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The PD-1 pathway and the complement system in systemic lupus erythematosus

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Abstract
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Autoimmune diseases occur in up to 3-5% of the general population and represent a diverse collection of diseases with regards to clinical manifestations. The unifying factor of autoimmune diseases is tissue and organ damage as a result of an immune response mounted against self-antigens.

Systemic lupus erythematosus (SLE) is considered a prototype of human systemic autoimmune diseases. The etiology of SLE is as yet largely unknown, but both epidemiological and genetic data suggest an interplay between numerous and varying genetic and environmental factors.

There is compelling evidence for a strong genetic component in SLE. The disease has a high λ_{sibs} value and familial clustering is apparent. Multiple susceptibility loci have been identified, some of which are syntenic between humans and mice and some of which overlap with other autoimmune diseases.

This thesis is based on analysis of Icelandic multicase SLE families and Swedish SLE patients.

Paper I is a study of the association of C4A protein deficiency (C4AQ0) with SLE in the multicase families and shows a significantly increased frequency of C4AQ0 in the families. The genetic basis for C4AQ0 varies and C4AQ0 is found on different MHC haplotypes, pointing to C4AQ0 as an independent risk factor for SLE.

Paper II describes the association of low MBL serum levels with SLE in the families and identifies low MBL as risk factor for SLE in families that carry the defect. Low MBL was furthermore found to mediate an additive risk when found in combination with C4AQ0.

In paper III cellular expression the PD-1 co-inhibitory receptor on T cells was studied. A polymorphism in the PDCD1 gene, PD-1.3A was previously associated with SLE in the multicase families. The polymorphism is thought to disrupt expression of the gene and may lead to decreased expression of the PD-1 receptor. The study demonstrates lower PD-1 expression in SLE patients and relatives in correlation to the PD-1.3A genotype.

Paper IV is a compiled analysis of the SLE families, including PD-1.3A, C4AQ0, low MBL, autoimmune diseases and autoantibody profiles. The study demonstrates clustering of different autoimmune diseases and autoantibodies in families that are heterogenic with regards to the genetic susceptibility factors, PD-1.3A, C4AQ0 and low MBL.

Keywords: SLE, autoimmune diseases, PD-1, C4AQ0, low MBL, multicase family

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Til Tóta, Össu og Sesselju.

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Kristjánsdóttir H**, Steinsson K. (2004) A study of the genetic basis of C4A protein deficiency. Detection of C4A gene deletion by long-range PCR and its associated haplotypes. *Scand J Rheumatol*, 2004;33(6):417-22.

II Sævarsdóttir S, **Kristjánsdóttir H**, Gröndal G, Víkingsdóttir T, Steinsson K, Valdimarsson H. (2006) Mannan-binding lectin and deficiency of complement C4A in Icelandic multicase families with systemic lupus erythematosus. *Ann Rheum Dis*, Nov;65(11):1462-7.

III **Kristjánsdóttir H**, Steinsson K, Gunnarsson I, Gröndal G, Erlendsson K, Alarcón-Riquelme ME. (2009) Lower expression levels of the PD1 receptor on CD4+CD25+ T-cells in SLE patients and correlation to the PD-1.3A genotype. *Revision submitted.*

IV **Kristjánsdóttir H**, Sævarsdóttir S, Gröndal G, Alarcón-Riquelme ME, Erlendsson K, Valdimarsson H, Steinsson K. (2008) Association of three systemic lupus erythematosus susceptibility factors, PD-1.3A, C4AQ0, and low levels of mannan-binding lectin, with autoimmune manifestations in Icelandic multicase systemic lupus erythematosus families. *Arthritis Rheum*, 58(12):3865-72.

Contents

Abbreviations

AS	Ankylosing spondylitis
ACR	American College of Rheumatology
ANA	Anti-nuclear antibodies
APC	Antigen presenting cell
BANK	B-cell scaffold protein with ankyrin repeats
BCR	B cell receptor
BLK	B lymphoid tyrosine kinase
C1-INH	C1 inhibitor
C2Q0	C2 protein deficiency
C4AQ0	C4A protein deficiency
C4BP	C4 binding protein
C4BQ0	C4B protein deficiency
CR1	Complement receptor 1
CR2	Complement receptor 2
CR3	Complement receptor 3
CR4	Complement receptor 4
CRD	Carbohydrate recognition domain
CTLA4	Cytotoxic T lymphocyte-associated antigen 4
DAF	Decay Accelerating Factor
DC	Dendritic cell
dsDNA	Double stranded DNA
EBV	Epstein Barr virus
FcγR	Fc gamma receptor
HLA	Human leukocyte antigen
IC	Immune complex
IFN	Interferon
IL	Interleukin
IRF5	Interferon regulating factor 5
ITGAM	Integrin Alpha M
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
LR-PCR	Long range PCR
Lyp	Lymphoid tyrosine phosphatase
MAC	Membrane-attack complex
MBL	Mannan-binding lectin
MCP	Membrane bound cofactor protein
MFI	Mean of fluorescence intensity

MHC	Major histocompatibility complex
MS	Multiple sclerosis
PBC	Primary biliary cirrhosis
PBMC	Peripheral blood mononuclear cell
PCR-SSP	PCR with sequence specific primers
PD-1, PDCD1	Programmed cell death 1
RA	Rheumatoid arthritis
RT-PCR	Real time polymerase chain reaction
RUNX1	Runt-related transcription factor
SLE	Systemic lupus erythematosus
SMA	Smooth muscle antibody
SSc	Systemic sclerosis
STAT4	Signal Transducer and activator of transcription
TCR	T cell receptor
TG	Thyroglobulin
TNF	Tumour necrosis factor
TPO	Thyroid peroxidase
T-regs	T regulatory cells
TYK2	Tyrosine kinase 2

Introduction

The role of the immune system is to guard the host against foreign organisms. Central to the development of a "healthy" immune response is self-tolerance or the ability of the immune system to discriminate between self-antigens (components of the body, such as nucleic acids and proteins) and foreign antigens. Useful cells that recognize foreign antigens are retained and recruited to mount an immune response while dangerous cells responsive to self-antigens are destroyed or inactivated.

