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Immunogenetic Studies in Multiple Sclerosis

by

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To my parents

Summary

Multiple sclerosis (MS) is an inflammatory, demyelinating and neurodegenerative disease of the central nervous system (CNS). Several genetic factors, rather than just one, are most likely involved in the etiology of MS.

At least two signals are required for T cell activation: an antigen specific signal mediated by human leukocyte antigens (HLA)/T cell receptor, and a co-stimulation signal mediated by B7/CD28 and CTLA-4. Applying newly developed PCR-amplification with sequence-specific primers (PCR-SSP) we found that certain polymorphisms in CTLA-4 gene are associated with susceptibility to MS in sporadic patients and MS families.

Several reports have suggested that a number of autoimmune diseases are associated with the CTLA-4 G^{49} allele. We found that T^{-318} and A^{49} alleles are associated with increased expression of cell-surface CTLA-4 after cellular stimulation and increased mRNA levels in non-stimulated cells. These results confirm earlier findings that cells homozygous for the A^{49} allele have a more profound inhibitory effect on the proliferation of T cells, regardless of the presence of an autoimmune disease. In conclusion, findings from our two studies on CTLA-4 support the hypothesis that increased CTLA-4 expression may decrease the risk of MS.

HLA-DR alleles are known to be associated with MS. Exceptions are MS patients in Sardinia and the optico-spinal form of MS in Japan. This inconsistency could be explained by additional independent and modifying genetic factors within the HLA region. We demonstrated that the association with HLA-DR15 in MS is modulated by the HLA class I alleles A2 and A3, which decrease and increase the risk of MS, respectively. These associations are independent of DR15, whereas a B7 association with MS is secondary to the DR15 association. In conclusion, interactions between several HLA complex genes influence the risk of MS.

Associations with HLA genes have been demonstrated in a number of autoimmune diseases. In a large set of sporadic MS patients we found that, in addition to DR15, DR17 is also positively associated with MS and that carriers of the DR15 allele are associated with a lower age at onset in MS. None of the HLA-DRB1 alleles influence the course or outcome in MS, cerebrospinal fluid or magnetic resonance imaging results. Identical DR15 phenotype frequencies were found for male and female patients, and for patients with familial and sporadic MS. In conclusion, results demonstrate that DRB1*15 carriers may have a genetically determined early start of MS but do not support a previous hypothesis that patients with bout-onset and primary chronic progressive disease course have different HLA-DRB1 allele associations.

We analyzed the importance of the HLA-DR locus for MS susceptibility in 542 MS sibling pairs and their families by nonparametric methods. Significant evidence for linkage was obtained in families carrying the associated allele DRB1*15, but surprisingly also in families lacking the DRB1*15 allele. This indicates either the presence of second susceptibility locus within the HLA region, independent from DRB1*15 or, alternatively, a single primary (non-DRB1) susceptibility locus in disequilibrium with the DRB1*15.

Table of Contents

Main References

This thesis is based on the following articles, which will be refereed to in the text by their roman numerals:

I Ligers A, Chun X, Saarinen S, Hillert J, Olerup O. (1999) The CTLA-4 gene is associated with multiple sclerosis. *J. Neuroimmunol* 97:182-190

II Ligers A, Teleshova N, Masterman T, Huang W-X and Hillert J. (2001) CTLA-4 gene expression is influenced by promoter and exon 1 polymorphisms. *Genes Immun* 2:145-152

III Fogdell-Hahn A, Ligers A, Gronning M, Hillert J, Olerup O. (2000) Multiple sclerosis: a modifying influence of HLA class I genes in an HLA class II associated autoimmune disease. *Tissue Antigens* 55:140-148

IV Masterman T, Ligers A, Olsson T, Anderson M, Olerup O, Hillert J. (2000) HLA-DR15 I associated with lower age at onset in multiple sclerosis. *Ann Neurol* 48:211-219

V Ligers A, Dyment D, Willer C, Sadovnick AD, Ebers G, Risch N, Hillert J and the Canadian Collaborative Study Group. (2001) Evidence for linkage with HLA-DR in DRB1*15 negative multiple sclerosis families. *Am J Hum Genet* In press

Abbreviations

APC	Antigen-presenting cell
CNS	Central nervous system
CTLA-4	Cytotoxic T lymphocyte antigen 4
EAE	Experimental autoimmune encephalomyelitis
ETDT	Extended transmission disequilibrium test
HLA	Human leukocyte antigens
IBD	Identical by descent
IBS	Identical by state
IDDM	Insulin-dependent diabetes mellitus
Ig	Immunoglobulins
IL	Interleukin
INF	Interferon
MG	Myasthenia gravis
MHC	Major histocompatibiliy complex
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
PBMCs	Peripheral blood mononuclear cells
PCP MS	Primary chronic progressive multiple sclerosis
PCR	Polymerase chain reaction
R/R MS	Relapsing/remitting multiple sclerosis
RFLP	Restriction fragment length polymorphism
SNP`s	Single nucleotide polymorphisms
SLE	Systemic lupus erythematosus
SSP	Sequence-specific primers
TCR	T-cell receptor
TDT	Transmission disequilibrium test
TGF	Tumor growth factor

Introduction

Basic immunological concepts

The immune system is able to efficiently protect against foreign infectious agents while sparing its own cells and tissues. The immune response that provides the first line of defense against pathogens without required prior exposure is called the innate immune response. The immune response that provides an increased level of protection upon later re-infection with the same pathogen is known as the adaptive immune response and confers life-long protective immunity against specific pathogens. These immune responses depend on leukocytes and lymphocytes. Innate immunity mainly involves granulocytes and macrophages, whereas adaptive immune responses depend on lymphocytes.

Antigen recognition is conferred through a trimolecular complex that includes a major histocompatibility molecule (MHC), a peptide bound to the MHC, and T-cell receptor (TCR). MHC type restricts antigen specificity for T-cells – a phenomenon known as MHC restriction. This initiates events resulting in destruction and clearance of the invading organism, antigen or infected cell.

T-cells fall into two major classes, depending on the expression of the CD4 and CD8 cell-surface proteins[1]. CD4 and CD8 molecules are components of the T-cell receptor – named co-receptors. T cells are activated by an interaction of the T-cell receptor, consisting of antigen-recognition proteins (TCR) and invariant signaling proteins (CD3), with the HLA-protein complex[2].

CD4 T-cells are specialized to activate other cells and are functionally divided in T_H1 cells, which activate macrophages, and T_H2 cells, which initiate a humoral immune response by activating B-cells. T_H1 cells enhance cell-mediated immunity, but T_H2 cells are mainly involved in antibody-mediated immune response[3]. T_H3 cells are immune response regulators via TGF- β_1, IL-10 and IL-4 production[4] and thereby mediate immune supression[5]. T_H0 is a T-cell subtype having the ability to differentiate into to ether T_H1 or to T_H2 cell subtypes. The CD4 molecule binds both α_2 and β_2 domain of HLA class II molecule and does not interact with peptide presentation[6].

The function of CD8 T cells is to kill cells infected with viruses or bacteria in the cytosol. CD8 cells can differentiate into Tc1 cells producing IFN-γ and Tc2 cells which in turn produce IL-4 and IL-5[7]. CD8 cells can recognize antigen and become activated by those cells that synthesize an appropriate antigen[8]. CD8 binds to the α_3 domain of the HLA class I molecule with an affinity lower than the of the TCR-peptide-MHC interaction but does not interact with peptide presentation[9].

Professional antigen presenting cells express both MHC class I and MHC class II molecules are able to deliver additional (co-stimulatory) signals to T cells for activation. Dendritic cells, macrophages and possibly B lymphocytes can act as professional antigen presenting cells.

Three iso-types of classical HLA class I molecules -A, -B and –C, and three non-

classical HLA class I molecules, -E, -F and –G, have been identified. These molecules form heterodimers with the non-HLA encoded β_2-microglobulin chain, are expressed ubiquitously and present peptides from intracellularly produced protein.

HLA class II molecules consist of one two-domain α-chain and one two-domain β-chain. The function of DR-, DQ- and DP- molecules is also antigen presentation, whereas the function of DM- molecule is to load peptide in class II molecule. The particular function of DN- and DO- molecules is less clear[10].

Immunoglobulins (Ig's) comprise the humoral part of immune system, and have diversified through somatic recombination[11]. CD4 T helper cells initiate the humoral part of innate immune response, by recognizing the antigen bound to the surface of antigen-specific B cells. With help from T-cells they differentiate into plasma cells secreting IgM antibodies, later switching to IgG, and antibodies become affinity maturated by somatic mutation. Thus B-cells express cell surface Ig's specific for a particular antigen. These cells persist as an immunological memory together with T-cell memory cells.

Natural killer (NK) cells are non-T and non-B lymphoid thymus-independent large granular cells of lymphoid lineage. NK cells mediate effector functions like cytotoxicity and cytokine release, depending on inhibitory or stimulatory signal contact with other cells[12,13,14]. NK cells can also be triggered by CD80 and CD86 molecules[15], but the particular contrareceptor molecule is unknown. Expression of Fc receptors allows them to mediate antibody-dependent cytotoxicity[16].

Tolerance and breaking of tolerance

The immune system's role is to fight foreign or altered self objects, but at the same time to not harm the body's own tissues. Tolerance to self antigens can be accomplished by positive or negative selection. After rearranging their antigen-receptor gene, T-cells are screened by positive and negative selection in the thymus. With a rearranged antigen-receptor gene, positive selection selects T-cells with low affinity to self antigens, whereas negative selection eliminates T-cells specific for self antigens.

It has been shown that T-cell tolerance can be regulated by clonal deletion, by anergy, immune ignorance or immune suppression[17,18]. It has also been shown that tolerance to peripheral antigens can be accomplished in the thymus[19] or by special regulatory cells demonstrated in diabetes[20], in anterior chamber of eye in mice[21] and autoimmune thyroiditis[22]. Orally administered antigen can induce peripheral immune tolerance by immune suppression or clonal anergy[23].

Self-tolerance of B-cells can be maintained by multiple mechanisms like clonal deletion, clonal anergy, receptor editing and maturation arrest, but tolerance breakdown in B cells can be accomplished by increased CD40 signaling, by defects in inhibitory co-receptors important for BCR signaling and by lack of Fas-induced survival of anergized self-reactive B cells[24].

Tolerance in autoimmunity can also be regulated by certain self-peptides derived from HLA molecules that potentially generate tolerance or autoimmunity depending on their binding affinity with HLA molecules. Strong data in mice models of

rheumatoid arthritis demonstrate that protective HLA alleles have high affinity binding peptide that can negatively select autoreactive T-cells expressing susceptibility allele, whereas low-affinity peptide from other alleles would positively select potential autoreactive T cells[25].

The immune "self" recognized by the immune system can be defined by everything encoded by the genome or any tissue or set of peptides in complex with HLA molecules accessible to lymphocytes during their maturation. Immune "non-self" can be defined by T-cell recognition of danger, i.e. special foreign antigens carrying "foreign-ness" marker[17]. Certain tissues, e.g., the eyes, brain, and testes, are traditionally considered immune-privileged because they are less accessible to the immune system and thus antigens would be rarely encountered during central and peripheral tolerance establishment. Following organ barrier damage these antigens might be more susceptible to autoimmune attack. Other factors like TGF-β, which assists the immune system to switch from Th1 to Th2 cytokine production, can also be of importance in this part of the body for T tolerance induction.

HLA molecules, genes, nomenclature and polymorphisms

The HLA region contains a variety of genes, many of them of importance for the immune response, and is located on chromosome 6p21.3 (Figure 1). Originally, it was identified and named for its role in transplantation rejection.

HLA class I molecules are peptide receptors contain a heavy α-chain with three extracellular domains linked to a β2-microglobulin. The function of these molecules is to capture intracellular peptides, transport them to the cell surface and display them in order to allow recognition by T cells[26]. These molecules are expressed on most nuclear cells of the body. Peptides presented by HLA class I molecules are mainly of endogenous origin, 8-12 amino acids in length and are located in the endoplasmatic reticulum. Such endogenous peptide presentation by HLA class I molecules helps the immune system to find infected cells, which thereby can be eliminated by cytotoxic CD8 T-cells. Molecules can also interact with natural killer cells (NK) to prevent NK-mediated cell lysis[27].

The HLA class II molecules consist of one α- chain with two extracellular domains and one β-chain with two extracellular domains. They have the ability to present slightly longer peptides in extended conformation, passing beyond antigen binding sites[28]. HLA class II peptides are loaded in class II peptide loading compartments and are mainly of extracellular origin[29,30]. They are expressed on professional antigen presenting cells (dendritic cells, macrophages, B cells) which are able to provide co-stimulatory signals for T-cell activation. Peptides presented by HLA class II molecules are recognized by CD4 positive T-cells. Some peptides from cytosolic proteins can be loaded in class II molecules and its been shown that the same HLA class II molecule can be used several times to present peptides[31].

The HLA class I region is 1800 kb long, and the genes coding for α-chain domains, the transmembrane region, the cytoplasmatic tail and 3' untranslated region consist of eight exons corresponding to the signal sequence. The majority of polymorphisms are located in the second and third exons, responsible for peptide binding and interaction with T-cell receptor. The gene for β_2-microglobulin is located on chromosome 15, and consists of four exons.

The HLA class II region is 800 kb long and is located centromeric of the class I region. Both the α- and β-chains have similar structures with six exons corresponding to signal sequence. The most polymorphic is the second exon, which encodes the portions responsible for peptide binding and T-cell receptor interaction[32].

HLA nomenclature is complicated because different typing techniques have their own nomenclature. Cellular assays are based on mixed lymphocyte reactions, comparing cells carrying different HLA haplotypes[33]. T-cells from HLA compatible individuals will not react, but cells from incompatible individuals will react with each others, thus defining allotypes. The resulting specificities make up the Dw-series (Dw1-Dw26) and formed the basis for the first functional test to identify HLA molecules.

