Identification and studies of MHC class III genes in animal models of rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis is an autoimmune disorder in which our own immune system attacks our own body tissues by mistake. It is a chronic inflammatory disease which primarily affects the joints in the body. The damage is caused in the joints in the form of inflammations [1]. The main parts affected by Rheumatoid arthritis are the peripheral joints which include finger joints, wrists, toes and knees. Though this disease is prevalent for a long time the cause of the disease is not known. It is believed that the genes present in the Major Histocompatibility Complex region might contribute to the onset of rheumatoid arthritis in one way or another [2] [3] [4]. Various animal models are used to study the between rheumatoid arthritis and the genes in Major Histocompatibility Complex. DA strains of rat are the widely used animal models for rheumatology studies. In our laboratory it is observed that rats carrying MHC class III congenic fragment, DA.1HR56 develop less severe diseases in our animal models of rheumatoid arthritis. The animal models used in our lab are pristine-induced arthritis (PIA) and oil-induced arthritis (OIA), both of which are T-cell mediate disease models. Based on testing different subcongenic fragments, we conclude that this protective phenotypes comes from a 220-kb region in chromosome 20 starting from 3649561 to 3864755; comprising approximately more than 20 genes like TNF, Lta, Ltb, Aif1, Bat2 and Lst1, a lot of which are potential candidate for immune regulation. Some of these are much better investigated while some are not so well-known. Analyzing the 20 genes in the congenic region for polymorphism and its comparative gene expression studies between DA strain and DA.1HR56 will help us to corner out the gene or group of gene which leads to this protective phenotype. Based on these data, functional studies of selected genes will be undertaken.

Keywords: Rheumatoid Arthritis, Major Histocompatibility Complex, Pristine Induced Arthritis, Oil Induced Arthritis.
**Abbreviation**

<table>
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<th>Definition</th>
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<tr>
<td>ACPA</td>
<td>Anti-Citrullinated Protein Antibodies</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>CII</td>
<td>Type II collagen</td>
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<td>CCP</td>
<td>Cyclic citrullinated peptide</td>
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<td>CIA</td>
<td>Collagen-induced arthritis</td>
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<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>IFA</td>
<td>Incomplete Freund’s adjuvant</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>MHC</td>
<td>Major Histocompatibility complex</td>
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<td>OIA</td>
<td>Oil-induced arthritis</td>
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<td>PIA</td>
<td>Pristane-induced arthritis</td>
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<td>RA</td>
<td>Rheumatoid arthritis</td>
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<td>RI</td>
<td>Recombinant inbred</td>
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<td>RF</td>
<td>Rheumatoid factors</td>
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<td>SE</td>
<td>Shared epitope</td>
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<td>TCR</td>
<td>T cell receptor</td>
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<td>Th</td>
<td>T helper cell</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>Treg</td>
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1. BACKGROUND

1.1 Introduction to Immune System:

Humans are generally prone to various types of infections. The cause and origin of infections varies widely which might be air, water till microorganisms. It is more than worthy to say that immune system is the key aspect for our survival. The immune system is a biological system which possesses a simple but important function, to fight against the foreign materials which enter our body. For this function the immune system should be able to distinguish self materials and non-self materials. For this the immune system should be able to detect the pathogen, act against them, store the antigen pattern in their memory and act against them in further attacks [8].

The foreign molecule may be a wide variety of agents starting from virus, bacteria to parasitic worms. They are generally termed as pathogens. These pathogens if they enter our body cause some sort of disease. Two line of defense acts against these pathogens. The first line of defense tries to restrict these pathogens from entering our body. For example the oil on our skin is acidic and it becomes difficult for some bacteria to break them and thrive in such an environment. Chemicals present in the mucous membrane always prohibit the entry of defense. When this line of defense is compromised or overpowered the pathogens enter our body. At this point of time the second line of defense comes into play.

The immune response is of two types, Innate and adaptive immunity. Innate immunity is a naturally occurring immunity which is based on PAMPs (Pathogen Associated Molecular Pattern). Innate immune response can also be referred to as Non-Specific immune response. In the case of innate immunity our immune system acts against any pathogens which enter our body. The detection of PAMPs evokes immune response. This leads to the production of proinflammatory cytokines and small mediator’s. These act on the pathogens and finally the pathogens are engulfed by the Phagocytes. Adaptive immunity is an acquired immunity. It can also be termed as specific immunity. In the case of adaptive immunity the immune system waits for the formation of many T-cells. The APC (Antigen Presenting Cells) present the antigen with MHC on their surface to the T Cells. The three main Antigen Presenting Cells are B Cells, Dendritic cells and Macrophages. The TCR’s (T Cell receptors) present in the T Cells help in the recognition of the antigens. Once the T Cells recognize them as a foreign material the immune response is initiated. As a result Antibodies are produced by B cells that have received “help” from the T cells. The pattern of the antigen is also saved in Memory B and T-cells. So when the antigen enters for second time into our body the memory T Cells remembers them and they initiate the production of Antibodies by B-cells immediately during further attacks [7].
1.2 Immune tolerance

The important task of the immune system is not only destroying the foreign peptides but also differentiating self and non-self peptides. Our T Cells and B Cells can recognize any microbes entering our body. At times the T and B Cells recognize self components too. In such a case they will recognize them as foreign peptides and act against them. This self reactivity should be prevented. This is achieved by immune tolerance [9].

1.3 Establishment of Immune tolerance:

Immune tolerance is of two levels

- Central tolerance
- Peripheral tolerance
Central tolerance is towards self particles and Peripheral tolerance is towards non-self particles. When this natural tolerance is bypassed it will lead to harmful effects. It is believed that both protective and harmful responses are mediated by T-cells and B-cells [10].