The complexity and potential destructive power of the human immune system requires meticulous mechanisms of regulation to prevent the immune response from turning against the self.

When the immune system mounts a hyperactive immune response against the host's own healthy cells and tissues, consequences can be severe. Such an autoimmune attack against 'self' normal antigens, resulting in inflammation and tissue damage, can lead to one of many forms of autoimmune diseases.

Autoimmune diseases

Autoimmune diseases occur in up to 3-5% (1, 2) of the general population and represent a diverse collection of diseases with regards to clinical manifestations. The unifying factor of autoimmune diseases is damage to tissue and organs as a result of an immune response mounted against self-antigens.

There is an autoimmune disease specific for nearly every organ in the body and autoimmune diseases vary widely with regards to the tissues targeted and the clinical symptoms portrayed. Autoimmune diseases can be classified as organ-specific or systemic autoimmune diseases (3) (Table 1).

In organ-specific autoimmune diseases, the immune response is directed against a single cell type. For example, against pancreatic β-cells resulting in type 1 diabetes. In systemic autoimmune diseases, the immune response is directed against antigens that are present in almost every cell of the body, thus targeting a broad range of cell types and tissues. Systemic lupus erythematosus (SLE) is an example of a systemic autoimmune disease.

Table 1. *Examples of systemic and organ specific autoimmune diseases.*

Systemic autoimmune diseases	**Organ specific autoimmune diseases**
Systemic lupus erythematosus	Vitiligo
Rheumatoid arthritis	Type 1 diabetes
Polymyositis/dermatomyositis	Graves´ disease
Sjögren´s syndrome	Celiac disease
Scleroderma	Thyroiditis
	Hashimoto´s disease
	Multiple sclerosis
	Pemphigus
	Hepatitis
	Myasthenia gravis
	Primary billiary cirrhosis

Etiology of autoimmune diseases

The etiology of autoimmune diseases is largely unknown, but is thought to be dependent on interplay between genetic and environmental factors (Figure 1).

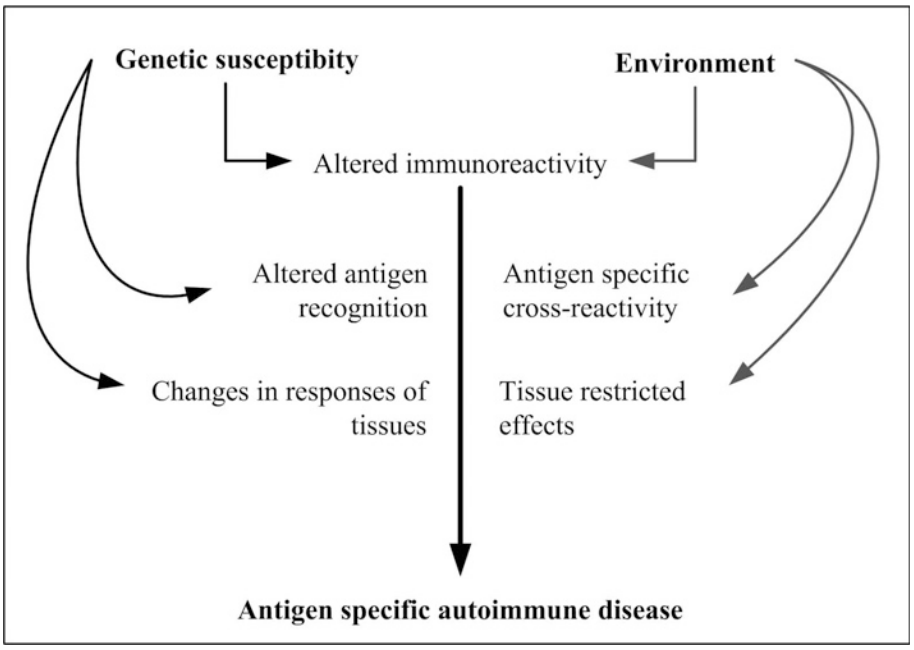

Figure 1. Pathways of autoimmune disease. (Adapted from Marrack 2001 (3)).

The genetic component in autoimmune diseases

The effects of a strong genetic component in autoimmune diseases is reflected in families multicase for specific autoimmune diseases, such as families multicase for systemic lupus erythematosus (SLE) (4-6) and rheumatoid arthritis (RA) (7-9) as well as in familial clustering of different autoimmune diseases (10-14).

A number of genes, both genes of the MHC and non-MHC are proposed to contribute to the predisposition of autoimmune disease. Current hypotheses concerning the pathogenesis of autoimmunity in general, have led to the inclusion of hundreds of genes as potential candidates and virtually any molecule involved in immune recognition, cell interaction, intracellular signalling, cytokine pathways or programmed cell death can be reasonably proposed (15, 16). These genes may act at different levels in diverse ways. Some genes influence the immune response or shape the immune repertoire, some genes play a role in regulating the immune response and some genes function at the level of the target organ affecting vulnerability. The clinical picture of autoimmune disease is the sum effect or interaction of a number of diverse genes (17).