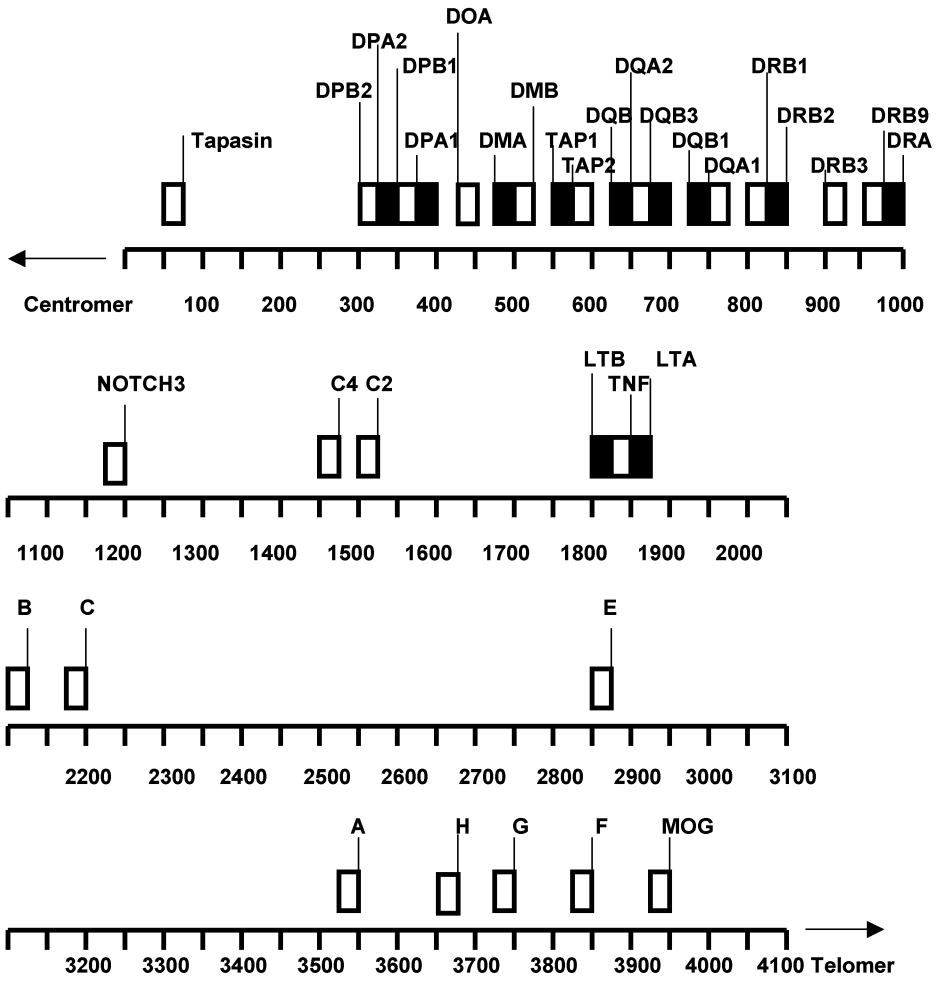
Serological typing uses antibodies. Using this method it is possible to detect more variability in the HLA region regarding separate –DR and DQ molecules. However, this method is limited, since antibodies are not able to detect all of the variability in the peptide binding groove.

Genomic typing techniques include the early restriction fragment length polymorphism (RFLP)[34] and later PCR methods. Utilizing PCR-based typing, it is possible to assign each unique HLA allele.

The first letters designating an allele is the locus separated by asterisk. The first two digits are named after the corresponding specificity defined by serology, and the last two digits give the particular allele.

A number of human diseases and their animal counterparts have shown to be associated with the HLA/MHC region. It's believed that HLA associations are explained by presentation of potential peptide autoantigens by HLA class I and class II molecules. This might lead to activation of specific T-cells and further activation of immune effector mechanisms which later lead to tissue damage[35]. Affected individuals carrying the susceptibility allele are two to three times more likely to develop disease than individuals who are negative for this allele. Thus, HLA association is not sufficient to cause disease, and only few diseases have almost complete HLA association[36,37,38,39,40]. Accordingly, penetrance of disease in individuals positive for associated allele is low. This may be explained by other genes and the importance of environmental factors. Because of strong linkage disequilibrium in the HLA region, its possible that disease predisposition is not explained by the HLA allele itself but by another gene[41] or several genes with modulatory role[42,43,44,45,46].

Figure 1 Structural organization of the human major histocompatibility complex adapted from Rhodes and Trowsdale[47].



CTLA-4 (signal one and signal two)

Two signals are required for the T-cell to proliferate and differentiate into an effector cell – an antigen dependent signal via TCR receptor (signal 1) and an antigen-independent signal provided by antigen presenting cells (signal 2). T-cells activated by a co-stimulatory signal clonally expand and initiate effector functions such as IL-2 production. Triggering TCR in absence of a second signal is not sufficient to activate T-cells. Several circumstances can influence the outcome of signal 1 – TCR has low affinity to its ligand, and MHC bind large numbers of peptides. Thus, specific ligands for TCR and MHC would be small, and the TCR receptor can be hidden by other larger molecules surrounding it[48]. T-cell activation is influenced also by competition between CD28 and CTLA-4 signaling pathways. In the absence of CD80 or CD86 expression on APC, T-cells would not became activated because there would be no ligands for CD28 positive signaling. Low-levels of CD80 and CD86 would also be unable to activate T-cells, because high affinity binding CTLA-4 molecule would mediate inhibitory signals. Also, antigens with low affinity for TCR would be unable to activate T-cells because decreased level of TCR signaling would be inhibited early by CTLA-4 signaling. Instead, high affinity antigen for TCR and/or high levels of CD80 or CD86 would promote T cell activation (See Figure 2).

The CTLA-4 gene is located on chromosome 2q33-q34 (Figure 3). This gene contains 4 exons coding for 186 amino acids. It is located close to and shares homology with the CD28[49]. The polymorphic $(AT)_n$ repeat was identified first[50] and contains at least 23 different alleles[51] with a heterozygosity rate of 0.93. The second polymorphism is a G to A transition located at position 49 in the leader peptide with a heterozygosity rate of 0.44. This results in an alanine or threonine amino acid substitution in codon 17. The third polymorphism is a transition of C to T located at position 318 in the promoter sequence[52] with heterozygousity rate of 0.15. There is linkage disequilibrium between the G^{49} allele and 102 bp microsatellite allele, the A^{49} allele and 84 bp microsatellite allele, and the T^{-318} allele and A^{49} alleles.

Murine and human CTLA-4 share 76% amino acid identity, but the cytoplasmatic domain is 100% conserved[49]. The CTLA-4 protein is expressed at low density but is up-regulated after cell stimulation, peaking after 48 hours and returning to background levels by 96 hours[53,54] and mainly expressed to CD4+ and CD8+ activated T-lymphocytes[53].

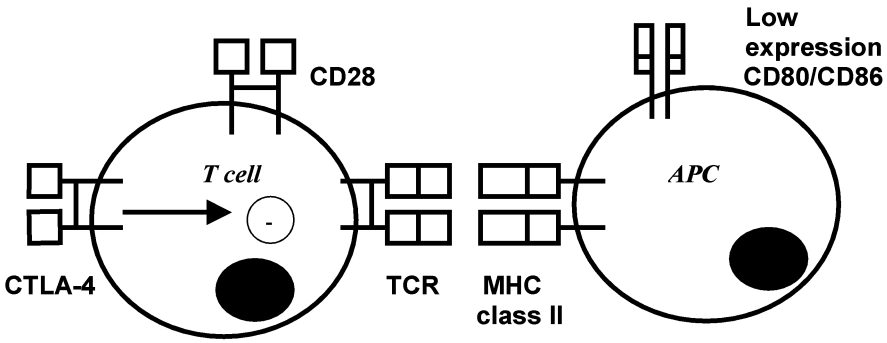
Ligation of CTLA-4 inhibits production of IL-2[55], while blockade of CTLA-4 increases production of IL-2, IFN-γ, IL-3, and TNF-α in Th_1 cell clones, and of IL-3, IL-4, IL-5 and IL-10 in TH_2 cell clones[56]. Also, its has been shown that signaling via CTLA-4 can modulate production of TGF-β[57], and suppress production of TNF-α and IL-1β[58]. CTLA-4 knockout T cells can produce TGF-β and IL-10[59] thus supporting the existence of alternative down-regulatory immune pathways.

mRNA expression can be detected without T-cell stimulation but cell surface dectection requires stimulation. It has also been demonstrated that CTLA-4 can be expressed on B-cells after activation with activated T-cells[60]. CTLA-4 is also localized in lysosomes and cycles between intracellular locations and the cell surface, where it is rapidly cleared[61]. The CTLA-4 molecule binds to CD80 and CD86 molecules on antigen-presenting cells with high avidity[62].

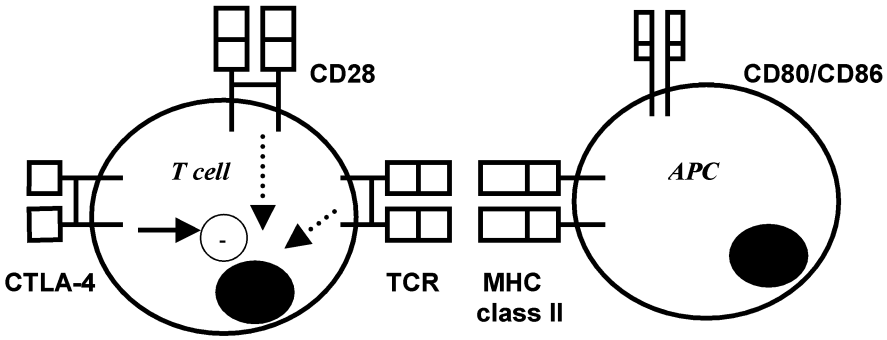
Recently, the alternatively spliced soluble CTLA-4 molecule has been detected in resting B-cells and T-cells of both subtypes. The soluble form lacks the transmembrane domain, but contains the binding capability for B7 molecules, and is more expressed by resting cells, in contrast to what is observed for the transmembrane form of the moleule[63,64]. Several possibilities arise as to how soluble molecule might regulate T cell responses – early binding of soluble CTLA-4 molecule to CD80/CD86 molecules block CD28 binding resulting in inhibition of T cell activation but late binding of soluble CTLA-4 molecule to CD80/CD86 molecules block CTLA-4 binding resulting in exacerbation of T cell activation.

The CTLA-4 gene has recently emerged as an important immunogenetic factor in a number of supposedly autoimmune disorders[65]. A number of studies have indicated linkage or association, although some findings are contradictory and still need further clarification. Most convincing evidence for an importance of the CTLA-4 gene has been demonstrated in Graves disease and Type I diabetes (IDDM). The first observation was that of an association in Graves disease the exon 1 microsatellite marker[66]. Subsequently, both linkage and association with exon 1 polymorphism was found in several populations[67,68]. In IDDM, linkage was first demonstrated in an analysis of the microsatellite marker in 48 Italian families[69]. Increased transmission of certain allele to IDDM was reported in Mediterranean populations[70]. In large UK and US studies, increased transmission of a supposed susceptibility allele was found, but not by identity by descent analysis failed to confirm this[71]. In the second generation genomic screen in IDDM, linkage was found in 2q31-q34 region but not with particular marker for which linkage was demonstrated previously[72]. Association studies of the promoter –318 C/T polmorphism are summarized in Table 1. In summary, although final consensus has not been reached, several indications suggest that the CTLA-4 gene is of general importance in autoimmunity. When this project was initiated, the CTLA-4 gene had not previously been studied in MS, nor was the functional importance of the CTLA-4 gene polymorphism known.

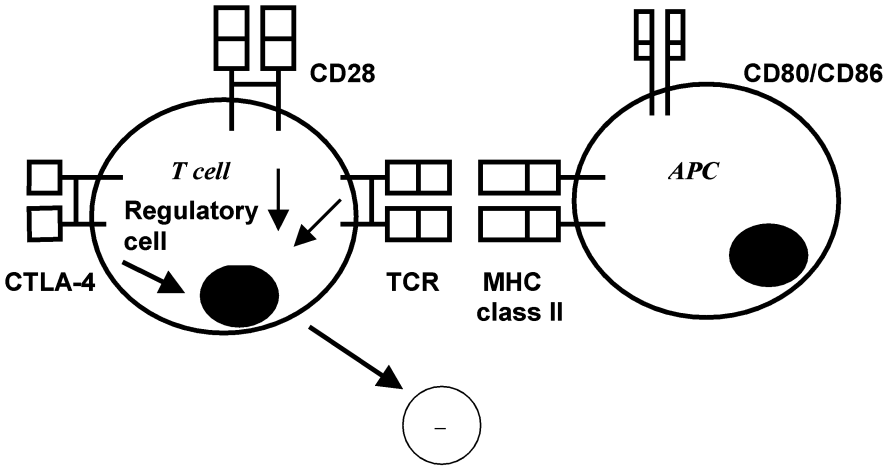
Figure 2. Model for role of CTLA-4 in maintenance of peripheral tolerance.



A. Low affinity antigen bind to TCR, low expression of CD80/CD86 molecule, CTLA-4 is expressed in levels able to complete with CD28 for ligants.