In central tolerance the generated lymphocytes are interacted with self peptides antigenic signals. At this stage the T-cells and B-cells will be in immature condition. T-Cells and B-Cells are both lymphoid cells. T-cells are produced in Thymus and B-Cells are produced in Bone marrow. During this interaction if a strong affinity signal is obtained the lymphocytes are considered to be self-reactive with self peptides and are destroyed by apoptosis. But this process is leaky as not all self peptides are available for the negative selection. This leads to the need of another screening level called peripheral tolerance [11].

Peripheral tolerance assures various safeguards like anergy, homeostatic control and ignorance which prevent the self-reactive lymphocytes to get activated [12].

So when this tolerance level is broke it leads to autoimmunity. Now the tolerance level is broken down and our immune system losses the capability to differentiate self and non-self peptides. Here our immune system recognizes our body peptides as foreign agents and act against them, which results in the destruction of the peptides. This is termed as autoimmunity [13]. The autoimmunity may be targeted towards a single organ or multiple organs. If the autoimmunity affects one organ it is termed as organ specific auto immunity and if it affects many organs it is termed as systemic autoimmunity

1.4 Rheumatoid Arthritis

One of the most common autoimmune diseases prevailing now is Rheumatoid arthritis. Rheumatoid arthritis can be defined as a chronic inflammatory auto immune disorder in which our own body tissues are targeted by our very own immune system. Rheumatoid Arthritis primarily affects the joints. The damage is caused in the joints in the form of inflammations. The joints involved are the joints of wrists, toes, knees and fingers. In addition to the joints it also has its effects on other tissues like muscles, blood vessels and ligaments around the joint. As it has the tendency to affect multiple organs it is also termed as systemic illness. The symptoms associated with the disease are swelling, stiffness and pain. At a more advanced stage the disease may lead to loss of function and mobility.
1.5 Symptoms:

The symptoms are

1. Morning stiffness
2. Soft tissue swelling of joint areas
3. Swelling of proximal interphalangeal or wrist joints.
4. Symmetric swelling.
5. Rheumatoid nodules
6. Rheumatoid factor
7. Radiographic erosion

The presence of rheumatoid arthritis can be confirmed if the symptoms 1 to 4 should be present at least for a span of 6 weeks. As well the out of the 6 symptoms mentioned a minimum of 4 should prevail for the confirmation of the disease [16].

1.6 Progression of the disease:

First stage: The onset of the disease begins at the joints, with inflammation of the synovial membrane. This inflammation occurs in the congestion and edema of the synovial membrane. As a result of inflammation there will be increase in cell count at this region (5000 to 6000 mm$^3$). At this stage diagnosis of the disease through X-ray becomes difficult as there is no destruction of the bone.

Second stage: This leads to accumulation of synovial fluid. This leads to the formation of pannus. The pannus formation reaches the joint capsule and destruction of the bone starts. Now the synovial tissue is inflamed. This inflammation spreads and proliferates more and forms into joint cavity. This growth takes place across articular cartilage. As a result the articular cartilage slowly gets destroyed.
Third stage: The spread of pannus occludes the joint surface resulting in bone atrophy and misalignment. This bone atrophy and misalignment disturb the movement of opposite bones and result in muscle atrophy and imbalance. At this stage if an X-ray is taken it shows extensive cartilage loss and deformities.

Final stage: At this stage the functional life of bony ankylosis of joints comes to an end. Total immobility occurs as a result of bony ankylosis [5].

The joint deformation is supported by the surrounding structures of the joint, like tendons and origins of muscles. The strains caused are generally irreversible. [6].

1.7 Epidemiology:

Almost 1 % of the world populations are affected by Rheumatoid arthritis. From the past studies it is believed that Native American populations like that of Pima Indians are highly susceptible to rheumatoid arthritis [15]. The disease is believed to be less prevalent in Asia and Africa. Women are more vulnerable to this disorder than men. The disease can occur at any age. But when analyzing the reported incidences it believed that the occurrence of the disease is more common as the age increases. The onset of the disease generally occurs between 35 to 50 years of age [14].
1.8 Etiology

Although this disease is prevalent for a very long time the cause of the disease is unknown. It is speculated that the onset of the disease might be induced by microorganisms like virus, bacteria [5] and fungi. It is also believed that hereditary factors also play an important role. Research has been carried out in vigorous manner throughout the world to find the onset which will be potent in producing a cure. There are lots of factors believed to play a hand in the onset of the disease.

1.9 Complement system

Complement system is the main part of innate immune system which can kill pathogen without the previous exposure [21]. The pathways involved in the complement cascade are classical, alternate and lectin pathway. Activation of classical pathway involves the presence of antigen-antibody complex and lectin pathway gets initiated in the presence of membrane binding lectins. But the activation of alternate pathway requires the presence of unstable complement factor C3. The activation of the complement system leads to the formation of C3 and C5 convertase which inturn recruits other complement factors like C5b, C6, C7, C8 and C9 to assemble Membrane Attack Complex (MAC).

This complement system is like a sword which is sharp at both edges. Excessive activation of the complement leads to various disease like rheumatoid arthritis, glomerulonephritis, multiple sclerosis and other inflammatory diseases. In order to prevent the excessive activation complement inhibitors are needed. The four main complement inhibitors are proteinase inhibitor, serum carboxypeptidase N inhibitor, CD59 inhibitor and inhibitors which acts on C3 and C5 convertase. Out of the four Proteinase inhibitors are generally C1 inhibitors which inhibits C1. Serum carboxypeptidase inhibitors acts on anaphylotoxins produced from C5 and C3. CD59 inhibitor acts on the Membrane Attack Complex and inhibits them [60].