Genetic studies have identified chromosomal regions and genes associated with different autoimmune diseases and several of the susceptibility loci originally identified in association or linkage with a particular autoimmune disease have been found to overlap in different autoimmune diseases (18, 19).

Environmental factors in autoimmune diseases

Although the nature of environmental factors in triggering autoimmune disease is not well understood, several potential factors have been associated with different autoimmune diseases, including infections, UV-radiation and smoking.

Infections have been implicated as potential triggers of autoimmunity (20, 21), either through molecular mimicry of antibodies against foreign antigens with self components (22, 23) or indirectly by influencing cytokine production. Infectious microorganisms including Epstein-Barr virus (EBV) have been suggested as plausible candidates for playing a role in the pathophysiology of SLE, RA and primary Sjögren´s syndrome (24).

Smoking has been linked to the development of SLE (25) and RA (26, 27). Tobacco smoke has been shown to interact with genetic factors to create a significant combined risk of disease. Smoking is known to modulate the immune response and may induce an inflammatory response, immune suppression, alter cytokine profiles, induce apoptosis, and damage DNA resulting in the formation of anti-DNA antibodies (28).

Systemic Lupus Erythematosus (SLE)

SLE is considered a prototype of human systemic autoimmune diseases because of the generalized autoimmune characteristics displayed (29). The clinical spectrum of SLE can vary from a mild disease affecting skin and joints to a severe life-threatening disease affecting internal organs.

SLE predominantly affects women and the prevalence varies considerably between ethnic groups and countries (30).

The diagnosis of SLE is based on the presence of clinical symptoms and autoantibodies. Due to the heterogeneity of the disease, The American College of Rheumatology (ACR) has established 11 criteria for classification of SLE (31). Table 2 lists the 1982 ACR criteria for SLE, which was used in this thesis. These criteria have since been revised and are still under study for further revision. The 1997 revised criteria, for instance, include anti-phospholipid antibodies, while LE cell preparations have been excluded (32).

Table 1. *The 1982 American College of Rheumatology criteria for the classification of SLE (31).*

	Criterion
1.	Malar rash
2.	Discoid rash
3.	Phosensitivity
4.	Oral ulcers
5.	Arthritis
6.	Serositis
7.	Renal disorders
	a. Persistent proteinuria
	b. Cellular casts of any type
8.	Neurological disorder
	a. Seizures (in the absence of other causes)
	b. Psychosis (in the absence of other causes)
9.	Hematological disorder
	a. Hemolytic anemia
	b. Leukopenia
	c. Lymphopenia
	d. Thrombocytopenia
10.	Immunological disorder
	a. Positive LE-cell preparation
	b. Anti-dsDNA
	c. Anti-Sm
	d. False positive serological test for syphilis
11.	Anti-nuclear antibodies (ANA)

A person shall be said to have SLE if 4 or more of the 11 criteria are present.

Immunopathology of SLE

The pathophysiology of SLE involves an autoimmune response mediated by autoreactive lymphocytes. Abnormalities in virtually all immune cell lineages have been reported in SLE including aberrant T and B cell interaction, T and B cell hyperactivity, abnormalities of the complement system, decreased phagocytic activity, increased death of cells through apoptosis and aberrant production of cytokines (29).

Autoantibodies

The presence of autoantibodies against cellular constituents is the hallmark of SLE. These autoantibodies are mainly directed against various nuclear components, such as double-stranded DNA (dsDNA) and histones, but also against cytoplasmic and cell membrane molecules. Antinuclear antibodies (ANA) are most characteristic for SLE and are present in over 95% of SLE patients (33).

B cells

SLE is associated with loss of B-cell tolerance, B-cell hyper-reactivity and autoantibody production. However, it remains controversial whether loss of B cell tolerance is primary and precedes other immune abnormalities or secondary to aberrant regulation by T cells and/or dendritic cells (DCs). Abnormalities of peripheral B cell homeostasis in SLE patients have been reported. Absolute numbers of B cells and memory B cells are reduced in SLE, but plasmablasts are expanded (34, 35).

The mechanisms underlying aberrant B cell function in SLE are still largely unknown, but there are studies reporting aberrant expression of receptors that regulate B cell activation and signaling pathway molecules controlling B cell proliferation and differentiation and abnormal apoptosis (36, 37). All may lead to increased autoantibody production.

Theoretically B cells may contribute to the immune dysregulation in SLE as antibody producing cells or as antigen presenting cells (APCs) by taking up and presenting autoantigens to T cells (34, 38).

T cells

Loss of self-tolerance also pertains to T cells. Autoreactive T cells in SLE characteristically exhibit a CD4+ T cell receptor (TCR) $\alpha\beta$ phenotype and are able to provide T cell help to antibody producing B cells, produce interferon-γ (IFN-γ) and moderate levels of IL-2 (39, 40).

Functional abnormalities that have been identified in T cells from SLE patients include increased intracellular calcium responses and increased intracellular phosphorylation, recruitment of Syk tyrosine kinase to the TCR complex, over expression of CD40L and decreased activation-induced cell death (40).

Data on the role of CD4+CD25+ T-regulatory cells (T-regs) in SLE is somewhat conflicting. Decreased numbers of peripheral blood T-regs have been reported by most studies on SLE patients with active disease, but non-impaired or even increased numbers of T-regs have also been described. In addition, both deficient and normal suppressive capacity of isolated T-regs has been observed in SLE patients (41).