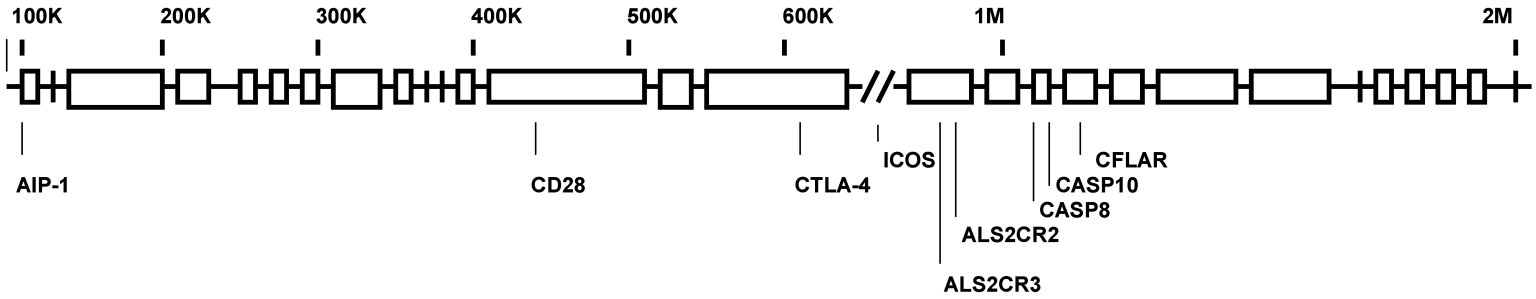


B. Under optimal costimulation, CTLA-4 expression is limiting. Surface expression of CTLA-4 later increase, competing with CD28 to disrupt TCR mediating signaling.



C. CTLA-4 stimulate regulatory cells to suppress other T cells[73].

Figure 3. Genomic organization of the CTLA-4 2q33-q34 region. AIP-1 – SH_2/SH_3 adaptor protein, ICOS – inducible co-stimulator, ALS2CR3 – amyotropic lateral sclerosis 2 chromosome region candidate 3, ALS2CR2 – amyotrophic lateral sclerosis 2 chromosomal region candidate 2, CASP8 – caspase 8, apoptosis-related cysteine protease, CASP10 – caspase 10, apoptosis-related cysteine protease, CFLAR – CASP8 and FADD like apoptosis regulator, K – kilo 10^3, M – mega 10^6



Autoimmunity and Multiple sclerosis

Autoimmunity

Autoimmune disease is initiated when the adaptive immune response reacts against self antigens. In this situation, the immune response is not able to eliminate antigen completely and response is therefore maintained. An adaptive immune response is initiated by activation of antigen-specific T-cells, and thus it is possible that autoimmunity might be initiated in the same way.

The human immune system is programmed not to react against self molecules but a certain degree of self-reactivity is present even after selection processes in ontogeny[74]. The factors initiating autoimmunity are unknown, but some individuals seem to be more at risk than others to develop an autoimmune disease possibly as a result of interactions of gene products with environment factors[75].

Multiple sclerosis

Multiple sclerosis is an inflammatory disease of central nervous system. It is characterized by loss of myelin, gliosis, axonal injury and neuronal dysfunction. MS pathogenesis can be grouped in different types or stages ranging from acute inflammation to neurodegeneration[76,77].

In northern Europe, this disease has a prevalence of about one in 1000 and an annual incidence of four in 100,000[78]. MS is uncommon in Japan (about two in 100,000) and other nations in Asia, in sub-Saharan Africa, the Indian sub-continent, in the native populations of Oceania and North America[79]. It affects people usually between the ages of 20 to 50 and more commonly affects females more than males. Prevalence is increased towards both poles, being lowest at the equator, and may differ between ethnic groups living in same area.

The clinical course of MS is variable, characterized by episodic worsening periods and, commonly later in the disease, progressive deterioration of neurologic functions[80]. Neurological signs in MS can be divided into several groups. Most MS patients do not display all the symptoms, and the most serious symptoms appear at later stages of the disease. Numerous attempts have been made to subdivide MS according to its clinical course. In the present work, we recognize the subdivision of two different disease courses in MS: relapsing/remitting (R/R) and primary chronic progressive (PCP) based on the early development of symptoms. The factors which determine disease course and biological markers defining clinical courses of MS are poorly known. Magnetic resonance imaging (MRI) of the brain and detection of oligoclonal bands in spinal fluid help to confirm diagnosis of the disease.

Immunosuppressive MS therapy in the form of corticosteroids is useful for shortening exacerbation but does not affect disease outcome. Established immuno-modulatory treatment include interferon-β (IFN- β1a and IFN- β1b) and glatiramer acetate (Copaxone). IFN-β has numerous effects including modulation of T-cell and macrophage function, as well as cellular traffic to the CNS and decreased expression of HLA molecules. Copaxone is thought to compete with myelin proteins for HLA class II binding on APC. Both agents affect relapse rate and relapse severity. Other tested immunosuppressive treatments include azathioprine, cyclophosphamide,

metotrexate and cyclosporine. However, results have generally been negative, with the possible exception of mitoxantrone, now licensed for MS in the US. Experimental strategies are based on blocking T-cell receptor and co-receptors, blocking and administration of cytokines, blocking adhesion molecule interactions and vaccination with altered myelin-reactive T-cells.

Genetic factors and MS

Genetic influence is indicated by an increased recurrence risk in family members of affected individuals[81]. First degree relatives of MS patients have a twenty times greater risk of developing MS than the general population. Modest concordance rates between monozygotic twins (about 30%) compared to smaller rates in dizygotic twins (about 4%) indicate a complex genetic etiology[82,83]. Rearrangement of genes coding for T-cell receptors and immunoglobulins, as well as somatic mutations, post transcriptional regulatory mechanisms and incorporation of retroviral sequences are not reflected in twin concordance rates but might also influence MS risk, as well as environmental factors and random chance.

The importance of genetic factors is also supported by a certain tendency of MS sibling pairs to cluster by age of onset, rather than year of onset[84], and by the increased ratio of lifetime risk of siblings of affected individuals versus population prevalence of the disease (λ_s). The high incidence of MS in populations of Northern European origin independently of geographic location[85], and the observation of resistant ethnic groups as Gypsies in Bulgaria[86], Japanese in United States[87], and Lapps in Norway[88] suggests that MS risk in different populations may well result primarily from genetic factors.

A simple model of inheritance for MS is unlikely. Thus, recurrence risk from multiplex families together with twin data suggests that MS results from multiple independent or interacting polymorphic genes. It is also likely that genetic heterogeneity exists where particular genes confer susceptibility in one family but not in other.

Immunopathogenesis of MS

MS is characterized by inflammatory infiltrates in CNS, leading to demyelination and axonal loss[89]. The loss of myelin results in neurological symptoms. Although the general assumption is that autoimmunity is the cause of damage, it may alternatively be a consequence of a disease process of other origin (genetic, toxic or secondary to an infectious agent). In addition, both healthy individuals and affected MS patients have autoreactive myelin-specific T-cells, which indicates that the existence of such cells is not confined to pathological situation[90].

Activated T-cells, are capable of entering the CNS in both MS patients and healthy individuals. Therefore, it is generally assumed that T-cells in MS are activated before entering the CNS, where they then become reactivated and trigger a sequence of inflammatory events. The majority of specific cells are CD4+ cells, which are supposed to confer damage to oligodendrocytes through activation of macrophages and microglia that express high levels of HLA class II molecules, resulting in the secretion of toxic molecules and damage to myelin and oligodendrocytes. Moreover, macrophages and microglia can lyse oligodendrocytes through antibody-dependent cytotoxicity when suitable antibodies and complement components are present[91].

Mature oligodendrocytes express CD59 that protects them from complement destruction, whereas developing oligodendrocytes express less CD59 explaining reduced numbers of oligodendrocytes in MS inflamation[92].

Environmental factors in MS

Support for environmental factors comes from migration studies, where children of parents who migrated from high-risk areas to low-risk areas of MS have a lower lifetime risk than their parents[93]. Clusters of MS described in the Faeroe Islands can indicate a potential infectious agent, but a recent study has been questioned due to the lack of investigative neurologists[94]. A decrease in MS prevalence with decreasing latitude might be related to sunlight exposure that catalyzes production of active form of vitamin D, however available data are contradictory and further studies are required[95,96].

Several other factors been proposed to be risk factors for MS[97]. Viruses have long been thought to induce disease, especially herpes virus 6[98], but no firm association for MS with a specific virus exists. Intrathecal Ig's are found to be virus antigen-reactive and are found in high numbers in MS patients, but there is little poof of virus persistence in the CNS of MS patients. Support for a viral etiology in MS also comes from the theory of molecular mimicry, where viruses or bacteria share peptide sequences with self peptides, resulting in crossreactive T-cell responses to self antigens. However, at present there is no clear evidence for participation of these factors in MS pathogenesis.

Another possible explanation for reduced concordance in monozygotic twins may be the necessity of stochastic events ("bad luck") for a predisposed individual to develop MS.

Studies arguing against environmental factors come from half-sibling data[99] and adoption data[100], where concordance in half-siblings and adopted siblings is not higher than prevalence in the general population.

HLA association and MS

The HLA chromosomal region has been consistently reported to be associated with MS and was first reported thirty years ago. Early studies described associations with A3 and B7 alleles[101], but later with HLA class II specific Dw2 (LD7a) haplotype[102]. The Dw2 association, or its corresponding serologic specificity, the haplotype consisting of DR15, DQ6, has been repeatedly found to be associated with MS in Caucasian population. Sequence identity between MS patients and controls shows that it is the normal Dw2 haplotype that increases the risk for MS[103]. In Europeans, only 60% of patients carry Dw2. MS susceptibility for the rest of patients might thus hypothetically be influenced by additional alleles and haplotypes. One possible additional class II association is with the DR17, DQ2 haplotype since a slight increase of this haplotype has been observed in several studies[104,105,106]. An exception for this Dw2 association is Sardinia, where disease prevalence is high but Dw2 is rare, though significant association with the DRB1*0301 allele and a lesser association with DRB1*0405 allele have been demonstrated[107].

It is not clear which part of the Dw2 haplotype accounts for the increased susceptibility to MS. When DRB1*1501 is removed from Dw2 haplotype, studies refute[108] or support[109] a primary role for DQA*0102; DQB1*0602 heterodimer. It was recently demonstrated in Afro-Brazilian MS patients that DQB*0602 segregates independently from DRB1*1501 and may have a primary susceptibility effect for MS[110]. In Japanese MS patients, two clinical forms of MS have shown different HLA associations - Western type MS was associated with DRB1*1501;DRB5*0101 haplotype, but not Asian type MS[111] which is associated with HLA-DPA*0501[112].

A few studies demonstrate linkage between HLA genes and MS either in multiplex families or in affected sibling pairs. Full genomic screening studies support linkage with HLA region, though not fulfilling stringent criteria[113,114,115], but at the same time no other major susceptibility were identified. Still, the particular role of HLA genes in MS is unclear, i.e., whether this association is due to the function of HLA molecules themselves or to other genes in this region.

Aims of the Study

The aims of the present investigation were to explore genes of potential relevance for MS and its treatment. The major emphasis was placed on HLA class I and class II genes and the co-stimulatory molecule CTLA-4.

Specific aims:

1. To investigate the possible role of the CTLA-4 gene in multiple sclerosis in sporadic MS patients and multiplex MS families.

2. To determine whether three CTLA-4 polymorphisms modulate protein and mRNA levels in MS patients, MG patients and healthy controls.

3. To investigate additional HLA class I and class II genes other than HLA-DRB, –DQA1 and –DQB1 for the possible genetic influence to MS.

4. To characterize the role of HLA-DRB1 alleles on MS disease course and outcome.

5. To characterize the influence of DRB*1 alleles in a large number of MS families, to investigate the role of the HLA gene region in absence of associate allele.

Methods

MS patients

Both sporadic cases of MS and families with multiple cases of MS were investigated. In paper I, all 378 sporadic MS patients showed presence of oligoclonal bands and/or an increased IgG index. In addition, 46 multiplex MS families and 55 singleton MS families (an MS patient with two parents) and four families with two affected offspring's were included. In paper II, of 38 patients, 15 were untreated, 21 were under treatment with interferon-β, one patient received Copaxone and one had recently received steroid treatment. In paper III, MS patients were divided into two groups, where the first group of patients were typed by high resolution protocol and new findings were corroborated in a second group of MS patients and in 62 Norwegian MS patients. In paper IV, 948 MS patients with definite, probable or possible MS were included. Four hundred-and-forty-one MS families containing 1,978 individuals were included in study V.

All MS patients fulfilled Poser criteria[116]. Sporadic MS cases and MS families were identified at the MS Center at Huddinge University Hospital except in paper IV where also patients from the neurological clinic at the Karolinska Hospital were included. All MS families in paper V were identified through the Canadian MS registry.

Controls

One hundred-and-sixty-two randomly selected blood donors and 108 organ donors were used as controls in Studies I and III. To corroborate findings, 101 Norwegian healthy controls were used in Study III. In Study II, peripheral blood mononuclear cells and DNA extraction was prepared from the blood of healthy medical students and hospital employees (n=10) or from blood donors (n=24). In Study IV, 202 blood donors, 103 organ donors, 100 employees at departments of biosciences and clinical immunology of Huddinge Hospital and 50 unrelated relatives of renal transplant candidates were used as controls. In Study V, control allele frequencies were estimated from 322 parental alleles not transmitted to affected individuals. All controls for the first four studies were of similar (Nordic) ethnical background. All individuals in study V were Caucasians.