In patients suffering from rheumatoid arthritis a large number of T and B cells, mast cells, macrophages and granulocytes are present in the synovial lining of the affected joints. These infiltrated activated macrophages, mast cells and granulocytes can cause joint erosion. The synovial fluids of the patients suffering from RA show high level of C4 and C5 complement factor [22-28]. It is believed that the classical pathway is activated by deposited antibodies in the inflamed joints. B cells producing autoantibodies and anaphylatoxin C5a also activates the complement during RA [17]. The activation of complement system in rheumatoid arthritis is not only limited to classical pathway. In the synovial fluid of the rheumatoid patients high number of Bb fragments which are generally produced during the formation of C3 convertase can be observed. It is still not clearly known which of the three pathways that play a significant role in rheumatoid arthritis.
1.10. Autoantibodies:

The antibodies produced during auto immune response are termed autoantibody. The three main groups of antibodies are

1. Rheumatoid factors
2. Anti-Citrullinated Protein Antibodies (ACPA)
3. Anti-Collagen antibodies

Rheumatoid factors act against the Fc region IgG. RF can be IgA, IgG and IgM. But the major isotype is IgM. IgM-RF is generally found during normal immune response too. So it will be
found in normal healthy person, but at low affinity. In case of RA patient’s high level of IgM-RF is found. Some studies suggest that IgA-RF is more specific as RA markers [18]. Anti-Citrullinated Protein Antibodies (ACPA) are antibodies which recognize the citrullinated epitopes. Filaggrin is a protein expressed in squamous epithelial cells. It is in this protein first citrullinated epitope was identified [19]. Other ACPA are Antiperinuclear factor, antikeratin, antifilaggrin and anticyclic citrullinated peptide (citrullinated fibrinogen, vimentin, fibronectin, α-enolase and CII) antibodies. The Anti-collagen antibodies are believed to be locally produced in the joints and are present in the synovial fluid [20].

1.11 Environmental factors:

Studies show that smoking and infections play a major role in the aetiology of rheumatoid arthritis. Microorganisms like Mycobacterium tuberculosis, Parovirus B19, Epstein-Barr virus (EBV) and Escherichia coli can act as triggers for Rheumatoid arthritis, although the results are not consistent [29-34]. We should understand that these environmental factors somehow act as a trigger, but they are not directly involved in disease process. The peptide sequence of some viruses and bacteria are almost identical to the specificity of some autoantibodies. So when these organisms enter our system an immune response is evoked. The immune response produced cross reacts with that of autoantigens and is referred to as antigen mimicry. Release of pro-inflammatory cytokines increases antigen-presenting capacity which results in an inflammatory cascade [35]. Hormonal factors also play a role in triggering the disease. High incidences are reported during post-partum or pre-menopausal period. Intake of contraceptive pills also might trigger the disease [36]. Diet and stress factors contribute to the disease. The immunomodulatory effect of Vitamin D makes it a potential candidate. High consumption of fruits, olive oil, fish, vegetables shows protective effect and low intake of antioxidant foods are associated with the increasing the possibility of the disease[37-40].

1.12 Role of T, B cells and Cytokines

In the synovial fluid of rheumatoid arthritis patient T-cells, B-cells and Macrophages are found in larger numbers than its normal level. In Rheumatoid Arthritis T Cells play an important role in the formation of Tissue destructive effector Cells by infiltrating into the synovial membrane and activating the synovial fibroblast. The increase in T-cell population means that the T-cells are activated by more than one pathway [52]. The T-cells are triggered by professional Antigen Presenting Cells like dendritic cells and Macrophages. B-cells can also perform as an Antigen Presenting Cells (APC) which activates the auto-reactive T-cells. T helper Cells are divided into two types referred to as Th1 helper Cells and Th2 helper cells [58]. Type 1 helper Cells on stimulation produce IL-2, Interferon gamma and Tumor Necrosis Factor (TNF). These cytokines activate macrophages and initiate cell mediate immunity. On the other hand type 2 helper cells stimulate the production of IL-4, IL-5, IL-10 and IL-13 cytokines. These cytokines initiate the
production of antibodies. The T cells differentiating into Type 1 or Type 2 subsets depend on the cytokines produced by Antigen Presenting Cells.

B-cells also produce antibodies against citrullinated protein [54]. The immunoglobulin receptors present in the B-cell can bind to even low level of antigen present. Once this antigen is bound it is degraded into antigenic peptides. The antigenic peptides are presented in the groove of the HLA-DR4 molecule, which in turn activates the T-cell, which in turn contribute to the pathogenesis factor. A large amount of pro and anti-inflammatory cytokines are found in the rheumatoid synovium. The main pro-inflammatory cytokines are TNF-α and IFN-γ. Other pro-inflammatory cytokines include IL-1, IL-6, IL-8, IL-12, IL-15, and IL-18. The anti-inflammatory cytokines found in the rheumatoid synovium are IL-4, IL-10, IL-11, and IL-13.
1.13 MHC

One region in our genome which is consistently linked with rheumatoid arthritis is the Major Histocompatibility complex (MHC). The MHC is 3.6 Mb in length. It is divided into 3 regions, MHC I, MHC II and MHC III. It is also termed as HLA (Human Leukocyte Antigen) region in man. The telomeric end of the MHC comprises the MHC I region and extends over 2000 kb. The MHC I region contains HLA class I genes. MHC II contains HLA-DR, HLA-DP and HLA-DQ loci. HLA-DR, HLA-DP and HLA-DQ loci encodes the α and β chains of different MHC II genes. The MHC III region is present between the MHC II and MHC I regions. The MHC complex is a gene rich complex and it contains 220 genes out of which many are involved in immune regulation [55].