Monocytes and dendritic cells

Increased apoptosis and dysregulated processing of apoptotic cells is seen in SLE. It has long been known that Fc and complement mediated phagocytosis is impaired in SLE patients. A loss in phagocytic ability has been correlated with complement deficiency (42).

Increased apoptosis has been suggested as a potential source of autoantigens for presentation by DCs to autoreactive T cells in SLE (43, 44).

Monocytes from SLE patients have been shown to stimulate proliferation of autologous CD4+ T cells in culture, thus displaying a functional phenotype typical of DCs. Furthermore addition of SLE serum to monocytes from controls has been shown to enable them to develop DC-function and phenotype with up-regulated expression of CD80 and CD86 and, in the presence of IFN-α, the ability to present antigen from apoptotic cells to autologous T cells (45).

Cytokines

Cytokines may be generally classified as Th1 or Th2 according to their cellular source and effector functions. In general, Th1 cytokines are pro-inflammatory and enhance cytokine responses and Th2 cytokines are anti-inflammatory and mediate antibody production (46).

A balance in cytokine production is necessary for immune homeostasis and both diminished and excessive production of cytokines can shift the balance. In general, overproduction of Th2 cytokines promotes B cell hyperactivity and humoral responses, while overproduction of Th1 and Th17 cytokines mediates T cell hyperactivity and inflammation (47).

Th1 cytokines include IFN-γ and TGF-β. IFN-γ increases production of IL-12 by DCs via a positive feedback loop and IL-12 stimulates production of IFN-γ, thereby promoting a Th1 cytokine profile. IFN-γ furthermore inhibits production of Th2 cytokines, such as IL-4.

Th2 cytokines include IL-4, IL-10 and IL-13. IL-4 acts on T helper cells to promote production of Th2 cytokines, including itself. IL-10 inhibits production of various cytokines, including T helper cell production of IFN-γ and IL-12 production in DCs and macrophages, thus promoting a Th2 cytokine profile.

There are limitations to the Th1/Th2 model. Th17 helper cells are considered distinct from Th1 and Th2 cells. It remains unclear which

cytokines exactly contribute to Th17 formation, but TGF-β, IL-6, IL-21 and IL-23 have been implicated (46).

Several cytokines belonging to more than one Th type have been implicated in SLE pathogenesis. In SLE there is tendency toward a Th2 cytokine response over a Th1 response.

Decreased levels of IL-2 are reported in SLE patients. IL-2 is a central cytokine required for the activation of T, B, and NK cells. Absence of a regulatory cytokine, such as IL-2 may prevent effective activation and functioning of T cells and may also induce activation-induced cell death in T cells (48, 49).

IL-10 has dual effects. It down-regulates the expression of Th1 cytokines, but also enhances B cell survival, proliferation, and antibody production (50). In SLE patients, high serum levels of IL-10 are reported that may promote antibody production of B cells and increased apoptosis (51-53). B cells from SLE patients have been shown to spontaneously produce IL-6, which drives proliferation and differentiation of B cells.

Significantly elevated IL-12, IL-17 and IL-23 levels and increased numbers of Th17 cells has been shown in SLE patients (54). Increased IL-12 levels promote IFN-γ production leading to polarization of peripheral cells toward a Th1 phenotype (55).

Serum levels of INF-α are elevated in SLE and genes regulated by interferon are upregulated in SLE patients (interferon signature) (56, 57). The ongoing IFN-α production in SLE can promote autoimmunity by stimulating key cells in the immune system, including lymphocytes and antigen-presenting DCs (58).

Etiology of SLE

It is widely acknowledged that the etiology of SLE combines genetic and environmental factors in ways still largely unknown.

The genetic component in SLE

Compiling evidence demonstrates the importance of genetic factors in SLE. Genetic predisposition in SLE is reflected in differences in the concordance rate for SLE in monozygotic vs. dizygotic twins. The concordance rate for SLE is estimated 24% - 69% for monozygotic twins, compared to 2% - 9% concordance for dizygotic twins (59, 60). The lack of complete concordance for SLE among monozygotic twins, however, highlights the importance of non-genetic factors. An important role for genes is furthermore supported by familial clustering of SLE with 10% - 12% of SLE patients having an affected first-degree relative (61).

The overall genetic contribution to SLE susceptibility is also reflected in the comparatively high λs (ratio of the risk to siblings of an affected individual divided by the background population prevalence of the disease

where “s” refers to sibling). For SLE the estimated λs is 10-20 and as high as 30 (4, 12, 14, 61) For comparison, estimated λs is 20for multiple sclerosis (MS), 15 for type 1 diabetes and 8 for RA (61, 62).

The genetics of SLE are complex and multiple susceptibility loci have been identified, some of which are syntenic between humans and mice (29) and some of which overlap with other autoimmune diseases (16, 18).

Through genome wide linkage and association studies (63-76) several genes have been associated with SLE. As may be expected there is a degree of ethnic heterogeneity. Some of the results have been replicated in different populations, but none have been replicated in all studies. The importance of studying genetically homogenous populations has been emphasized (5, 77-79).

The MHC region - HLA alleles and complement

Like in most human autoimmune diseases, genes within the MHC region exhibit strong association with SLE; the HLA class I and II genes that encode membrane glycoproteins that present peptides for recognition by T lymphocytes and genes within the HLA class III region, particularly complement components C4 and C2 (80).

Papers I and II describe the association of partial deficiency of C4 or deficiency of C4A (C4AQ0) with SLE.

PDCD1

Fine mapping of the 2q37 SLEB2 locus in Icelandic, Swedish, Norwegian and Mexican SLE patients led to identification of the PD-1.3A polymorphism in the PDCD1 gene (81).