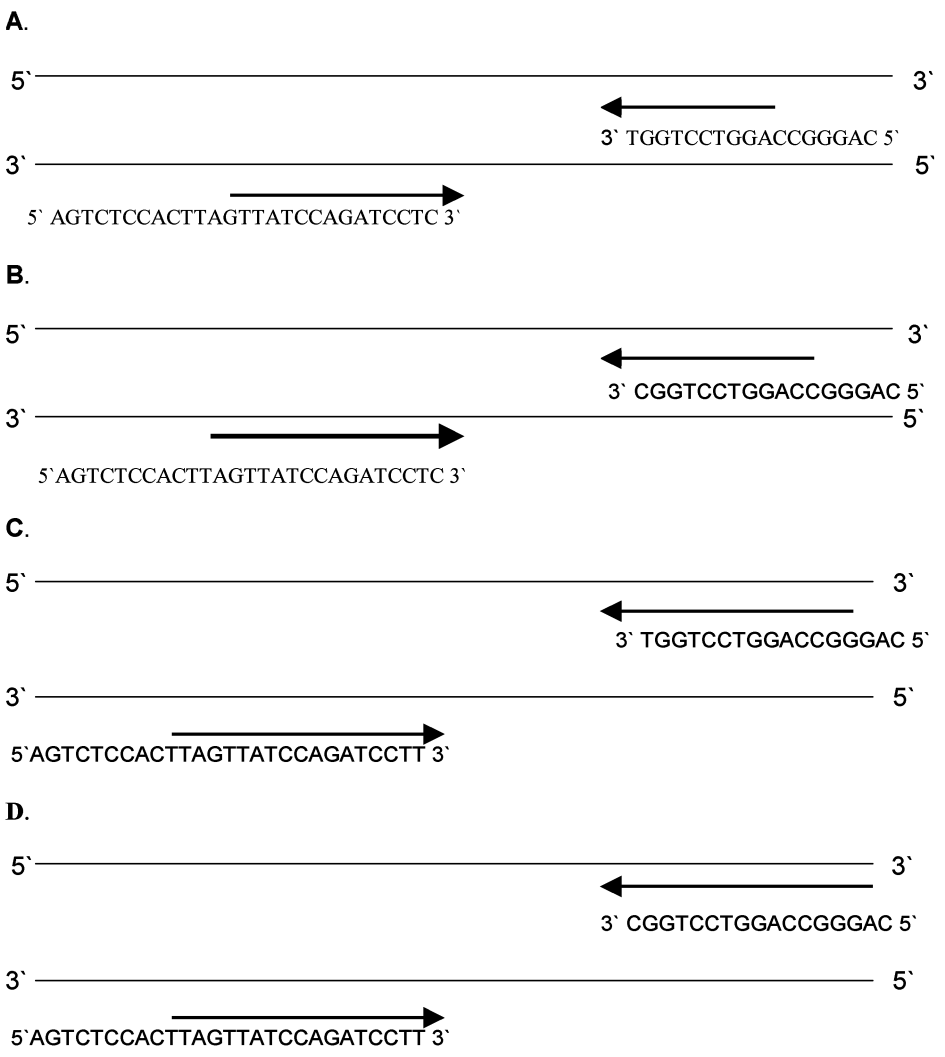
PCR amplification with sequence-specific primers

The polymerase chain reaction[117] (PCR) method was used to amplify a segment of DNA between known sequence. The requirements for PCR are a DNA template, a DNA polymerase enzyme (*Thermus aquaticus*), four nucleotides (dNTPs), primers complementary to the starting point of the template, and variation in temperature to separate, extend and re-anneal the DNA strands. Primers are complementary to sequences that lie on opposite strands of the template DNA and flank the amplified segment of DNA.

PCR amplification with sequence-specific primers allows efficient amplification with completely matched primers (Figure 4). When the difference between two alleles is only one nucleotide, a primer can be modified to be more specific by making it longer or by introducing additional mismatches a few nucleotides from the 3`-end. Sequence polymorphisms are detected in the cis position, uninfluenced by what

allele the other chromosome carries. An internal positive control by primers specific for a non-allelic gene (e.g. human growth hormone) ensures that no specific HLA allele will be missed due to failure of amplification.

Figure 4. Illustration of principle for allele-specific PCR amplification for CTLA-4 polymorphisms used in study I and II. Completely matched primers were used for amplification A. $C^{-318} – A^{49}$ allele, B. $C^{-318} – G^{49}$ allele, C. T^{-318}-A^{49} allele, D. T^{-318}-G^{49} allele of the gene.



Nested PCR

To allow typing from small amounts of genomic DNA, a nested PCR procedure was performed in study V whereby an outer primer pair amplified the second exon of the DRB1 and DRB5 genes and then samples were run a second round of PCR amplifications by sequence-specific primers. One potential complication of a nested amplification strategy is possible contamination between samples. However, in no instance did we observe evidence for such contamination, for instance, as the presence of three alleles.

Sequencing

DNA sequencing in Study I was used to identify new polymorphisms. Sequencing was performed by dye terminator sequencing, using an enzymatic procedure to synthesize DNA chains of varying lengths, stopping DNA replication at one of the four bases and then determining the resulting fragment lengths. Dideoxynucleotide triphosphate terminates the growing DNA chain where deoxynucleotide triphosphates are incorporated. Fragments of varying length were separated by electrophoresis and the positions of the nucleotides analyzed to determine sequence. Sequencing was performed using Applied BioSystem 377 DNA equipment.

mRNA analysis

In Study II, total RNA was isolated from 5 million cells by using guanidine thiocyanate and phenolchloroform extraction. cDNA was synthesized using oligo-d$(T)_{16}$ as primer according to the instructions of the manufacturers of the reagents (Perkin-Elmer). cDNA was quantified by a competitive PCR method described elsewher[118]. In this assay, the sample cDNA is amplified in completion with an artificially prepared internal standard DNA which is in itself a amplificate of a genomic DNA sequence to which a primer motif is added[119]. The difference between internal standard and template in competitive PCR was designed to be less than 15%. The forward primer for the competitive PCR was fluorescently labeled. The competitive PCR reaction system contained two µl internal standard with a suitable concentration. The PCR-amplified product was mixed with formamide, loading buffer and size standard.

Denatured amplificates were loaded onto polyacrylamide gel, separated by electrophoresis and fluorescence assessed on a ABI 377 sequencing system. Results were further analyzed by Genotyper 1.1 software to assess the peak areas of target gene cDNA and internal standard. By co-amplification of different amount of target cDNA with a fixed amount of internal standard, a linear standard curve was obtained. Tested samples were co-amplified with same amount of internal standard and their content was calculated based on the peak area ratio between target cDNA and internal standard by the linear regression formula from the standard curve calculated previously.

To compensate for relative differences between sample preparation and for variations in reverse transcription, RNA sample β-actin cDNA was amplified and quantified for test sample. The estimated amount of target cDNA/mRNA was divided by the amount of β-actin in the sample. Standard curves showed high correlation coefficients, typically $r>0.97$.

Flow cytometry

In Study II, all monoclonal antibodies were titrated to optimal concentrations. All cells used in the study were stimulated with Con-A for 72 hrs. Direct immunostaining with flourochrome-conjugated mAbs of CD3, CD4, CD8 and CTLA-4 in blood was examined. To confirm staining specificity, a blocking experiment with unlabeled anti-CTLA-4 mAb was performed. The percent values presented were the fractions of cell populations that stained positively for the specific antigen.

Data Analysis

Linkage analysis: In study I we used several methods to look for genetic linkage. We performed parametric two-point linkage analysis with MLINK and FASTLINK of the linkage package. In comparison to non-parametric methods, these methods permitted us use marker information from all individuals and to include large and complex pedigrees in the analysis. These methods have a disadvantage: genetic model parameters must be specified and misspecification will generate misleading results. Thus, we also performed various non-parametric analysis. The non-parametric linkage analysis (NPL) of GeneHunter is based on an identical-by-descent (IBD) test. The affected pedigree member (APM) analysis is based on an identity-by-state (IBS) test. The IBD and IBS sib-pair analysis methods are very sensitive, but has a drawback: the inability to use additional information from distantly related individuals. Finally, we also applied a transmission disequilibrium test (TDT) which measures the increased frequency of a particular disease-marker haplotype over that expected based on the Hardy-Weinberg equilibrium[120], and the ETDT (extended transmission disequilibrium) analysis, which examined the possibility of significance in overall allele transmission deviation to patients. In study V linkage nonparametric analysis was made by ASPEX program, allowing us to test for TDT, IBD sharing and IBS sharing. This program reconstructed missing parental genotype from unaffected offsprings.

In studies I, III and IV we used association analysis for categorical variables with Fisher's exact test for independence in contingency tables. In study II we used nonparametric rank randomization Mann-Whitney test where no assumptions about specific probability of data distribution were made. In study II, tests of mean differences in one- and two-way analysis of variance (ANOVA) were calculated from square-root values. In studies II and IV, t-tests were calculated for continuous variables and corrected for multiple comparisons, where the probability (p) value was multiplied by the number of comparisons done in the study minus one. In study IV, to avoid misleading deviations in association analysis, the relative predispositional effect (RPE) analysis was used[121].

To distinguish primary and secondary HLA associations in study III, we used the method described by Svejgaard and Ryder[122]. These statistics identify the strongest HLA association and are based on a two-by-four table, giving four phenotypic combinations of factors A and B in patients and controls and later these combinations are analyzed in various two-by-two tables involving each of the two factors against the other. Through comparison with control data, this method allows us to identify how linkage disequilibrium may influence secondary associations and also investigates the interaction between the HLA factors.

In study IV, survival to different end-points was computed by life table analysis. The comparison between two or more survival curves was performed with a log-rank test. This test compares whether the relationships between the independent variables and survival are identical in different groups based on regression analysis.

In study V, correlation analysis for transmission for a particular allele between two groups of MS families was performed. In this study, expected allele sharing was calculated by the method described previously by N. Risch[123], where odds ratios were taken from our study, allele frequencies were taken from the Canadian population and a multiplicative disease model was assumed.

Results and Discussion

CTLA-4 gene and multiple sclerosis (paper I)

Autoimmune diseases have certain features in common. Studies indicate that peripheral mechanisms of T-cell tolerance are essential to control self-reactive T-cells, and elimination of peripheral mechanisms of self-tolerance may therefore result in the development of autoimmune disease[124,125,126]. In MS its has been shown that inflammatory infiltrate in plaque contains only a few auto reactive T-cells but a multitude of non-antigen-specific lymphocytes[89].

It has been shown that CTLA-4 manipulation *in vivo* has important effects on T-cell responses in experimental autoimmune encephalomyelitis, often referred to as the autoimmune model for multiple sclerosis[127,128,129]. Our interest and approach was supported by association and linkage results reported in Type I diabetes[130,131] as well as association results with thyroid diseases[66,68]. In this study, we wanted to determine whether susceptibility to MS can be influenced by the three previously known CTLA-4 gene polymorphisms.

In addition to MS patients and controls, 27 singleton MS families and four multiplex families were genotyped for promoter and exon 1 polymorphisms, and 46 multiplex MS families and 28 singleton MS families were genotyped for the microsatellite markers. MS patients were not stratified according to clinical course of disease at this stage. We found that homozygosity for the G^{49} allele was increased in MS patients ($p=0.047$). The G^{49} allele was transmitted more often than non-transmitted from heterozygous parents to affected offspring ($p=0.02$). Frequency for the promoter T^{-318} allele was reduced among MS patients. Stratification for HLA-Dw2 revealed no significant differences, indicating that two associations are independent of each other.

Significant linkage disequilibrium was observed between the 102 bp microsatellite allele and the exon 1 G^{49} allele, and between the 84 bp allele and exon 1 A^{49} allele. No chromosome was found to carrying T^{-318} allele together with the G^{49} allele indicating that the mutation in position 49 occurred earlier than the mutation in position 318.

This study supports previous findings that the region on chromosome 2q within or close to CTLA-4 gene influences genetic susceptibility to MS (Table 1), that the G^{49} allele is a risk allele in MS, that the T^{-318} allele may have a protective effect for MS. In parallel to our study, a report from Norway demonstrated that MS patients and group of relapsing/remitting MS patients had higher frequency of A^{49}/G^{49} genotype in the first exon 1[132], supporting a role for the G allele in disease susceptibility. However, a report from Japan[133] demonstrated a similar distribution of CTLA-4 genotypes between MS patients and controls, whereas certain differences were indeed found after subgrouping of patients according to the clinical profile.

We concluded from these results that the CTLA-4 gene influences genetic susceptibility to MS, that the G^{49} allele is a risk allele in MS, or that the T^{-318} allele may have protective effect for MS. Furthermore, we hypothesize from these findings that a CTLA-4-driven down-regulation of T-cell activation can be deregulated by one

of the studied gene polymorphisms and influence the risk of in MS in an non-antigen specific manner.

Table 1. Linkage results of in published genome screens for markers close to the chromosomal region 2q 33-34 which harbors the CTLA-4 gene.

Country (ref)	Numbers of families	Sibling pairs	Closest Marker	Lod score	Distance from CTLA-4
Canada[113]	61	100	D2S155	MLS 0.69	7.3 cM
USA[114]	52	81	-	-	-
UK[115]	129	143	D2S126	MLS 0.5	28.1cM
Finland[134]	21	-	D2S1384	LS 0.8	3.9 cM

CTLA-4 polymorphisms and gene expression (paper II)

Possibly due to the low density expression of the CTLA-4 protein on non-stimulated cells, little attention has been given to functional involvement of CTLA-4 molecule in human diseases[135]. It has been shown in mice that inducible expression of CTLA-4 is controlled by the promoter region including position –318[136]. Results from Alegre et al[56] suggested that different cell-surface expression of CTLA-4 is partly regulated by mRNA abundance. However, a soluble form of the molecule has been identified[63,64], generated by alternative splicing of the primary transcript, and this is likely to complicate the analysis of mRNA expression data of CTLA-4. Due to constitutive expression, the soluble form might partly regulate immune homeostasis by blocking B7 receptors during the different stages of the immune response. In MS, CTLA-4 cell surface protein expression was shown not to be influenced by INF-β treatment or disease status[137].

We were interested in clarifying the role of CTLA-4 in MS by investigating the importance of CTLA-4 gene polymorphisms for mRNA and protein expression levels. Therefore, patients typed for CTLA-4 gene polymorphisms had their peripheral blood mononuclear cells analyzed for gene expression by flow cytometry or mRNA analysis.