It is believed that HLA-DRB1 locus plays a significant role in disease development. This locus consists of an amino acid sequence which is highly homologous (QKRAA, QRRAA, and RRRAA) [53]. The amino acid sequence is termed as shared epitope. Several allelic variants of HLA-DRB1 are associated with Rheumatoid arthritis. HLA-DR B1*01 alleles are found in patients with RF-Negative factor. HLA-DR B1*04 is found in both RF-Positive and RF-Negative patients. HLA-DR B1*04 and HLA-DR B1*01 when combined increases the risk of formation of rapidly progressive synovial lesions [56]. Much research is concentrated towards the genes present in the MHC region which might prove to be an important candidate in influencing the disease. A study conducted by Harney SM et al clearly demonstrates the association of AIF1 and rheumatoid arthritis [57]. TNF is also considered to be a potential candidate.

Lot of studies are focused on TNF gene to find out if there is any association of the gene with that of Rheumatoid Arthritis. Haplotypic studies were carried out in the TNF gene to find out its association with Rheumatoid arthritis. In case of the *0404 haplotypes (P=0.007) it was over expressed and in case of *0401 haplotypes (P=0.007) it was under expressed. This study showed that there are some other MHC susceptible loci outside that of the LTA-TNF region [61]. Ota et al tried to establish the role of a region telomeric to TNF with that of the disease by designing microsatellite markers which covered 3.6Mb of the MHC. This included 5 TNF SNP’s in them. But the study failed to establish any Linkage Disequilibrium with DRB1 alleles which was confirmed in a study by Okamato et al. [62].

As all believed that the HLA-DR molecules play a significant role in rheumatoid arthritis there are contradictory reviews saying that DQ molecules are really associated with the disease and not the DR molecules. Richard Holmdal et al. came up with an opinion that although everyone believe that the DR molecules are programmed to deliver peptides which are supposed to bind to the DQ molecule. But the shared epitopes never bind to DQ molecule and in the process bind with the self-peptides causing rheumatoid arthritis [63].
2. Introduction

2.1 Animal models in rheumatoid arthritis

Animal models are of extreme importance in the research of rheumatoid arthritis. It forms the backbone of inflammation research. This is mainly due to the reason that such research cannot be carried out in humans as such. Using animal models the pathogenesis of rheumatoid arthritis can be studied which will lead to the discovery of anti-arthritic drugs. The three main models used in Rheumatoid arthritis research are

- Oil Induced Arthritis (OIA)
- Pristine Induced Arthritis (PIA)
- Collagen Induced Arthritis (CIA)

2.1.1 Oil Induced Arthritis:

In case of Oil Induced Arthritis (OIA), arthritis is induced by injecting Incomplete Freunds Adjuvant (IFA) in DA rats [44]. IFA has 85% paraffin oil and 15 % emulsifier ArlacelA. The intradermal injection of IFA induces arthritis within 12-14 days [43]. The arthritis caused by OIA is T-Cell-Dependent polyarthritis. This polyarthritis can be induced not only by IFA, but also by other oils like mineral oils with adjuvant properties [45]. The onset of disease is from 14 days after intradermal injection of IFA with symptoms of joint inflammation and it decreases within 45 day with no ankylosis. This disease is T-cell dependent and influenced by both MHC and non-MHC genes.

2.1.2 Collagen Induced arthritis:

In this model the disease is induced in the rats with type II collagen as antigen adjuvants. Type II collagen is a major structural protein which is found exclusively in cartilage. When administered in native form this Type II collagen has the tendency to induce disease. After injecting the type II collagen within 2-5 weeks, swelling and redness is seen in peripheral joints [42]. There are a lot of pathological similarities between CIA and RA [41].

2.1.3 Pristine Induced Arthritis

Pristane-Induced arthritis (PIA) in rats fulfills the criteria necessary for diagnosis of RA in humans. Pristane induced Arthritis in rat is induced in susceptible strains such as DA rat by an intradermal injection of non-antigenic pristane oil (2, 6, 10, 14-tetramethylpentadecane) resulting in development of severe relapsing arthritis disease course with an erosive and symmetric destruction of peripheral joints after two weeks [46]. The PIA rat model resembles the human disease such as development of symmetrical disease, presence of serum rheumatoid factors, radiographic changes and chronicity. PIA is also T cell driven and
dependent on MHC Class II. The dependency on MHC was only observed in the chronic phase of disease. The arthritogenic T cells recognize both DQ and DR MHC class II alleles suggesting that T cells activated after Pristane injection recognize a self-peptide in the MHC class II COMPLEX but no self-antigen has been identified so far. The arthritis can be transferred with αβ cd4+ T cells [46, 47].

Figure 8: MHC Class III Congenic Strains

The animal model used in my study was the Oil Induced Arthritis (OIA) in Rats. The adjuvant used to induce the disease is mineral oil.

In our study the main focus is on the rat strain DA.1HR56T. DA.1HR56T is a congenic rat. Congenic strains are generally produced by repeated backcrossing of F1 generation animals. During such backcrossing a part of the genome from one strain is introgressed on the genetic background of the other strain. DA.1HR5T strain developed less severe disease when compared to other rat strains. This particular rat strain DA.1HR56 carries a MHC class III congenic fragment. Different sub congenic fragments like DA.1HR5, DA.1HR4, DA.1HR7, DA.1HR2 and DA.1HRT have been tested and we believe that the protective phenotype comes from the DA.1HRT congenic fragment. Recently two more congenic strains were obtained: DA.1HR56A and DA.1HR56B.
3. Objective:

1. To find out whether there are further congenic fragments formed due to repeated backcrossing other than DA.HR56A and DA.HR56B
2. To find out the border between DA.HR56A and DA.HR56B.
3. Comparative gene expression studies of DA strain and DA.1HR56 strain to find the genes responsible for the protective phenotype.