PDCD1 encodes the co-inhibitory immunoreceptor PD-1 (programmed death 1), which is expressed on activated T and B cells. PD-1 and its ligands, PD-L1 and PD-L2, on antigen presenting cells (APCs) deliver inhibitory signals that regulate T cell activation. PD-1.3A is suggested to affect transcription of the PDCD1 gene leading to decreased expression of the PD-1 receptor and inappropriate activation of autoreactive T and B cells (82).

PD-1.3A has been associated with SLE in different cohorts as well as with other autoimmune diseases (81, 83-93).

CTLA4

The CTLA4 (cytotoxic T lymphocyte-associated antigen 4) gene maps to chromosome 2q33 and encodes for the CTLA4 co-inhibitory receptor. CTLA4 is expressed on activated CD4+ and CD8+ T cells and binds to the same ligands as the T cell co-stimulatory receptor CD28. CTLA4 downregulates T cell activation and function and polymorphisms in the CTLA4 gene have been associated with SLE (94) and other autoimmune

diseases (95-101). Aberrant function of CTLA4 may lead to inappropriate activation of autoreactive T cells.

FcγR

Functional polymorphisms in the genes for FcγR 2A and FcγR 3A (Fc receptor for IgG) have been associated with risk for SLE by several groups (102-111). FcR are expressed on phagocytes and mediate clearance of apoptotic cells, immune complexes and pathogens and defective function of FcγR has been implicated in the pathogenesis of SLE (109, 112-114).

IRF5

Polymorphisms in the gene encoding the IRF5 (interferon regulating factor 5) transcription factor have been associated with SLE (115, 116). Association of the gene encoding IRF5 was first identified in an association screen of genes related to type I interferon in SLE cohorts, among them Icelandic multicase SLE families (117). An independent study found strong association with a haplotype containing SNPs affecting expression levels. High expression of type I interferon and type I-inducible genes is commonly observed SLE patients (57).

The IRF5 gene has been associated with RA (118-122), inflammatory bowel disease (123) and MS (124).

STAT4

The signal transducer and activator of transcription 4 (STAT4) is a cytoplasmic transcription factor involved in cytokine signal transduction. STAT4 transduces IL-12, IL-23, and type 1 interferon cytokine signals in T cells and monocytes, leading to Th1 and Th17 differentiation, monocyte activation, and IFN-γ production (125).

Different polymorphisms in the STAT4 gene have been associated with SLE (126-128) and additive effects with IRF5 have been suggested (129, 130). Association has also been reported with other autoimmune diseases, such as RA (127, 131-135), Sjögren´s syndrome (136, 137) and type 1 diabetes (138).

BANK1

The B-cell scaffold protein with ankyrin repeats (BANK1) is a substrate of tyrosine kinases and is phosphorylated upon B-cell antigen receptor (BCR) stimulation. BANK1 regulates BCR-induced calcium mobilization by promoting tyrosine phosphorylation of IP_3Receptor leading to calcium mobilization and B cell activation (139).

A functional variant of the BANK1 gene was recently associated with SLE. The variant may contribute to sustained BCR signaling and B cell hyperreactivity (140).

PTPN22

A SNP in lymphoid tyrosine phosphatase (Lyp), encoded by the PTPN22 (protein tyrosine phosphatase non-receptor 22) gene (R620WC/T), has been associated with SLE (75, 141-148). PTPN22 polymorphisms have also been associated with other autoimmune diseases, including type 1 diabetes (149-158), RA (147, 159) and Graves' disease (160-163). A meta-analysis demonstrated that the PTPN22 1858T allele confers susceptibility to RA, SLE, Graves´ disease, type 1 diabetes and juvenile arthritis, supporting evidence of association of the PTPN22 gene with autoimmune diseases (146). Lyp, is a powerful inhibitor of T cell activation.

BLK

Association of SLE with the BLK gene (B lymphoid tyrosine kinase) has recently been reported (75, 76). A risk allele localized between BLK and the C8orf13 (unknown function) was associated with reduced expression of BLK, and increased expression of C8orf13 (76) and both genes may be risk factors. BLK is a B-cell specific member of the Scr family of tyrosine kinases and may influence proliferation and differentiation of B cells (164).

ITGAM

The ITGAM (Integrin Alpha M, CD11b) gene has been associated with SLE (75, 76, 165, 166). ITGAM encodes a subunit of complement receptor 3 (CR3) on macrophages, dendritic cells and neutrophils. CR3 mediates phagocytosis of pathogens opsonized with complement iC3b, and mediates leukocyte adhesion and emigration from the bloodstream via ICAM-1 and ICAM-2 adhesion molecules (167).

MBL2

The MBL2 gene on chromosome 10 encodes for mannan-binding lectin (MBL), a component of the complement system, which mediates clearance of pathogens and apoptotic cells. Variant MBL2 gene alleles are associated with low serum MBL levels are associated with increased infections (168) and have been associated with SLE (169-174) and other autoimmune diseases (172, 175-177).

Paper II describes the association of low MBL with SLE in Icelandic multicase SLE families.

IL-10

Promoter polymorphisms in the IL-10 gene on chromosome 1q31-32 have been associated with SLE (178-187) and increased serum levels of IL-10 have been reported in SLE (51-53, 182, 188, 189). High IL-10 levels have been correlated with increased apoptosis in SLE and a synergistic effect reported in combination with apoptosis related polymorphisms (179, 190).