We observed that cell-surface expression of the CTLA-4 protein was affected by, or at least associated with, both promoter T^{-318} and exon 1 A^{49} polymorphisms. Furthermore, mRNA levels were found to be higher in individuals carrying T^{-318}allele. Treatment or disease status was not shown to influence the CTLA-4 protein levels. Polymorphisms seemed to influence protein cell-surface expression both in unsorted PBMCs, in unsorted $CD3^+$ cells and in the $CD4^+$ T-cell subset but not in the $CD8^+$ T-cell subset.

The results indicate that CTLA-4 genotypes influence mRNA and protein expression regardless of the presence of an autoimmune disease. The similar pattern in MS and controls indicates that CTLA-4 expression in MS was not altered by treatment.

Furthermore, the results elucidate the intracellular and cell surface expression pattern of this molecule Its has been showed that the molecule`s cytoplasmic tail contains a specific motif which can regulate intracellular CTLA-4 protein levels through its interaction with clatrin adaptor complex AP-1 and AP-2[138](Figure 5).

Moreover, there is a possibility that different CTLA-4 genotypes may be associated with different clinical forms of the same disease entity, i.e. that CTLA-4 genotype may influence clinical characteristics. Recently, it has been shown that cells carrying the A^{49}/A^{49} genotype exert a more profound inhibitory effect on cell proliferation than T-cells carrying G^{49}/G^{49} genotype [177]. Studies in autoimmune diseases for promoter region –318 are summarized in Table 2.

Previously, we found that T^{-318} frequency is lower among MS patients than among controls[paper I]. Thus, specific CTLA-4 genotypes may decrease the risk of MS, by increasing CTLA-4 expression.

In conclusion, we believe we have identified the CTLA-4 gene to be an important immunogenetic factor in MS, as has been similarly shown for several other "autoimmune" disease. In addition, since it seems likely that the polymorphisms studied are of importance in CTLA-4 expression, we propose a functional basis for the observed associations. Therefore, our findings strongly support the concept of the CTLA-4 gene as important in MS as well as in immunogenetics in general.

To more directly study the importance of the promoter polymorphism for expression, we extensively attempted to acquire expression data from transfection experiments using the two alternative sequences. However, for reasons as yet unclear, transfected sequences did not provide adequate information. Hence, our analysis had to rely on indirect rather than direct evidence.

Figure 5. Schematic representation of CTLA-4 (CD152) molecule adapted from Thompson and Allison[48]. (A) Variable region domain, (B) transmembrane domain, (C) cytoplasmic tail. Clathrin adaptor complexes AP-1,AP-2 binding sites (D) shown to be important for controlling intracellular accumulation of CTLA-4.

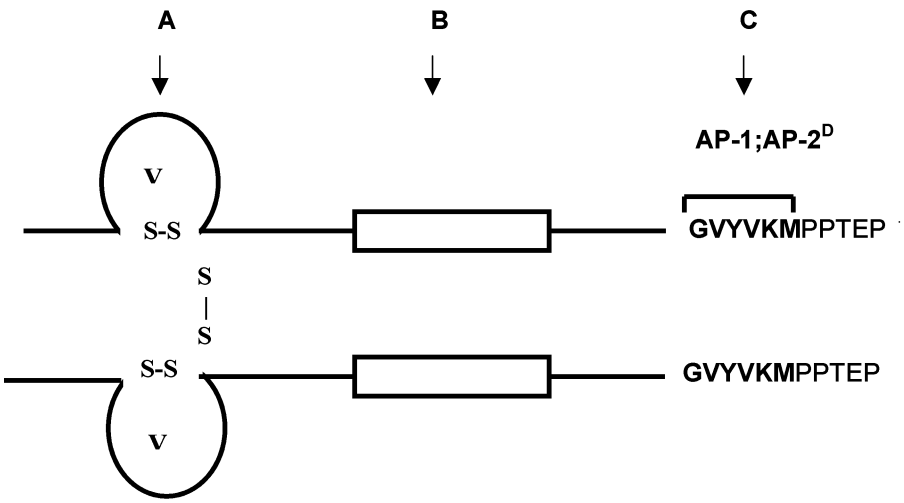


Table 2. Phenotypic frequencies (%) for T^{-318} allele in autoimmune diseases and controls of various ethnic backgrounds.

	N	In affecteds	N	In controls
Hashimoto`s thyroiditis (Germany)[139]	45	7(16%)	142	35(25%)
Hashimoto`s thyroiditis (Canada)[139]	19	3(17%)	31	11(36%)
Graves` disease (Germany)[139]	85	13(15%)	142	35(25%)
Graves` disease (Canada)[139]	40	5(13%)	31	11(36%)
Rheumatoid arthritis (Span)[140]	138	30(22%)	305	62(20%)
MS (Norway)[132]	296	47(16%)	227	41(18%)
MS (Sweden)[paper I]	378	57(15%)	237	54(23%)
Graves` disease (UK)*[141]	188	37(10%)	335	55(8%)
Hashimoto`s thyroiditis (UK)*[141]	90	19(11%)	335	55(8%)
SLE (UK)*[141]	124	18(7%)	335	55(8%)
Graves` disease (China)*[141]	98	16(8%)	82	28(17%)
SLE (Japan)[142]	113	18(16%)	200	43(22%)
Graves` disease (Koreans)[143]	97	14(14%)	199	53(27%)
Hashimoto`s thyroiditis (Koreans)[143]	110	20(16%)	199	53(27%)

*allele frequencies

HLA class I and class II genes and MS (paper III)

Several supposedly autoimmune diseases have been found to be HLA-associated. Association in MS with HLA class II was first described by Jersild et al[102] and later was confirmed to be associated with the HLA class II haplotype DRB1*15,DQB1*06[106]. Some exceptions have been found in Sardinian MS patients[144] and in the optico-spinal form of MS in Japan[112]. A potentially protective effect of MHC class I genes has been observed in experimental autoimmune encephalomyelitis (EAE)[145]. Inconsistencies in HLA association in MS might be explained by additional modifying genes within the HLA gene region.

In this study, we investigated the influence of HLA class I and class II alleles for genetic susceptibility to MS by a new, more accurate genomic typing technique[146]. We divided patients and controls in two groups, where high-resolution typing was performed in the second group for alleles which differed between patients and controls in first group, or have been previously associated with MS.

We found that HLA class II DRB1*15,DQB1*06 haplotype effect can be modified by presence of HLA class I alleles. Both increased (HLA-A*03) and decreased (A*02) frequencies of HLA-A alleles found in MS patients were independent of DRB1*15,DQB1*06 haplotype. In addition, HLA-B7 allele was shown to be associated with, but secondary to, DRB1*15,DQB1*06, being part of the DRB1*15-carrying haplotype. We found that the telomeric side of DRB1*15,DQB1*06 haplotype can be disease-promoting or protective, although the centromeric side was only disease promoting.

In addition, we found that the DPB1*0401 allele was carried significantly more often by patients with DRB1*1501, DQB1*0602, DPA1*0103 alleles than by controls with the same alleles. There was no association between MS patients and controls for DPB1*0401 allele. Association with DPB1*0401 has been reported previously[147], but not confirmed by others with high resolution HLA typing. Thus association might be considered as combined effect of the DPB1*0401 and the DRB1*1501 allele.

Based on our data, we propose several ways through which a disease promoting effect of DRB1*15,DQB1*06 may be reduced by a negatively associated HLA-A allele. Myelin-derived peptides may down-regulate T-cells restricted to an HLA-A allele. Alternatively, HLA class I molecules may delete potentially autoaggressive T-cells by being presented as peptides by DRB1*15,DQB1*06 haplotype-derived molecules. Another possibility would be competition between myelin-derived peptide and HLA-A-derived peptides to bind DRB1*15- or DQ*06-containing heterodaimers, thus decreasing the risk of MS. It is also possible that HLA-A molecules act as restriction elements for viruses that activate cross-reacting T-cell clones. In addition, the negative association of HLA-A*02 allele could be due to a protective allele of a gene encoded on the haplotype which is not carried on the HLA-A*03 haplotype. Recently, it has been shown that HLA haplotypes may extend 5-6 megabases telomeric of the HLA-A gene, thus increasing the possibility for non-HLA genes being of importance[148].

Taken together, our results might explain the low penetrance shown for the DRB1*15;DQB1*06 haplotype in MS and the difficulty in establishing genetic linkage.

HLA class II genes and lower age at onset in MS (paper IV)

The cause of MS is uncertain, though there is ample evidence that both genetic and environmental factors play a major role for susceptibility[81]. The MS susceptibility factor remains to be determined, and there is variability in clinical and pathological phenotypes. Sisters of MS index cases are at two times higher risk for MS than for brothers, and sibs who have a parent with early onset of MS have shown to be at higher risk for MS[149].

There is also no clear consensus that MS represents a homogeneous entity[150]. Clinical, pathological and MRI features have been reported differ between primary progressive, relapsing/remitting and secondary progressive MS. The age of onset in MS can span five decades[151] and disability can range from nonexistent (discovered at autopsy) to a disease fatal in a few months.

Previously, primary chronic progressive MS was shown to be associated with the DR4,DQ8 haplotype, while relapsing-remitting and secondary progressive MS forms were associated with DR17,DQ2[152]. Although a report partly confirmed these findings in a group of Norwegian patients[153], results have been inconsistent in later reports by other authors[154,155,156]. Since MS is a heterogeneous group of disorders, susceptibility may be difficult to identify by standard methods. Identification of subgroups of MS (by genetic or non-genetic factors) would later have implications for identification of other genetic and non-genetic factors implicated in disease outcome.

In this study, we investigated the influence of HLA-DRB1 alleles in MS by sub-grouping patients according to sex, ethnic background, family history of MS, diagnostic category, disease course, age at disease onset, results of cerebrospinal fluid analysis and magnetic resonance imaging, disability and disease duration, in a large, well-defined population-based sample.

We confirmed that the DR15 allele increases the disease risk of MS patients. The only additional susceptibility allele was DR17. No protective alleles were observed. No differences for HLA-DR allelic distribution were found between primary progressive or relapsing remitting clinical forms of disease, and no alleles were observed to affect disease severity. As expected, the DR15 allele frequency was found to be lower in groups of less certain diagnosis, probably signifying contamination of non-MS cases. However, mean age at onset for DR15-positive MS patients was significantly lower than for DR15 negative MS patients ($p=0.00012$).

The latter finding was supported by a report from Norway where it was observed that the frequency of DR15 among MS patients decreases with increasing age at diagnosis of disease[157]. The frequency of DR15 in that study was significantly higher among female than male patients, a difference not present in our material. Recently, a large data set from Northern Ireland[158] also confirmed that the DR15 allele is associated with early age of onset. Moreover, it also confirmed that the DR17 allele is associated with MS.

Our observations support the hypothesis that there are no definitive genetic disease subgroups in MS, since all such groups studied shared the same immunogenetic association with DR15. The effect on age-at-onset can be easily explained if

presence of the DR15 allele is regarded as evidence for genetic loading. It is possible that DR15-positive MS patients are more sensitive to a hypothetical environmental factor, whereas DR15-negative MS patients are more resistant and might need a longer exposure time to a single factor and/or exposure to multiple environmental factors. Alternatively, the DR15 allele might be important for shortening the time from onset of the pathogenic process until clinical symptoms of MS.

HLA-DRB1 alleles in multiple sclerosis families (paper V)

The analysis of HLA genes in relation with a disease is complicated by the presence of many highly polymorphic genes and strong linkage disequilibrium. In this study, we investigated the influence of the HLA gene region in a large, well-defined number of MS families. This gave us the opportunity to investigate the possible importance of all common DRB alleles by transmission analysis, for once avoiding the problem of ethnic stratification inherent to case-control studies. In addition, because of the low frequency of MS families lacking DRB1*15 allele, this study provided the opportunity to assess the influence of the HLA gene region in non-DRB*15-bearing MS families.

The importance of the HLA class II region in MS has not been elucidated. Overall, non-parametric sib-pair analyses have provided less evidence for linkage than parametric analyses of multiplex families (Table 3). These discrepancies may depend on study design or on relative importance of these genes in different populations (a marker for the population at risk for MS rater than a gene which influences susceptibility). Whereas a clear association with HLA –DRB1*15 allele has been consistently observed, the combined observations have been interpreted as indicating the legitimate but minor importance of HLA genes in MS[159,160]. Since the clinical course of index cases is often different from the clinical course of affected relatives[161,162], different clinical subtypes of MS seem to share genetic background.

We observed increased overall sharing of DRB1* alleles (in all families) and significantly increased allele-sharing in the group of DRB1*15-positive MS families as well as in DR15-negative MS families. Transmission analysis confirmed the importance of DRB1*15 (69% transmission rate) where lack of significant transmission distortion was observed to unaffected offspring. In the full data set, as in the DR15-negative families, we observed some additional alleles to be increased and others decreased in transmission to patients. One such additional association was with the DRB1*17 allele. A slight increase in frequency of this allele has been observed in several studies in MS[106, paper IV]. However, other deviating alleles had previously not been observed to have a role in MS, suggesting that the present observations were likely due to random variation.

Furthermore, after removal of the DRB1*15 allele from the overall data set, positive correlation for allele transmission was found between DRB1*15 positive and negative families. By calculating the expected allele sharing we found that the observed sharing in overall families cannot be explained only by DRB1*15 allele or other DRB1* alleles.

Taken together, we revealed a linkage with HLA-DRB1 alleles even in absence of the previously identified associated DRB1*15 allele. Our findings indicate that there may

be a second MS susceptibility locus within the HLA region, or primary, not identified (non-DRB1) susceptibility locus in disequilibrium with the DRB1*15-DQB1*06 haplptype.