4. Materials & Methods:

To find out whether there was further congenic fragments formed due to repeated backcrossing other than DA.HR56A and DA.HR56B

4.1 Animals:

The DA.HR56T strain was bred and maintained in polystyrene cages with water and chow. Each cage contains 2 female and 1 male rats. Pathogen free environment was maintained along with 12-h light/dark cycles.

4.2 Toe marking:

The gestation period of rats differ between 21-23 days. As soon as the pups were born they were toe marked. The toe marking was done to find new recombinants. Toe marking was done by taking toe biopsy from the pups. While toe marking, the pups should be between 7 to 12 days old due to ethical permits. Not more than ¼ of a toe should be cut. By cutting more than ¼ of a toe we not only violate the ethical permits, unnecessary harm and damage will be caused to the animal. Furthermore, tail biopsies were taken and placed in the wells of a PCR plate. A list was made to know exactly which pup biopsy was placed in the corresponding PCR wells. The maintained list contain all information like Cage Id, breeding strain, toe cut date, Male or female and Date of birth.

4.3 Genotyping using microsatellite markers:

Taking tail biopsies

↓

Extracting DNA from the tissue

↓

Setting up PCR reaction
Pooling and diluting PCR products

Performing a run on ABI3730 machine

Analyzing the data in Gene Mapper

The biopsies taken were centrifuged. In order to separate the DNA from the tissue, diluted NaOH was added to the PCR plates. Now the tissues were boiled at 95 degree for 90 minutes. Tris HCl was added to the plate (Tris Plate). PCR reaction was set up using the sample from the Tris plate. For every strain there were different primers to be tested. These primers were designed and validated previously. For DA.HR56T strain the primers to be checked were 8, 12 and 13. The primer names were given based on their location in the fragment. The primers were given name in increasing order from the telomere. PCR amplification was done. The PCR product was diluted. The diluted PCR product was added to formaldehyde and Liz 600 standard. Now the plates were denatured using a PCR machine and later kept in ABI3730 machine for genotyping. Genotyping was done and the result was analyzed using Gene mapper software. On analyzing the result the homozygous and heterozygous pups were identified. The reason for having more than 2 primers for a strain was to cross check if there was any new recombinant fragment formed. The genotyping was followed my weaning of the animals. The pups needed for further experimental studies were maintained and grown. The rest of the pups were killed by subjecting them to carbon dioxide gas.

4.4 Result:

During my entire project genotyping was done every week. Until I finished my work in the lab no new recombinants were found in addition to DA.HR56A and DA.HR56B. This experiment was not only carried to find out further recombinants but it was also very important to have animals for further experiments. This experiment helps us to differentiate the homozygous rats and the heterozygous rats. Homozygous HR56T, HR56A, HR56B and DA pups were kept alive for further experiments while all the other heterozygous pups were killed as all these pups cannot be kept alive because of ethical and space constraint.
5. To find out the border between DA.HR56A and DA.HR56B

5.1 Animals:

The DA.HR56A and DA.HR56B strains were maintained separately in polystyrene cages with water and chow. Each cage shouldn’t contain more than 5 rats. Pathogen free environment was maintained along with 12-h light/dark cycles.

Tissue collection
↓
DNA preparation
↓
Designing Primers
↓
Checking the primers and Templates
↓
Micro-capillary direct sequencing on ABI 3730 using BigDye terminators

5.2 Tail biopsy:

The small fraction of the tail segment were dissected aseptically from the rats and placed in 1ml eppendorf tube which in turn was kept in dry ice. Later the eppendorf tubes containing the Tail fragment were stored in -80 Degree freezer. It was so important to mark the tubes with the appropriate strain name in order to avoid confusion. When needed the tail biopsies were taken and centrifuged. In order to separate the DNA from the tissue diluted NaOH was added to the PCR plates. Now the tissues were boiled at 95 degree for 90 minutes. It was not mandatory that DNA should be prepared from the tail biopsy alone. It can be obtained from any tissue part of the rat like toe, lymph node, liver, blood etc.

5.3 DNA preparation:

DNA was prepared from tail tips. It was done by adding lysis buffer and proteinase k to each sample. Incubate overnight. Then the tubes were vortexed for 10 minutes and the supernatant was collected in new tube to which isopropanol was added. When the isopropanol was added the solution become no longer viscous and the DNA strand was visible. The DNA was collected in the form of pellet and stored in -20 degree Celsius.
5.4 Designing Primers:

The sequence information was obtained using the UCSC database (http://genome.UCSC). For each gene at least 2 sets of forward and reverse primers were designed based on the length of the gene. The obtained genome sequence from the UCSC database was copied and pasted in the space provided in the primer 3 website (http://frodo.wi.mit.edu/primer3/). This program automatically picks the sets of forward and reverse primers. When compared to other applications, Primer 3 generates good and consistent results but it might be necessary to alter the primer conditions slightly. The conditions to be altered were

i. Target - should include repeats
ii. Product Size ranges – should be between 110 and 600bp
iii. Primer Size - between 19 and 21
iv. Primer temperature – 59 and 61 degrees
v. Primer GC% - should be between 40 and 60 %

It was important to make sure that the primers overlap at least 100 bp and the PCR product was no longer than 600 bp. We should also make sure that there were enough nucleotides before and after our sequence of interest, which means at least 80 bp.