Environmental and hormonal factors in SLE

Environmental factors and hormones are considered important in the etiology of SLE. Various environmental hazards have been proposed to be important in triggering disease or possibly also in triggering the effect of genes that otherwise would be kept silent.

Environmental exposures include infectious agents, chemicals or other compounds capable of modulating immune responses such as occupational/environmental pollutants or drugs, and behavioural factors such as smoking and diet (191).

Hormonal factors

SLE predominantly affects females, particularly in their reproductive years, and the female to male ratio is approximately 9:1 (192, 193). Disease activity is sometimes modified by menstrual cycle and pregnancy, suggesting hormonal effects. Estrogens have been implicated in increased disease activity and have been shown to repress tolerance (194, 195). In men with SLE, low testosterone and high estrogen levels have been detected (196) and men with Klinefelter´s syndrome (XXY) are more prone to SLE (197).

Infections

Viruses and bacteria have been suggested as having a role in SLE. Among them, Epstein Barr virus (EBV) infection has been hypothesized to cause loss of self-tolerance via molecular mimicry of EBV-specific antibodies with SLE autoantigens (198). Exposure to viruses also increases antibody titers, which may be the result of polyclonal B cell activation.

Drugs, occupational and life-style hazards

UV-radiation appears to be a risk factor and trigger onset of SLE and photosensitivity is one of the clinical manifestation of SLE (191). Several drugs have been associated with drug-induced lupus, such as procainamide and hydralazine, which are aromatic amines or hydrazines. These drug are metabolized through an acetylation pathway and patients who are genetically slow acetylators are more prone, suggesting that the free amine or hydrazine moiety is a trigger (199, 200).

Occupational risk factors have been reported in SLE, such as exposure to crystalline silica, solvents and pesticides. Silica exposure has been shown to increase the generation of apoptotic material (201, 202).

Other potential risk factors are life-style factors such as cigarette smoking, lipstick and hair-dye. Cigarette smoke contains hundreds of potentially toxic components, including tars, nicotine, carbon monoxide and polycyclic aromatic hydrocarbons. Whether cigarette smoking increases the risk of developing SLE remains controversial and both significantly

increased odds ratios for SLE in smokers and unclear associations have been reported (25). Lipstick use has been hypothesized to be a risk factor for developing SLE and a recent study found a trend of greater risk for SLE with increased frequency of use and with a younger age of beginning lipstick use. Biologic effects of chemicals found in lipsticks absorbed across the buccal mucosa could explain this association (203).

The major histocompatibility complex (MHC)

The classical MHC region spans ~4 Mb on the short arm of chromosome 6 and is the most gene-dense region of the human genome comprising over 160 protein-coding genes and is, moreover, highly polymorphic with multiple allelic variants at each locus (204, 205). The MHC region is divided into three classes, class I, II and III (Figure 2).

It is estimated that about 40% of the expressed genes within the MHC have immune system function and the MHC has been associated with various inflammatory and autoimmune conditions (205).

Genes within the MHC are in strong linkage disequilibrium and are inherited “en bloc” as MHC haplotypes, making it difficult to determine which gene or allele is the real susceptibility gene and which are innocent bystanders. The high level of allelic variation further complicates dissection of disease alleles.

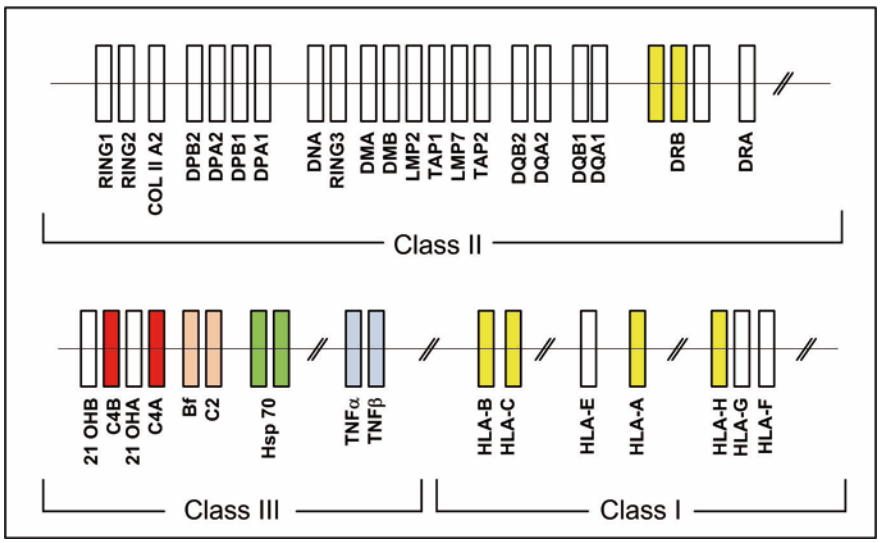

Figure 2. Gene organization of the human MHC on chromosome 6.

MHC class I, II and III genes

MHC class I genes

Within the MHC class I region lie genes that encode for HLA-A, B and C molecules, which present endogenously synthesized antigens to CD8+ cytotoxic T cells. HLA class I molecules are heterodimers consisting of a single transmembrane polypeptide chain (the α-chain) and a ß2-microglobulin (not encoded in the MHC) (204). The transmembrane α-chain has three polymorphic domains (α1, α2, α3) and each alpha-chain gene can be any one of 90–500 different alleles. HLA class I molecules are expressed on most nucleated cells of the body.

