.4. Immunoglobulins' genes:

A particular immunoglobulin kappa genotype appears to confer a risk of RA. Although it cannot be considered an immunogenetic determinant, deficient galactosylation of immunoglobulin might also be a risk factor for the development of autoimmune diseases, including RA. The immunoglobulin G (IgG) glycosylation defect is present before the onset of RA and is especially prominent on the IgG_1, IgG_2, and IgG_4 isotypes of RF **(Tsuchiya *et al.*, 1994; Axford, 1999)**. The cause might be reduced galactosyl transferase activity in RA B cells. Deficient galactosylation is also thought to be predictive, in some settings, for patients with early synovitis who

will progress ultimately to full-blown RA **(Rademacher *et al.*, 1994; Axford, 1999)**.

1.1.1.5. Cytokine polymorphisms:

Single nucleotide polymorphisms (SNPs) in promoter regions or coding regions have been extensively investigated in RA. SNPs in promoter regions could lead to altered gene regulation due to variable binding of transcription factors to promoters, whereas SNPs in coding regions directly changes the amino acid sequence of the encoded protein. A second method for assessing genetic associations involves the evaluation of microsatellite sequences near key genes that are implicated in the disease. Microsatellites are tandem repeated sequences in the DNA that are primarily (but not exclusively) located in noncoding regions. Considerable heterogeneity exists in the length of each microsatellite, which can indirectly alter gene expression or be in linkage disequilibrium with other undefined genetic polymorphisms. Many SNPs and microsatellites have been studied with RA. The relative contribution of each is still poorly defined, and variations in technique, stage of disease, and patient populations result in some disagreement among various reports **(Firestein, 2005)**.

Associations with polymorphisms or microsatellites have been identified for several cytokines, inflammatory mediators, and chemokines, including interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), CCR5, RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted) and CTLA4 (Cytotoxic T lymphocyte-associated antigen) **(Rodriguez *et al.*, 2002; Prahalad 2006; Yao *et al.*, 2009; Lee *et al.*, 2009)**. One SNP for the T cell

costimulatory molecule CTLA4 is also associated with susceptibility **(Rodriguez *et al.*, 2002)**. The contribution of each gene is relatively small compared to class II MHC, but combinations might provide an appropriate genetic background to influence the course of arthritis.

1.1.1.6. Gender:

RA is one of many chronic inflammatory diseases that predominates in women with a ratio of female to male patients 2:1 to 4:1. The basis of the gender differences is not known but presumably is related to effects of the hormonal milieu on immune function. Pregnancy is often associated with remission of the disease in the last trimester. More than three quarters of pregnant patients with RA improve, starting in the first or second trimester; but 90 percent of these experience a flare of disease associated with a rise in RF titers in the weeks or months after delivery. The mechanism of protection is not defined but might be due to the expression of suppressive cytokines such as IL-10 during pregnancy or alterations in cell-mediated immunity. A possible relationship between the alleviation of RA symptoms during the last trimester of pregnancy and immunogenetics may be supported by the observation that during pregnancy, alloantibodies in the maternal circulation develop against paternal HLA antigens **(Nelson *et al.*, 1993)**. Therefore, suppression of maternal immune responses to paternal HLA haplotypes might be protective. This question remains unsettled because a recent study failed to find a correlation between the HLA disparity and clinical improvement during pregnancy **(Brennan *et al.*, 2000)**.