5.5 Checking our primers and templates:

It was absolutely necessary to check both the primers and the templates. This was done by amplifying the templates using the primers designed. Then the amplified product was run on gel electrophoresis. Appearance of band confirms the quality of the template and primers. It was good to use the same Taq, water and buffers that we intend to use later for sequencing. The reason for checking primers and templates before sequencing were not only to check whether they work or not, but also to know whether there were any primers which amplifies several products.

5.6 Micro-capillary direct sequencing on ABI 3730 using BigDye terminators:

Target Sequence Amplification

↓

Gel Electrophoresis

↓

Purification of PCR products

↓
Quantification of PCR products

↓

Sequencing reaction

↓

Post sequencing clean up

↓

ABI 3730 set up

↓

Result analysis in Seqman

Target sequence amplification was carried out by adding 1.0 µl template to 14 µl PCR mixture which contain 10x PCR buffer w/o MgCl₂, MgCl₂, dNTP, Platinum Taq, Water, Forward primer and Reverse primer. It was always advisable to use duplicates or triplicates. The reason was even if amplification was not good in one well, the amplification from the other well can be considered for analysis. The amplified products were run in Gel Electrophoresis.

Purification of the product was done by adding the PCR product to the gel filter prepared by adding 300 µl of P100 gel solution. The excess water in the gel filter was removed by spinning at 1620 rpm. The excess water should be removed before adding the PCR product for purification. Once the PCR product was added the PCR plate was spun at 1620 rpm for 4 minutes to collect the purified final product. If excess water not is carefully removed before the purification process it will dilute the PCR product. Then quantification of the purified PCR product was done using Nano drop. The A260/280 ratio should be 2.00 for 100 % nuclei acid.

Now sequencing reaction was set up by adding 1 µl purified PCR product to 9 µl of sequencing mixture consisting of Big Dye v3.1, Buffer 5x, Water and Sequencing primers (forward or reverse). Even here use of duplicates or triplicates was advisable. The plate was run at BigDye Standard_8 PCR program.

Post sequencing clean-up was carried out. This was done by adding 1.8 µl of SA/EDTA mixture and then washing them with 96% and 70% ethanol. Then the PCR product was speed vaced for 15 minutes in dark to remove the ethanol precipitate if present after centrifugation. After the post sequencing clean-up, 10 µl HiDi was added to all wells and septa was placed on the plates.
With the lids the plates with septa were placed in ABI 3730 and sequencing analysis program was initiated.

5.7 Results & Analysis:

Once the run was completed in ABI 3730 the result was analyzed using Seqman software. The genes in the fragment were in the order Lta, Tnf, Ltb, Lst1, Ncr3, Aif1, Bat2, Bat3, Apom, G4, Bat4, Csnk2b, Ly6g5b, Ly6g5c, Bat5, Ly6g5f, Ly6g5e, Ly6g5d, Ly6g6c, G6b, and Ddah2. Out of all genes present in the fragment we believed the border should lay between Bat5 and Ly6g5e. Primers were designed for Lta, Tnf, Aif1, Ltb, Lst1, Msh5, Ng23, Ly6g5c, bat5, Ly6g5f, Ly6g5e, and Ly6g5d. 51 pairs of forward and reverse primers were designed and checked. They were numbered from 1001 to 1103. Each of the primer pairs were checked for its efficiency.

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**Table 1 - HR56A & HR56B Border**

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</table>

**Figure 9 - HR56T, HR56A & HR56B fragments with the genes**
While running the sequencing experiment all the four strains HR56T, HR56A, HR56B and DA were sequenced with the sets of primers designed. By including HR56T and DA it’s easy for us to compare whether that particular position lies in the fragment or not. The nucleotides at various positions were closely studied. From the above table we can conclude that the border lies between 3805744 and 3808884. This shows that the gene Ly6g6f was in the HR56A fragment and Ly6g6e was in the HR56B fragment. It was also important to make sure that the entire Ly6g6f gene was in the HR56A fragment and entire Ly6g6e gene was in the HR56B fragment. The gene position of Ly6g6f starts at 3801118 and extends till 3806163. Similarly the gene position of Ly6g6e starts at 3808707 and extends till 3810443. The nucleotide positions close to that of 3806163 and 3808707 were checked and it was confirmed that the entire Ly6g6f gene was in the HR56A fragment and the entire Ly6g6e gene was in the HR56B fragment.

Sorting out the border of the HR56A and HR56B fragment was important in more than one way. Now we were sure about the genes which lie in HR56A fragment and about the genes which lie in the HR56B fragment. Later experiments can be carried out to find out whether both the strains are resistant to Oil Induced Arthritis or not. If either of the strains is not resistant to the disease then we can ignore the genes present in those fragment and concentrate more on the genes present in the other fragment as we will be sure that the protective phenotype lies in that fragment. But if both fragments show resistance to the disease it makes our work difficult as it will have more than one meaning. It might mean that the protective phenotype may be provided by more than one gene which is present in both fragments. If we find out that both fragments not confer resistance to the disease then it leads to the suggestion that the combination of more than one gene might be responsible for the protective phenotype.

Although all this is just assumptions right now we have some results which can help us to proceed further and minimize or maximize our efforts to find the protective phenotype. In this case we strongly believe that the protective phenotype should lie in the HR56A fragment that contains more potential candidates for immune regulation.
6. Comparative gene expression studies of DA strain and DA.1HR56 strain by ABI7900HT to find the genes responsible for the protective phenotype.