MHC class II genes

MHC class II genes include the genes for HLA-DR, DQ and DP molecules, which present exogenously derived antigens to CD4+ T cells. HLA class II molecules are expressed on cell surfaces as heterodimers consisting of α and ß chains. Both the α and ß can be polymorphic, although the ß chains are more polymorphic. HLA class II molecules are expressed B cells, DCs and macrophages.

MHC class III genes

The MHC class III region lies between class II and class I. It does not contain genes encoding HLA molecules, but contains genes encoding molecules with immunological function, including the genes for complement components C4A and C4B, C2 and factor B, and the genes for tumor necrosis factor (TNF).

The complement system

Complement was first identified as a heat-labile component of serum that augmented or "complemented" the anti-bacterial activity of antibody. The complement system consists of more than 30 plasma and cell-surface bound proteins, which react with one another to opsonize pathogens and induce inflammatory responses.

Complement contributes to host defence by providing a rapid response to bacterial infections, by bridging innate and adaptive immune responses and by promoting clearance of apoptotic cells and immune complexes (ICs) (206, 207) (Table 3).

Table 2. *Main biological functions of the complement system.*

Physiological role of complement	
1. Host defence against infections	- Opsonization
	- Lysis of bacterial cells
	- Chemotaxis and activation of leukocytes
	- Anaphylatoxin effects
2. Bridging innate and adaptive immunity	- Augmentation of antibody responses
	- Enhancement of immunological memory
3. Disposal of waste	- Clearance of apoptotic cells
	- Clearance of immune complexes

Activation pathways of the complement system

The complement system can be activated by different stimuli through three pathways; the classical pathway, the mannan-binding lectin or MBL pathway and the alternative pathway (Figure 3).

Activation of the first component of each pathway initiates an enzymatic cascade through which complement components are sequentially cleaved, generating a large and a small fragment, that mediate biological functions. The large fragment remains surface bound and mediates two functions. It has proteolytic activity and continues the enzymatic activation cascade, and serves as an opsonin that marks the target for uptake by phagocytes. The smaller fragments are released and mediate chemotaxis and anaphylatoxin effects (208, 209).

The classical pathway of complement activation

The classical pathway is initiated by binding of the C1 complex to antibody (IgG and IgM) or directly to pathogen surfaces. C1 is a complex consisting of a single C1q molecule which has six globular heads with long collagen tails that combine to bind to two molecules of C1r, and two molecules of C1s, which are zymogens (210).

Binding of C1q to the Fc portion of antibody causes a conformational change in C1r enabling cleavage of C1s, thus initiating activation of the classical pathway enzymatic cascade. Once activated C1s cleaves the next components of the classical pathway, C4 and C2.

C4 is cleaved to form C4b and C4a. C4b is covalently bound to the target surface and recruits C2 making it susceptible to cleavage by C1s. C2 is cleaved to generate C2a and C2b. C2a is joined to C4b on the target surface to form the C3 convertase, C4bC2a, which cleaves C3 generating C3b and C3a. C3b is deposited onto the target surface to join the C4bC2a convertase to from the C5 convertase, C4bC2aC3b, which cleaves C5 to generate C5b

and C5a. Formation of C5b initiates the terminal complement pathway. The small fragments C4a, C3a and C5a are released.

The MBL pathway of complement activation

The MBL pathway is initiated by binding of the MBL complex to certain sugar residues on the pathogen surfaces and to apoptotic and necrotic cells (211).

MBL belongs to the collectin family of C-type lectins that function as pattern recognition proteins and provide first line of immune defence. Collectins are characterized by a carbohydrate recognition domain (CRD) and a collagenous tail (212). The structure and function of MBL is similar to C1q.

MBL consists of 2 to 6 carbohydrate-binding globular heads, containing CRD and a collagen stalk connected to 2 MBL-associated serine proteases, MASP-1 and MASP-2. Binding of MBL to carbohydrates activates MASP-2, which cleaves C4 and C2. At this point the MBL pathway converges with the classical pathway (213).

The alternative pathway of complement activation

The alternative pathway is initiated by spontaneous hydrolysis of C3 leading to formation of C3(H_20) and the binding of C3b to hydroxyl groups on cell surface carbohydrates and proteins. C3(H_20) forms a complex with factor B, a protein homologous to C2 and making factor B susceptible to cleavage by Factor D. Factor B is cleaved to generate Bb and Ba.

Bb remains bound to C3b on the target surface to form the alternative pathway C3 convertase, C3bBb, which is able to cleave many C3 molecules to C3b forming an amplification loop for generation of C3b. C3b joined to C3bBb forms the alternative pathway C5 convertase, C3bBbC3b.

The C3 convertases

The C3 convertases, C4bC2a and C3bBb are powerful amplifiers of complement activation and up to 1000 C3 molecules are cleaved to C3b by each molecule of active C3 convertase.

The terminal complement pathway

All three pathways lead to cleavage of C5 and initiation of the terminal pathway. The C5 convertases, C4bC2aC3b and C3bBbC3b, cleave C5 to generate C5b and C5a.

C5b is deposited on the pathogen surface and recruits C6, C7 and C8 exposing hydrophobic domains that insert into the lipid bilayer of the cell. The C5b678 complex catalyses polymerization of the final component, C9, to form the membrane-attack complex (MAC) leading to lysis of the cell.