Our results also support the hypothesis that MS is polygenetic disorder, since overall λ_s was suggested to be approximately 20[163], whereas in our study λ_s for HLA –DRB was 1.56 in DRB1*15-positive families and 1.78 in DRB1*15-negative families. In addition, our results support the assumption that DRB1*15 has low disease penetrance, thus making significant linkage data difficult to obtain.

Table 3. Results of linkage studies of HLA class II genes in multiple sclerosis.

Country (ref)	Number of families	Sib Pairs	DR15 positive families	Overall families	DR15 negative families	Type of study
UK[160]	109	115	negative	negative	-	sib-pairs
Scandinavia[164]	106	117	-	positive	-	sib-pairs
USA[165]	98	150	positive	positive	negative	families and sib-pairs
Sweden[166]	9	9	positive	positive	-	families
USA[167]	9	35	positive	positive	positive	families and sib-pairs
Sweden[168]	49	19	positive	positive	positive	families
Sardinia[169]	-	57	-	negative	-	sib-pairs
Canada[170]	36	40	-	negative	-	sib-pairs
Scotland[159]	14	11	-	negative	-	sib-pairs
Finland[171]	21	-	-	positive	positive*	families
Italy[172]	28	-	-	positive	positive*	families
Australia[173]	17	100	-	positive	-	sib-pairs

*with other than DR15 allele in overall dataset.

Concluding Discussion

We have added support to the hypothesis that MS is polygenetic disorder, where disease susceptibility depends on several genes with different magnitude of their individual effects.

We observed the influence of the CTLA-4 gene in sporadic MS cases and in MS families. This effect mapped to promoter and exon 1 polymorphisms: promoter T^{-318} allele showed negative association, while G^{49} allele showed positive association with disease, independent of previously observed HLA-Dw2 association.

The CTLA-4 gene alleles showed different influence to mRNA and protein expression, where T^{-318} allele and A^{49} allele-bearing individuals had higher molecular expression, but it is unknown whether this was primarily associated with the T^{-318} or A^{49} alleles due to strong linkage disequilibrium between alleles. In addition, it cannot be excluded that other polymorphisms exist in linkage disequilibrium with the studied polymorphisms, although we failed to observe such polymorphisms by sequencing covering promoter, exon 1 and part of exon 2. Our data suggest that CTLA-4 expression is modulated by promoter and exon 1 polymorphisms rather than by disease itself.

We hypothesize that T^{-318} and A^{49} alleles can be more commonly observed in relapsing/remitting disease where, under certain conditions, high CTLA-4 molecule expression would promote relapses by mediating recession of inflammation. We hypothesize that such phenomena are common in other autoimmune diseases, where increases in the number of accumulated activated T-cells are observed.

Our observations for HLA class I and class II associations in MS revealed a hitherto undiscovered complexity for disease susceptibility. We confirmed the previously demonstrated association with DR15 in MS, while at the same time demonstrated secondary HLA associations where additional HLA susceptibility alleles increase or decrease the risk of MS strongly enough to be able modulate DR15 association. It is unclear if susceptible or protective HLA alleles in MS are important by themselves or are simply markers for other disease-causing genes.

We observed that DR15 allele can be associated with early age at onset, but the particular mechanism is unclear. Possible mechanisms include a greater genetic susceptibility from potential disease-causing factors, and/or a shorter time from the start of the disease's pathogenetic processes until appearance of clinical symptoms. Moreover, the data demonstrated that the DR15 allele is over-represented in definitive MS and is therefore worthy of inclusion as an additional independent marker of MS to improve the diagnostic criteria for this disease, at least at the group level.

Observed linkage with the HLA-DRB1 gene, even in absence of the previously identified associated DRB1*15 allele, and increased allele sharing in HLA-DR15-negative families tend to support a second susceptibility gene in the same region, or a primary, not identified (non-DRB1) susceptibility factor in disequilibrium with the DRB1*15-DQB1*06 haplotype.

The observed negative association might be due to a protective allele encoded on the same haplotype but not observed on susceptibility haplotypes. This might explain a number of observations, namely, this disease's incomplete penetrance for the DR15 allele observed in families, the more pronounced additional HLA associations (in the Sardinian population), the low disease susceptibility in isolated populations (Laps, Gypsies,

Japanese) and the discrepancies observed in linkage studies by different frequencies and combinations of HLA alleles in haplotypes in these populations.

Support for this hypothesis comes from the observation that DNA from DR and DQ genes of the Dw2 haplotype[103] and flanking sequences[174] in MS patients and controls have identical sequences. This might also be supported by the fact that forty percent of healthy people who carry HLA Dw2 haplotype do not develop MS.

Susceptibility to HLA genes for MS might be modulated by other non-HLA, background genes. This has been observed in experimental autoimmune encephalomyelitis (EAE) in rats[175]. By changing MHC haplotype on constant non-MHC background and by changing non-MHC genes on a constant MHC haplotype background, these data demonstrate that MHC haplotypes are not strictly defined as disease permissive or resistant. They determine disease susceptibility, clinical course and pathology but MHC disease susceptibility is graded may be overcome by effects of non-MHC genes and micro environment.

Several polymorphic genes might influence disease susceptibility. In humans, gene variation has been observed among people of different ethnic backgrounds[176]. From sequencing 313 genes, roughly fourteen different haplotypes per gene were observed. It was suggested that haplotypes are more likely to determine phenotype than single nucleotide polymorphism (SNP`s) (26% of SNP`s were located in coding regions, from them 55% coded for amino acid change).

In addition, unknown micro-environmental factors might influence and modulate genetic susceptibility. Thus MS can be caused by several genes with relatively minor risk factors, where genes might have a normal DNA sequence but only their product interaction and exposure to micro environmental factors predicts disease outcome.

Summary of Findings:

1. CTLA-4 gene polymorphisms influence susceptibility to MS. The main susceptibility determining CTLA-4 polymorphisms are promoter T^{-318} allele having protective role and exon 1 G^{49} allele having a susceptibility-conferring role. This influence does not extend to microsatellite marker at exon 4. These associations are independent from the HLA-DR15.

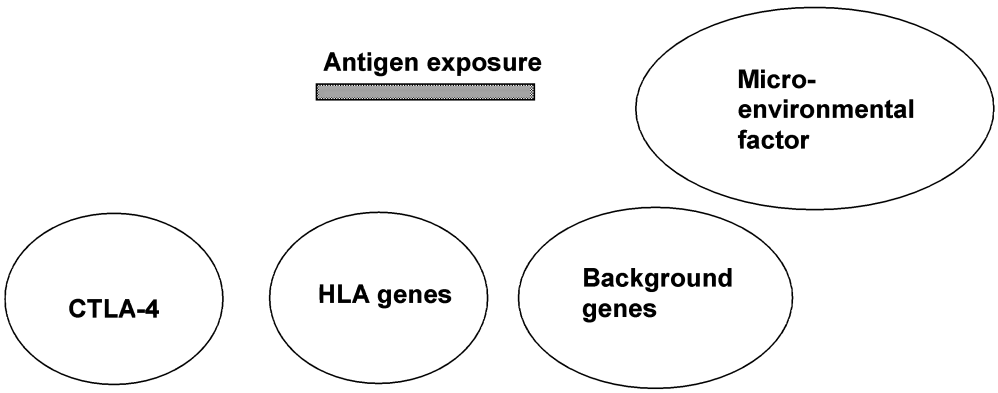
2. Cell surface expression of CTLA-4 protein is affected by promoter T^{-318} and A^{49} polymorphisms. The two alleles might act synergistically to determine CTLA-4 protein levels. The -318 polymorphism was shown to influence CTLA-4 mRNA levels in unsorted PBMC, CD3+ cells and CD4+ cells.

3. A*0201 allele decreases and A*0301 allele increases the risk of MS. These associations are independent from DRB1*15,DQB1*06-associated haplotype. A*0201 allele and A*0301 allele associations also modulate the risk in DRB1*1501 allele carriers.

4. The DR17 allele is associated with MS. No HLA-DR allele is associated with particular clinical form of disease and no alleles have an impact on disease severity. No HLA-DR resistance gene for MS have been identified. DR15 allele frequency is associated with the diagnostic certainty of MS at the group level. DR15 allele is associated with early age of onset in MS patients.

5. There is increased HLA-DRB1*allele sharing in overall families as well as in DR15-negative MS families. The DRB1*15 and DRB1*17 alleles are significantly more transmitted to patients. DRB1* allele transmission correlates between the groups of overall families and DR15-negative families. Allele sharing cannot be explained only by the DRB1*15 allele or by other DRB1* alleles.

Figure 8. Hypothetical interaction of possible factors that may trigger MS.



HLA phenotype may determine the target specificity for immune response and the amount of antigen required to initiate the immune response. The HLA haplotype sequences are normal and epitope specific. Several background genes as CTLA-4, immunoregulatory molecules as cytokines and other genes products regulate T cell responses. Background genes modify importance of HLA molecules for disease susceptibility.

The background gene's DNA sequences are likely to be normal and frequent in population. Different microenvironmental factors might interact, becoming danger signal that modulates antigen presentation by HLA and recognition by T cells. Particular combinations of these genes and microenvironmental factors determine breakthrough of MS.

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References:

1. Basson MA, Zamoyska R. The CD4/CD8 lineage decision: integration of signaling pathways. *Immunol Today* 2000,21(10),509-514
2. Judd BA, Koretzky GA. Antigen specific T lymphocyte activation. *Rev Immunogenet* 2000,2(2):164-174
3. Seder RA. Acquisition of lymphokine-producing phenotype by CD4+T cells. *J Allergy Clin Immunol* 1994,94(6):1195-1202
4. Prud`homme GJ, Piccirillo CA. The inhibitory effects of transforming growth factor-beta-1 (TGF-β1) in autoimmune diseases. *J of Autoimmun* 2000,14(1):23-42
5. Mason D, Powrie F. Control of immune pathology by regulatory T cells. *Curr Opin Immunol* 1998,10(6):649-655
6. Gaubin M, Houlgatte R, Dettin M et al. Definition of the alpha 2 region of HLA-DR molecules involved in CD4 binding. *Hum Immunol* 1999,60(4):273-281
7. Mosmann TR, Li L, Sad S. Functions of CD8 T-cell subsets secreting different cytokine patterns. *Semin Immunol* 1997,9(2):87-92
8. Kurts C. Cross-presentation: inducing CD8 T cell immunity and tolerance. *J Mol Med* 2000,78(6):326-332
9. Gao GF,Willcox BE, Wyer JR et al. Classical and nonclassical class I major histocompatibility complex molecules exhibit subtle conformational differences that affect binding to CD8 alphaalpha. *J Biol Chem* 2000,275(20):15232-15238
10. Kropshofer H, Vogt AB, Thery C et al. A role for HLA-DO as a co-chaperone of HLA-DM in peptide loading of MHC class II molecules. *EMBO J* 1998,17(11): 2971-2981
11. Meffre E, Casellas R, Nussenzweig MC. Antibody regulation of B cell development. *Nat Immunol* 2000,1(5):379-385
12. Ljunggren HG, Kärre K. In search of the “missing self”: MHC molecules and NK cell recognition. *Immunol Today* 1990,11(7):237-244
13. Lanier LL. NK cell receptors. *Annu Rev Immunol* 1998,16:359-393
14. Raulet DH. Recognition events that inhibit and activate natural killer cells. *Curr Opin Immunol* 1996,8(3):372-377
15. Wilson JL, Charo J, Martin-Fontecha A et al. NK cell triggering by the human costimulatory molecules CD80 and CD86. *J Immunol* 1999,163(8):4207-4212
16. Perussia B. Signaling for cytotoxicity. *Nat Immunol* 2000,1(5):372-374
17. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* 1994, 12:991-1045
18. Schwartz R. Acquisition of immunologic self-tolerance. *Cell* 1989,57(7):1073-1081
19. Sospedera M, Ferrer-Francesch X, Dominguez O et al. Transcription of a broad range of self-antigens in human thymus suggests a role for central mechanisms in tolerance toward peripheral antigens. *J Immunol* 1998,161(11):5918-5929
20. Saoudi AB, Seddon B, Fowell D, Mason D. The thymus contains a high frequency of cells that prevent autoimmune diabetes on transfer into prediabetic recipients. *J Exp Med* 1996,184(6):2393-2398
21. Wang Y, Goldschneider I, Foss D et al. Direct thymic involvement in anterior chamber-associated immune deviation: evidence for a nondeletional mechanism of centrally induced tolerance to extrathymic antigens in adult mice. *J Immunol* 1997,158(5):2150-2155
22. Seddon B, Mason D. Peripheral autoantigen induces regulatory T cells that prevent autoimmunity. *J Exp Med* 1999,189(5):877-882