6.1 Animals

10 DA rats and 10 DA.HR56T rats were maintained in polystyrene cages with water and chow. Each cage should hold only 5 rats. Out of the 20 rats, 10 rats (5 DA rats + 5 DA.HR56T) were immunized with OIA and the other 10 rats were naive rats. The immunization was done by injecting 300ul I.F.A. at the base of the tail intradermally.

Lymph node collection
↓
Designing primers
↓
Validating primers
↓
mRNA extraction
↓
cDNA synthesis
↓
Expression studies using qPCR

6.2 Lymph node Collection:

Ten days after immunization the lymph nodes were removed from both sets of rats. The lymph nodes were removed and placed in an eppendorf tubes which in turn were placed in dry ice. Later the lymph nodes were stored in -80 degree freezer until further use.

6.3 Designing primers:

Gene expression primers are designed by following the steps in section 5.4.
6.4 Validating primers

This was the most time taking part of the experiment. Validation of the ordered primers was the most time taking task of the experiment. It was absolutely necessary that a validation experiment was done to test both primers and template quality. According to the ABI manual, a primer titration test should also be performed in order to check for optimal primer concentration. However, a primer titration was normally not necessary and should only be done when qRT validation was okay but the efficiency does not reach 100 % and the reason cannot be addressed to the template.

The primer tubes were centrifuged so that the lyophilized primer gets settle in the bottom of the tube. Dilute the primers with the HPLC water according to the 100 µM. The stock was further diluted by adding 975 µl water to 25 µl primer. Then the primers were diluted to 1X, 2X, 3X, 4X and 5X concentration. It was so important that we don’t waste the template cDNA which we were going to use for the experiments so it was always better to use stored template cDNA. In my case I used cDNA from CD4T+ which was stored by my supervisor for a different experiment. The primers were added to the template and SYBR green. It was also important to use duplicates or triplicates for each primer.

Once the RT was complete the Ct data was examined. The amplification was the foremost thing to be checked. While checking the result in the system if all the wells are in green colour it means that the entire wells were amplified. The dissociation curve and the melting temperature were checked. The slope should be within -3.1 and -3.6. A slope of less than -3.6 means that the reaction contains a PCR inhibitor which could be everything from contaminating DNA, suboptimal primers or bad templates. The R2 value should be greater than 0.99. The Ct can be everything between 14 and 30. It was also important to add some housekeeping primers Hmbs, Arbp and Actb. This was to make sure that the quality of the template was good. If these housekeeping genes were not included then if something was wrong with the template cDNA we would assume that the primer efficiency was not good. In order to avoid it the housekeeping genes were also included. The reaction also needs to contain a non-template control (NTC), that is a PCR reaction with water instead of cDNA template to check for unspecific amplification and contamination. If the primers prove their efficiency in this validation step then they can be stored for use. Primers of no use were discarded to avoid later mistakes.

6.5 mRNA extractions:

The lymphnodes which were stored in the -80 degree freezer were transferred to RNA stabilizer immediately. It was also necessary to check that the tube only contained lymphnode. While removing lymph nodes from the rats there were high chances that it might contain some fat along with the lymph node. In such case it was important to remove the fat from the lymph
node before extracting mRNA. The lymph nodes were disrupted using RLT buffer and beads as disruptor. By subjecting the lysate to ethanol, RW1 buffer and RPE buffer mRNA was extracted from the lymphnodes. The mRNA concentration was measured using Nanodrop and the A260/280 should be around 2.0 to 2.2. Then the extracted mRNA was stored in -80 degree freezer.

6.6 cDNA synthesis:

While preparing cDNA it was important that all samples were of equal concentration. The mRNA concentration may differ based on the size of the lymph node taken. Although it was not possible to take exact equal size of lymph node for mRNA preparation, we can try and make the size taken to be approximately similar. Doing so will help us in the cDNA synthesis step. cDNA synthesis was carried out by using High-Capacity cDNA reverse transcription kit.

6.7 Expression studies using qPCR:

For expression studies 25 µl of primers were mixed with 975 µl of water. 5.7 µl of template cDNA was mixed with 1.8 µl of primer and 7.5 µl of SYBR green and loaded in a barcoded plate. Samples were run as triplicates. The plate was then placed in ABI7900HT and run. Once the run was complete the results were imported for analysis.

6.8 Result and Discussion

Primer designing and primer validation took most of the time as designing 53 pairs of primer took quite a long time. As there were 53 pairs of primers to be validated and some problem with the 10th, 11th and 12th columns of the ABI7900Ht machine. As we were using triplicates only three primes could fit in a plate. So a total of 18 plates were required. Each run takes approximately 3 hours. So a total of 54 hours of running time was required. Also the machine was used by everyone in the lab and pre booking of time slots came with some limitation. This part of designing and validating of primers alone took a major part of my thesis time. Primers for testing some cytokines in immunized samples were designed and ordered beforehand. Those primers were tested well ahead of my project start. When all the primers were approved the gene expression analysis was done in ABI7900HT. In case of samples from naïve animals only the genes were tested. But in case of samples from immunized animals also expression of cytokines like IL4, IL17A, IL10, IL12A, IL6, GATA3 and TNF-α were tested. Again to study the expression analysis only 3 genes can be placed in a plate and each run takes 3 hours. Two batches of immunized rats were tested and one batch of naive rats. The results were calculated using the comparative CT method of relative quantification. This will yield a precise fold change value for each gene of interest in the sample. The Ct values were noted down from the finished run. Ct value represents the PCR cycle at which the SDS software first detects a noticeable
increase in reporter fluorescence above a baseline signal. From the Ct values $\Delta$Ct, $\Delta\Delta$Ct of the samples were calculated followed by Fold change values.

$$\Delta$Ct = Ct (target gene) – Ct (reference gene)$$

$$\Delta\Delta$Ct = $\Delta$Ct (sample) – $\Delta$Ct (Calibrator)$$

$$\text{Fold Change} = 2^{-\Delta\Delta$Ct}$$

Out of all the gene and cytokines tested genes G$ and Ncr3 show significant difference in gene expression between DA rats and DA.HR56T rats. In the case of G4 two primers 1014 and primers 1043 shows significant difference in gene expression. With regards to Ncr3 gene primer 1006 shows significant difference in gene expression.

**Figure 10: Comparison of G4 gene expression in Immunized rats and naive rats.**

The function and role of the G4 gene in autoimmune disease is unknown. But in samples from both naïve and immunized animals the gene expression between both DA.HR56T and DA strains varied significantly. If the P Value calculated was less than 0.05 it means there was significant difference. In case of G4 gene the P values were 0.0028, 0.0025 and 0.0033 respectively.
Figure 11: Comparison of Ncr3 gene expression in Immunized rats and naive rats.

NCR stands for Natural Cytotoxicity receptors. NCR3 is a human NK cell receptor. NCR3 has a significant cytotoxic function including antitumor responses as it was directly involved with NK cells. When this NCR3 was activated it leads to the production of IFN-γ. Activated T-cells proliferate and differentiate into either Type-1 helper cells or Type-2 helper cells. Type-1 helper cells produce IFN-γ and TNF. This IFN-γ is mainly released when the NCR3 receptor is engaged and mediates activation of NK cells. This IFN-γ drives Type 1 helper cells production and inhibits Type 2 helper cells production. So we believed the gene expression seen with respect to NCR3 gene might lead to some interesting conclusion.

Gene sequencing primers were designed and ordered for G4 and NCR3 gene. Now both genes were sequenced and the two strains HR56T and DA were checked for polymorphism in them. We believed that if a polymorphism was found in the HR56T strain and if it doesn’t exist in the DA strain we might conclude that it was responsible for the change in expression and that it might be the reason for the protective phenotype. To our disappointment this wasn’t the case, we didn’t find any such polymorphism.

7. Further research

Experiments were initiated to find out which of the two fragments, HR56A or HR56B that were correlated to resistance to rheumatoid arthritis. As both G4 and NCR3 gene were located in the HR56A fragment we believe that the other fragment not was involved in the resistance to the disease. As a profound gene expression difference exists with NCR3, we can proceed by checking the gene expression of IFN-γ, IL-2 and TNF-β just to make sure if such a difference exists. In that case we can be sure it has something to do with the Type 1 helper cells. As these were a very lengthy process and careful steps were to be taken right from the selection of the animal we believe that the results obtained could have been more satisfying if the possibilities of errors were minimized. Repeated backcrossing will lead to the formation of more subcongenic fragments which will make this research more interesting.
8. Appendix

8.1 Housekeeping genes:
In case of qPCR it is important to validate that the cDNA extracted from the lymph nodes are good. The normalizing is done by the housekeeping genes. The house keeping genes selected by us are β-Actin, Arbp and Hmbs.

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<th>Forward primer (5’ - 3’)</th>
<th>Reverse primer (5’ - 3’)</th>
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<td>β-Actin</td>
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<td>Hmbs</td>
<td>TCTAGATGGCTCAGATACGT</td>
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8.2 qRT PCR primers
The primers designed for the expression analysis are

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<th>Target</th>
<th>Forward Primer (5’ - 3’)</th>
<th>Reverse Primer (5’ - 3’)</th>
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<td>ACGTGGAAGGCACTGCC</td>
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### 8.3 Sequencing primers:

Sequencing primers for G4 and NCR3 are

<table>
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<th>Gene</th>
<th>Forward primer (5’ - 3’)</th>
<th>Reverse primer (5’ - 3’)</th>
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<tr>
<td>Ncr3</td>
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</tr>
<tr>
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<tr>
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<td>CTCTGAGGTCAGCAG</td>
</tr>
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<td>GACACCTGCAACCACCTCAA</td>
<td>TGGGAAACCCAGATTGAAAC</td>
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Figure 12: Graph showing that the ct values of both the strain of animals almost remains the same for the house keeping gene with respect to immunized rats.
Figure 13: Graph showing that the ct values of both the strain of animals almost remains the same for the house keeping gene with respect to naive rats.

8.5 Fold Change Calculation:

\[ \Delta Ct = Ct \text{ (target gene)} - Ct \text{ (reference gene)} \]
\[ \Delta \Delta Ct = \Delta Ct \text{ (sample)} - \Delta Ct \text{ (Callibrator)} \]
\[ \text{Fold Change} = 2^{-\Delta \Delta Ct} \]
9. ACKNOWLEDGEMENT

It's my pleasure and honor to thank my supervisors Anthony Yau and Jonathan Tuncel, who introduced me to the field of rheumatology and its importance. I am grateful to both of them for guiding and assisting me in every step of this thesis work. I would also like to thank the laboratory personnel's and other thesis students who shared their experience and knowledge with me. Finally, Thanks to the laboratory head Dr. Rikard Holmdahl for providing me with an opportunity to work in the laboratory.

I would also like to thank my Student Corordinator Dr. Volkamar Passoth, my Internal Examiner Dr. Caroline Fossum who helped me by supervising me and the supervisor Prof. Magnus.
10. References


20. Dr Günter Steiner, Autoantibodies in rheumatoid arthritis, Issue 7- November 2006