23. Weiner HL, Friedman A, Miller A et al. Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu Rev Immunol* 1994,12:809-837
24. Tsubata T, Honjo T. B cell tolerance and autoimmunity. *Rev Immunogenet* 2000,2(1):18-25
25. Taneja V, David CS. Autoimmunity versus tolerance: analysis using transgenic mice. *Hum Immunol* 2000,61(12):1383-1389
26. Natarajan K, Li H, Mariuzza RA, Margulies DH. MHC class I molecules, structure and function. *Rev Immunogenet* 1999,1(1):32-46
27. Reyburn HT, Mandelboim O, Vales-Gomez M et al. Human NK cells: their ligands, receptors and functions. *Immunol Rev* 1997,155:119-125
28. Nelson CA, Fremont DH. Structural principles of MHC class II antigen presentation. *Rev Immunogenet* 1999,1(1)47-59
29. Tulp A, Verwoerd D, Dobberstein B et al. Isolation and characterization of the intracellular MHC class II compartment. *Nature* 1994,369(6476):120-126
30. Peters PJ, Neefjes JJ, Oorschot V et al. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* 1991,349(6311):669-676
31. Watts C. Capture and processing of exogenous antigens for presentation on MHC molecules. *Annu Rev Immunol* 1997,15:821-850
32. March SG, Bodmer JG. HLA class II nucleotide sequences. *Tissue Antigens* 1992,40(5) :229-243
33. Olerup O, Möller E, Persson U. HLA-DP incompatibilities induce proliferation in primary mixed lymphocyte culture in HLA-A, -B, -C, -DR and –DQ identical individuals. *Transplant Proc* 1990,22(1):141-142
34. Mytilineos J, Scherer S, Opelz G. Comparison of RFLP-DR beta and serological HLA-DR typing in 1500 individuals. *Transplantation* 1990,50(5):870-873
35. Hammer J, Gallazzi F, Bono E et all. Peptide binding specificity of HLA-DR4 molecules: correlation with rheumatoid arthritis association. *J Exp Med* 1995,181(5)1847-1855
36. Brewerton DA, Hart FD, Nicholls A et al. Ankylosing spondylitis and HL-A27. *Lancet* 1973,1(7809):904-907
37. Juji T, Satake M, Honda Y, Doi Y. HLA antigens in Japanese patients with narcolepsy:All the patients were DR2 positive. *Tissue Antigens* 1984,24(5):316-319
38. Lin L, Hungs M, Mignot E. Narcolepsy and the HLA region. *J Neuroimmunol,* 2001,117(1-2):9-20
39. Reveille JD. The genetics of ankylosing spondylitis. *Bull Rheum Dis* 2001,50(2):2-3
40. Hohjoh H, Terada N, Honda Y et al. Negative association of the HLA-DRB1*1502-DQB1*0601 haplotype with human narcolepsy. *Immunogenetics* 2001 52(3-4): 299-301
41. Goldwurm S, Powell LW. Haemochromatosis after the discovery of HFE (HLA-H) *Gut* 1997:41(6):855-856
42. Reveille JD, Moulds JM, Ahn C et al. Systemic lupus erythematousus in three ethnic groups: I The effects of HLA class II, C4 and CR1 alleles, socioeconomic factors and ethnicity at disease onset. LUMINA study groups. Lupus in minority populations, nature versus nurture. *Arthritis Rheum* 1998,41(7):1161-1172
43. Skarsvag S. The importance of C4 A null genes in Norwegian patients with systemic lupus erythematosus. *Scand J Immunol* 1995,42(5):572-576
44. Todd JA, Bell JI, McDevitt HO. HLA-DQ beta gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. *Nature* 1987,329(6140): 599-604

45. Nishimaki K, Kawamura T, Inada H et al. HLA DPB1*0201 gene confers disease susceptibility in Japanese with childhood onset type I diabetes, independent of HLA-DR and DQ geneotypes. *Diabetes Res Clin Pract* 2000,47(1):49-55
46. Clot F, Babron MC. Genetics of celiac disease. *Mol Genet Metab* 2000, 71(1-2):76-80
47. Rhodes D A, Trowsdale J. Genetics and molecular genetics of the MHC. *Rev Immunogenet* 1999,1(1):21-31
48. Thompson CB, Allison JP. The emerging role of CTLA-4 as an immune attenuator *Immunity* 1997,7(4):445-450
49. Harper K, Balzano C, Rouvier E et al. CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. *J Immunol* 1991,147(3):1037-1044
50. Polymeropoulos MH, Xiao H, Rath DS, Merril CR. Dinucleotide repeat polymorphism at the human CTLA-4 gene. *Nucleic Acids Res* 1991,19(14):4018
51. Larsen ZM, Kristiansen OP, Mato E et al. IDDM 12 (CTLA-4) on 2q33 and IDDM 13 on 2q34 in genetic susceptibility to type 1 diabetes (insulin dependent). *Autoimmunity* 1999, 31(1):35-42
52. Deichmann K, Heinzmann A, Bruggenolte E et al. An Mse I RFLP in the human CTLA-4 promoter. *Biochem Biophys Res Commun* 1996,225(3):817-818
53. Linsley PS, Greene JL, Tan P et al. Co expression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. *J Exp Med* 1992,176(6):1595-604
54. Lindsten T, Lee KP, Harris ES. Characterization of CTLA-4 structure and expression on human T cells. *J Immunol* 1993,151(7):3489-3499
55. Krummel MF, Allison PJ. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J Exp Med* 1996,183(6): 2533-2540
56. Alegre ML, Shiels H, Thompson CB, Gajwski TF. Expression and function of CTLA-4 in Th1 and Th2 cells. *J Immunol* 1998,161(7):3347-3356
57. Chen W, Jin W, Wahl SM. Engagement of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) induces transforming growth factor-β (TGF-β) production by murine CD4(+) T cells. *J Exp Med* 1998,188(10):1849-1857
58. Liu MF, Yang CY, Li JS et al. Increased expression of down-regulatory CTLA-4 molecule on T lymphocytes from rheumatoid synovial compartment. *Scand J Immunol* 1999,50(1):68-72
59. Bachmann M F, Köhler G, Ecabert B et al. Cutting edge: lyphoproliferative disease in the absence of CTLA-4 is not T cell autonomous. *J Immunol* 1999,163(3): 1128-1131
60. Kuiper HM, Brouwer M, Linsley PS, Lier RA. Activated T cells can induce high levels of CTLA-4 expression on B cells. *J Immunol* 1995,155(4):1776-1783
61. Iida T, Ohno H, Nakaseko C et al. Regulation of cell surface expression of CTLA-4 by secretion of CTLA-4 containing lysosomes upon activation of CD4+ T cells. *J Immunol* 2000,165(9):5062-5068
62. Peach RJ, Bajorath J, Brady W et al. Complementarity determining region 1 (CDR1) – and CDR3 – analogous regions in CTLA-4 and CD28 determine the binding to B7-1. *J Exp Med* 1994,180(6):2049-2058
63. Oaks MK, Hallett KM, Penwell RT et al. A native soluble form of CTLA-4. *Cell Immunol* 2000,201(2):144-153
64. Magistrelli G, Jeannin P, Herbault N et al. A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells. *Eur J Immunol* 1999, 29(11):3596-3602

65. Kristiansen OP, Larsen ZM, Pociot F. CTLA-4 in autoimmune diseases-a general susceptibility gene to autoimmunity ? *Genes Immun* 2000,1(3):170-184
66. Yanagawa T, Hidaka Y, Guimaraes V et al. CTLA-4 gene polymorphism associated with Graves` disease in a Caucasian population. *J Clin Endocrinol Metab* 1995, 80(1):41-45
67. Heward JM, Allahabadia A, Armitage M et al. The development of Graves disease and the CTLA-4 gene on chromosome 2q33. *J Clin Endocrinol Metab* 1999,84(7): 2398-2401
68. Vaidya B, Imrie H, Perros P et al. The cytotoxic T lymphocyte antigen-4 is a major Graves` disease locus. *Hum Mol Genet* 1999,8(7):1195-1199
69. Nistico L, Buzzetti R, Pritchard LE et al. The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. *Hum Mol Genet* 1996,5(7): 1075-1080
70. Marron MP, Raffel LJ, Garchon HJ et al. Insulin-dependent diabetes mellitus (IDDM) is associated with CTLA-4 polmorphisms in multiple ethnic groups. *Hum Mol Genet* 1997, 6(8):1275-1282
71. Esposito L, Hill NJ, Pritchard LE et al. Genetic analysis of chromosome 2 in type 1 diabetes: analysis of putative loci IDDM 7, IDDM 12, and IDDM 13 and candidate genes NRAMP1 and IA-2 and the interlukin-1 gene cluster. IMDIAB Group. *Diabetes* 1998,47(11):1797-1799
72. Concannon P, Goglin-Ewens KJ, Hinds DA et al. A second-generation screen of the human genome for susceptibility to insulin-dependent diabetes mellitus. *Nat Genet* 1998,19(3):292-296
73. Salomon B, Lenschow DJ, Rhee L et al. B7/CD28 costimulation is esential for the homeostasis of the CD4+ CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 2000,12(4):431-440
74. Lehmann PV. The fate of T cells in the brain. Veni, vidi, vici and veni, mori. *Am J Pathol* 1998,153(3):677-680
75. Vyse TJ, Todd JA. Genetic analysis of autoimmune disease. *Cell* 1996,85(3):311-318
76. Lucchinetti CF, Bruck W, Rodriguez M, Lassmann H. Distinct patterns of multiple sclerosis pathology indicate heterogeneity on pathogenesis. *Brain Pathol* 1996,6(3):259-274
77. Lucchinetti C, Bruck W, Parisi J et al. Heterogeneity of Multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol* 2000,47(6):707-717
78. Compston A, Sadovnick AD. Epidemiology and genetics of multiple sclerosis. *Curr Opin Neurol Neurosurg* 1992,5(2):175-181
79. Kurtzke JF. MS epidemiology word wide: one view of current status. *Acta Neurol Scand* 1995,161:23-33
80. Lublin FD, Reingold SC. Defining the clinical course of multiple sclerosis. National multiple sclerosis society (USA). Advisory comitte on clinical trails of new agents in multiple sclerosis. *Neurology* 1996,46(4):907-911
81. Ebers GC, Sadovnick AD. The role of genetic factors in multuple sclerosis susceptibility. *J Neuroimmunol* 1994,54(1-2):1-17
82. Mumford CJ, Wood NW, Kellar-Wood H et al. The British Isles survey of multiple sclerosis in twins. *Neurology* 1994,44(1):11-15
83. Sadovnick AD, Armstrong H, Rice PG, et al. A population-based study of multiple sclerosis in twins: update. *Ann Neurol* 1993,33(3):281-285
84. Bulman DE, Sadovnick AD, Ebers GC. Age of onset in siblings concordant for multiple sclerosis. *Brain* 1991,114:937-950

85. Compston A. Genetic epidemiology of multiple sclerosis. *J Neurol Neurosurg Psychiatry* 1997,62(6):553-561
86. Milanov I, Topalov N, Kmetski T. Prevalance of multiple sclerosis in Gypsies and Bulgarians. *Neuroepidemiology* 1999,18(3):218-222
87. Lauer K. The risk of multiple sclerosis in the USA in relation to sociogeographic features: a factor-analytic study. *J Clin Epidemiol* 1994,47(1):43-48
88. Gronning M, Mellgren SI. Multiple sclerosis in the two northernmost counties of Norway. *Acta Neurol Scand* 1985,72(3):321-327
89. Steinman L . A few autoreactive cells in an autoimmune infiltrate control a vast population of non-specific cells: a tale of smart bombs and the infantry. *Proc Natl Acad Sci USA* 1996,93(6):2253-2256
90. Martin R, Voskuhl R, Flerlage M et al. Myelin basic protein-specific T-cell responses in identical twins discordant or concordant for multiple sclerosis. *Ann Neurol* 1993,34(4):524-535
91. Ozawa K, Suchanek G, Breitschopf H et al. Pattern of oligodendroglia pathology in multiple sclerosis. *Brain* 1994,117:1311-1322
92. Zajicek J, Wing M, Skepper J, Compston A. Human oligodendrocytes are not sensitive to complement. A study of CD59 expression in human the central nervous system. *Lab Invest* 1995,73(1):128-138
93. Gale CR, Martyn CN. Migrant studies in multiple sclerosis. *Prog Neurobiol* 1995 47(4-5):425-448
94. Kurtzke JF, Hyllested K. Multiple sclerosis in the Faroe Islands: 1. Clinical and epidemiological features. *Ann Neurol* 1979,5(1):6-12
95. Fukazawa T, Yabe I, Kikuchi S et al. Association of vitamin D receptor gene polymorphism with multiple sclerosis in Japanese. *J. Neurol. Sci* 1999,166(1):47-52
96. Steckley JL, Dyment DA, Sadovnick AD et al. Genetic analysis of vitamine D related genes in Canadian multiple sclerosis patients. *Neurology* 2000,54(3): 729-732
97. Bachmann S, Kesselring J. Multiple sclerosis and infectious childhood diseases. *Neuroepidemiology*. 1998,17(3):154-160
98. Soldan SS, Berti R, Salem N et al. Association of human herpes virus 6 (HHV-6) with multiple sclerosis: increased IgM response to HHV-6 early antigen and detection of serum HHV-6 DNA. *Nat Med* 1997,3(12):1394-1397
99. Sadovnick AD, Ebers GC, Dyment DA, Risch NJ. Evidence for genetic basis of multiple sclerosis. The Canadian collaborative study group. *Lancet* 1996,347(9017):1728-1730
100. Ebers GC, Sadovnick AD, Risch NJ. A genetic basis for familial aggregation in multiple sclerosis. Canadian Collaborative study group. *Nature* 1995,377(6545):150-151
101. Jersild C, Svejgaard A, Fog T. HL-A antigens and multiple sclerosis. *Lancet* 1972,1(7762): 1240-1241
102. Jersild C, Fog T, Hansen GS et al. Histocompatibility determinants in multiple sclerosis, with specific reference to clinical course. *Lancet* 1973,2(7840):1221-1225
103. Cowan EP, Pierce ML, McFarland HF, McFarlin DE. HLA-DR and DQ allelic sequences in multiple sclerosis patients are identical to those found in the general population. *Hum Immunol* 1991, 32(3):203-210
104. Cullen CG, Middleton D, Savage DA , Hawkins S. HLA-DR and DQ DNA genotyping in multiple sclerosis patients in Northen Ireland. *Hum Immunol* 1991,30(1):1-6
105. Francis DA, Thompson AJ, Brookes P et al. Multiple sclerosis and HLA: is the susceptibility gene really HLA-DR or –DQ? *Hum Immunol* 1991,32(2):119-124

106. Olerup O, Hillert J. HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation. *Tissue Antigens* 1991,38(1):1-15
107. Marrosu MG, Murru MR, Costa G et al. Multiple sclerosis in Sardinia is associated and in linkage disequilibrium with HLA-DR3 and -DR4 alleles. *Am J Hum Genet* 1997,61(2):454-457
108. Allen M, Sandberg-Wollheim M, Sjogren K et al. Association of susceptibility to multiple sclerosis in Sweden with HLA class II DRB1 and DQB1 alleles. *Hum Immunol* 1994,39(1): 41-48
109. Spurkland A, Celius EG, Knutsen I et al. The HLA-DQA1*0102,DQB1*0602 heterodimer may confer susceptibility to multiple sclerosis in the absence of the HLA-DRA1*01, DRB1*1501 heterodimer. *Tissue Antigens* 1997,50(1): 15-22
110. Caballero A, Alves-Leon S, Papais-Alvarenga R et al. DQB1*0602 confers genetic susceptibility to multiple sclerosis in Afro-Brazilians. *Tissue Antigens* 1999,54(9): 524-526
111. Kira J, Kanai T, Nishimura Y at al. Western versus Asian types of multiple sclerosis: immunogenetically and clinically distinct disorders. *Ann Neurol* 1996,40(4):569-574
112. Fukazawa T, Kikuchi S, Sasaki H et al. Genomic HLA profiles of MS in Hokkaido, Japan: important role of DPB1*0501 allele. *J Neurol* 2000,247(3):175-178
113. Ebers GC, Kukay K, Bulman DE et al. A full genome search in multiple sclerosis. *Nat Genet* 1996,13(4):472-476
114. Haines JL, Ter-Minassian M, Bazyk A et al. A complete genomic screen for multiple sclerosis underscores a role for the major histocompatibility complex. The Multiple Sclerosis Genetic Group. *Nat Genet* 1996,13(4):469-471
115. Sawcer S, Jones HB, Feakes R et al. A genome screen in multiple sclerosis reveals susceptibility loci on chromosome 6p21 and 17q22. *Nat Genet* 1996,13(4):464-468
116. Poser CM, Paty DW, Scheinberg L et al. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol* 1983,13(3):227-231
117. Mullis KB, Faloona FA. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol* 1987,155:335-350
118. Huang W-X, Huang P, Link H, Hillert J. Cytokine analysis in multiple sclerosis by competitive RT-PCR: A decreased expression of IL-10 and an increased expression of TNF-α in chronic progression. *Mult Scler* 1999,5(5):342-348
119. Celi FS, Zenilman ME, Shuldiner AR. A rapid and versatile method to synthesize internal standards for competitive PCR. *Nucleic Acids Res* 1993,21(4):1047
120. Spielman RS, McGinnis RE, Ewens WJ et al. Tramsmission test for linkage disequilibrium: The insulin gene region and insulin-dependent Diabetes mellitus (IDDM). *Am J Hum Genet* 1993,52(3):506-516
121. Payami H, Joe S, Farid NR et al. Relative predispositional effects (RPEs) of marker alleles with disease: HLA-DR alleles and Graves disease. *Am J Hum Genet* 1989,45(4):541-546
122. Svejgaard A, Ryder LP. HLA and disease associations: detecting the strongest association. *Tissue Antigens* 1994,43(1):18-27
123. Risch N. Assessing the role of HLA-linked and unlinked determinants of disease. *Am J Hum Genet* 1987,40(1):1-14
124. Bluestone JA. Is CTLA-4 a master switch for peripheral T cell tolerance? *J Immunol* 1997,158(5):1989-1993
125. Jenkins MK. The ups and downs of T cell costimulation. *Immunity* 1994, 1(6):443-446
126. Sansom DM. CD28, CTLA-4 and their ligands: who does what and to whom? *Immunology* 2000,101(2):169-177

127. Perrin PJ, Maldonado JH, Davis TA et al. CTLA-4 blockade enhances clinical disease and cytokine production during experimental allergic encephalomyelitis. *J Immunol* 1996,157(4):1333-1336
128. Karandikar NJ, Vanderlugt CL, Walunas TL et al. CTLA-4: a negative regulator of autoimmune disease. *J Exp Med* 1996,184(2):783-788
129. Hurwitz AA, Sullivan TJ, Krummel MF et al. Specific blockade of CTLA-4/B7 interactions results in exacerbated clinical and histologic disease in an actively-induced model of experimental allergic encephalomyelitis. *J Neuroimmunol* 1997,73(1-2):57-62
130. Donner H, Rau H, Walfish PG et al. CTLA-4 alanine-17 confers genetic susceptibility to Graves` disease and to type 1 diabets mellitus. *J Clin Endocrinol Metab* 1997,82(1):143-146
131. Marron MP et al. Genetic and physical mapping of a type 1 diabetes susceptibility gene (IDDM 12) to a 100-kb phagemid artificial chromosome clone containing D2S72-CTLA-4-D2S105 on chromosome 2q33. *Diabetes* 2000, 49(3):492-499
132. Harbo FH, Celius EG, Vartdal F, Spurkland A. CTLA-4 promoter and exon 1 dimorphisms in multiple sclerisis. *Tissue Antigens* 1999,53(1):106-110
133. Fukazawa T, Yanagawa T, Kikuchi S et al. CTLA-4 gene polymorphism may modulate disease in Japanese multiple sclerosis patients. *J Neurol Sci* 1999,171(1):49-55
134. Kuokkanen S, Gschwend M, Rioux JD et al. Genomewide scan of multiple sclerosis in Finnish multiplex families. *Am J Hum Genet* 1997,61(6):1379-1387
135. Vandenborre K, Delabie J, Boogaerts MA et al. Human CTLA-4 is expressed in situ on T lymphocytes in germinal centers, in cutaneous graft-versus host disease, and in Hodgkin's disease. *Am J Pathol* 1998,152(4):963-973
136. Perkins D, Wang Z, Donovan C et al. Regulation of CTLA-4 expression during T cell activation. *J Immunol* 1996,156(11):4154-4159
137. Mena E, Rohowsky-Kochan C. Expression of costimmulatory molecules on peripheral blood mononuclear cells in multiple sclerosis. *Acta Neurol Scand* 1999,100(2):92-96
138. Schneider H, Martin M, Agarraberes FA et al. Cytolytic T lymphocyte-associated antigen-4 and the TCR zeta/CD3 complex, but not CD28, interact with clathrin adaptor complexes AP-1 and AP-2. *J Immunol* 1999,163(4):1868-1879
139. Braun J, Donner H, Siegmund T et al. CTLA-4 promoter variants in patients with Graves` disease and Hashimoto`s thyroiditis. *Tissue Antigens* 1998,51(5):563-566
140. Gonzalez-Escribano MF, Rodriguez R, Valenzuela A et al. CTLA-4 polymorphisms in Spanish patients with rheumatoid arthritis. *Tissue Antigens* 1999,53(3):296-300
141. Heward JM, Allahabadia A, Carr-Smith J et al. No evidence for allelic association of a human CTLA-4 promoter polymorphism with autoimmune thyroid disease in either population-based case-control or family-based studies. *Clin Endocrinol* 1998,49(3):331-334
142. Ahmed S, Ihara K, Kanemitsu S et al. Association of CTLA-4 but not CD28 gene polymorphisms with systemic lupus erythematosus in the Japanese population. *Rheumatology* 2001,40(6):662-667
143. Park YJ, Chung HK, Park DJ et al. Polymorphism in the promoter and exon 1 of the cytotoxic T lymphocyte antigen-4 gene associated with autoimmune thyroid disease in Koreans. *Thyroid* 2000,10(6):453-459
144. Marrosu MG, Murru MR, Costa G et al. DRB1-DQA1-DQB1 loci and multiple sclerosis predisposition in the Sardinian population. *Hum Mol Genet* 1998,7(8):1235-1237

145 Mustafa M, Vingsbo C, Olsson T et al. Protective influences on experimental autoimmune encephalomyelitis by MHC class I and class II alleles. *J Immunol* 1994,153(7):3337-3344
146 Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 1992,39(5):225-235
147. Odum N, Hyldig-Nielsen JJ, Morling N et al. HLA-DP antigens are involved in the susceptibility to multiple sclerosis. *Tissue Antigens* 1988,31(5):235-237
148 Worwood M, Raha Chowdhury R, Robson KJ et al. The HLA A1-B8 haplotype extends 6Mb beyond HLA-A: association between HLA-A,B,F and 15 microsatellite markers. *Tissue Antigens* 1997,50(5):521-526
149. Sadovnick AD, Yee IM, Ebers GC. Factors influencing sib risks for multiple sclerosis. *Clin Genet* 2000,58(6):431-435
150 Thompson AJ, Polman CH, Miller DH et al. Primary progressive multiple sclerosis. *Brain* 1997,120:1085-1096
151 Weinshenker BG, Bass B, Rice GP et al. The natural history of multiple sclerosis: a geographically based study. 1. Clinical course and disability. *Brain* 1989,112: 133-146
152 Olerup O, Hillert J, Fredrikson S et al. Primary chronic progressive and relapsing/remitting multiple sclerosis; two immunogenetically distinct disease entities. *Proc Natl Acad Sci USA* 1989;86(18):7113-7117
153 Hillert J, Gronning M, Nyland H et al. An immunogenetic heterogeneity in multiple sclerosis. *J Neurol Neurosurg Psychiatry* 1992,55(10):887-890
154 Weinshenker BG, Santrach P, Bissonet AS et al. Major histocompatibility complex class II alleles and the course and outcome of MS: a population-based study. *Neurology* 1998,51(3):742-747
155 Runmarker B, Martinsson T, Wahlstrom J, Andersen O. HLA and prognosis in multiple sclerosis. *J Neurol* 1994,241(6):385-390
156 McDonnell GV, Mawhinney H, Graham CA et al. A study of the HLA-DR region in clinical subgroups of multiple sclerosis and its influence on prognosis. *J Neurol Sci* 1999,165(1):77-83
157 Celius EG, Harbo HF, Egeland T et al. Sex and age at diagnosis are correlated with the HLA-DR2, DQ6 haplotype in multiple sclerosis. *J Neurol Sci* 2000,178(2): 132-135
158 Weatherby SJ, Thomson W, Pepper L et al. HLA-DRB1 and disease outcome in multiple sclerosis. *J Neurol* 2001,248(4):304-310
159 Francis DA, Batchelor JR, McDonald WI et al. Multiple sclerosis in north-east Scotland. An association with HLA-DQw1. *Brain* 1987,110:181-196
160 Kellar-Wood HF, Wood NW, Holmans P et al. Multiple sclerosis and the HLA-D region:linkage and association studies. *J Neuroimmunol* 1995,58(2):183-190
161. Ebers GC, Koopman WJ, Hader W et al. The natural history of multiple sclerosis: a geographically based study: 8: familial multiple sclerosis. *Brain* 2000,123:641-649
162. Weinshenker BG, Bulman D, Carriere W et al. A comparison of sporadic and familial multiple sclerosis. *Neurology* 1990,40(9):1354-1358
163. Sadovnick AD, Dyment D, Ebers GC. Genetic epidemiology of multiple sclerosis. *Epidemiol Rev* 1997,19(1):99-106
164. Oturai A, Larsen F, Ryder LP et al. Linkage and association analysis of susceptibility regions on chromosomes 5 and 6 in 106 Scandinavian sibling pair families with multiple sclerosis. *Ann Neurol* 1999,46(4):612-616

165. Haines JL, Terwedow HA, Burgess K et al. Linkage of the MHC to familial multiple sclerosis suggests genetic heterogeneity. The Multiple Sclerosis Genetics Group. *Hum Mol Genet* 1998,7(8):1229-1234
166. Hillert J, Käll T, Vrethem M et al. The HLA-Dw2 haplotype segregates closely with multiple sclerosis in multiplex families. *J Neuroimmunol* 1994,50(1):95-100
167. Voskuhl RR, Goldstein AM, Simonis T et al. DR2/DQw1 inheritance and haplotype sharing in affected siblings from multiple sclerosis families. *Ann Neurol* 1996,39(6):804-807
168. Fogdell A, Olerup O, Fredrikson S et al. Linkage analysis of HLA class II genes in Swedish multiplex families with multiple sclerosis. *Neurology* 1997,48(3):758-762
169. Marrosu MG, Fadda E, Mancosu C et al. The contribution of HLA to multiple sclerosis susceptibility in Sardinian affected sibling pairs. *Ann Neurol* 2000,47(3):411-412
170. Ebers GC, Paty DW, Stiller CR et al. HLA-typing in multiple sclerosis sibling pairs. *Lancet* 1982,2(8289):88-90
171. Tienari PJ, Wikstrom J, Koskimies S et al. Reappraisal of HLA in multiple sclerosis: close linkage in multiplex families. *Eur J Hum Genet* 1993,1(4):257-68
172. Eoli M, Pandolfo M, Amoroso A et al. Evidence of linkage between susceptibility to multiple sclerosis and HLA-class II loci in Italian multiplex families. *Eur J Hum Genet* 1995,3(5):303-11
173. Stewart GJ, McLeod JG, Basten A, Bashir HV. HLA family studies and multiple sclerosis: A common gene, dominantly expressed. *Hum Immunol* 1981,3(1):13-29
174. Hillert J, Olerup O. Multiple sclerosis is associated with genes within or close to the HLA-DR-DQ subregion on a normal DR15,DQ6,Dw2 haplotype. *Neurology* 1993 43(1):163-168
175. Weissert R, Wallström E, Storch MK et al. MHC haplotype-dependent regulation of MOG-induced EAE in rats. *J Clin Invest* 1998,102(6):1265-1273
176. Stephens JC, Schneider JA, Tanguay DA et al. Haplotype variation and linkage disequilibrium in 313 human genes. *Science* 2001,293(5529):489-493
177. Kouki T, Sawai Y, Gardine C A et al. CTLA-4 gene polymorphism at position 49 in exon 1 reduces the inhibitory function of CTLA-4 and contributes to the pathogenesis of Graves` disease. *J Immunol* 2000, 165:6606-6611