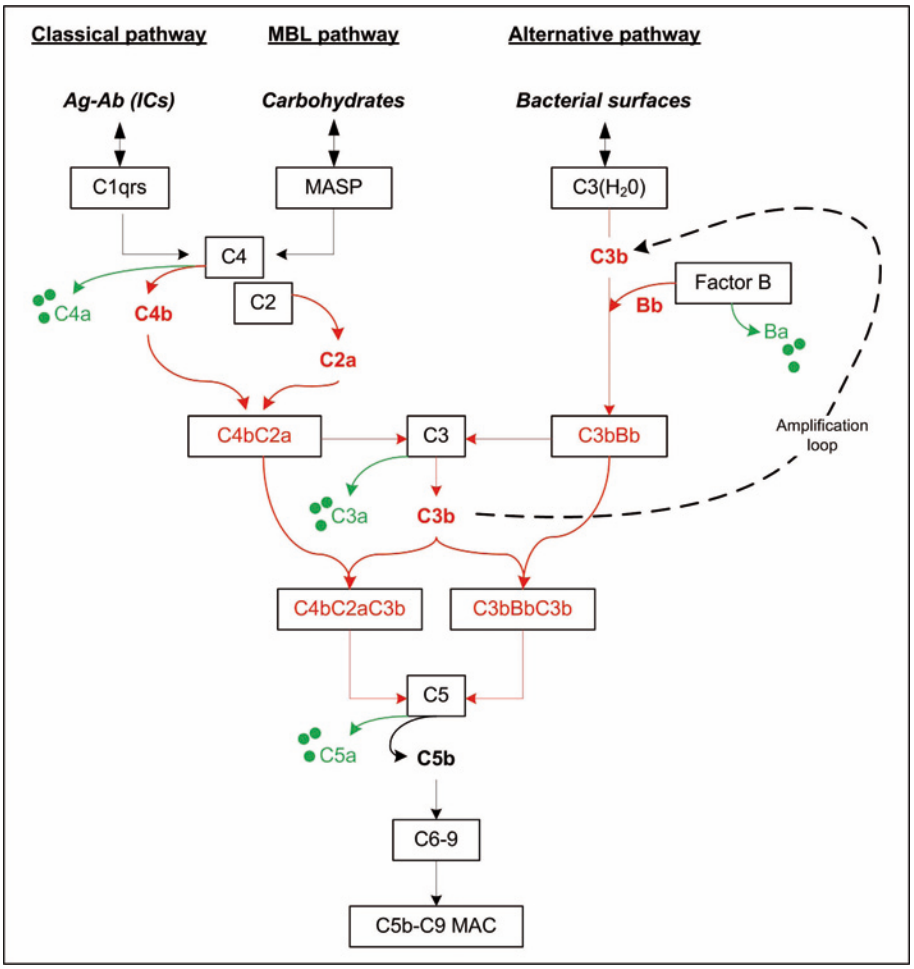

Figure 3. The complement system.

Biological effects of complement

C3b and C4b deposited on pathogen surfaces and ICs are opsonins that mark the targets for clearance from circulation and uptake by phagocytes. C3b and C4b are ligands for complement receptors expressed on phagocytes and red blood cells.

On the target surface, C3b undergoes further cleavage to generate iC3b (inactive) and C3d and C3dg. C3b, iC3b, C3d and C4b are ligands for complement receptors on phagocytes and red blood cells.

The smaller fragments generated, C4a, C3a and C5a, are peptide mediators of inflammation. They are all anaphylatoxins. C5a is more potent than C3a, which is more active than C4a. They act directly on local blood vessels, stimulate increased blood flow and vascular permeability, induce smooth muscle contraction, and increase binding of phagocytes to

endothelial cells. C5a acts directly on neutrophils and monocytes to increase their adhesion to vessel walls, their migration towards antigen and their ability to ingest particles and increases expression of CR1 and CR3 on these cells. C5a furthermore activates mast cells to release mediators such as histamine and TNFα (214).

Complement receptors

Specific complement receptors provide the link between activated complement components and various cell types.

Complement receptor 1 (CR1)

CR1 (CD35) is widely expressed and is found on erythrocytes, macrophages and monocytes, neutrophils, leukocytes, B cells and DCs. CR1 binds to C3b, C4b and iC3b and probably also C1q (215).

CR1 on erythrocytes mediate clearance of ICs via binding to C3b and C4b on ICs facilitating their transport to the reticuloendothelial system where ICs are taken up by phagocytes.

CR1 plays a role in regulating complement activation by inhibiting C3 convertase activity (216).

CR1 on macrophages and neutrophils can trigger phagocytosis on cells recruited by C5a.

Complement receptor 2 (CR2)

CR2 (CD21) is expressed on B cells, follicular DCs and some epithelial cells. CR2 binds iC3b, C3d or C3dg.

On B cells, CR2 is part of the B cell co-receptor complex (CR2-CD19-CD81) and binding of iC3b, C3d and C3dg to CR2 is thought to provide a link between the BCR and opsonized ICs, resulting in an enhanced B cell response to the antigen. CR2 binding to iC3b or C3dg opsonized ICs traps them in germinal centers where the antigen is displayed to activated B cells. Immature B cells in the marrow encounter antigen bound to C3d via binding to CR2 inducing B cell anergy. CR2 serves as a receptor for EBV (217).

Complement receptor 3 (CR3) and complement receptor 4 (CR4)

CR3 and CR4 are related receptors which bind iC3b and are found on monocytes/macrophages and neutrophils. They trigger phagocytosis of opsonized particles, either in concert with Fc receptors or independently. Phagocytosis of microorganisms via CR3, CR1 and FcR, is a major defence mechanism against bacterial and fungal infection.

Regulation of complement activation

Because of the potent inflammatory effect of activated complement, the regulatory mechanisms of complement activation are finely balanced to ensure that activation is restricted to surfaces of opsonized targets and to hinder deposition of complement fragments on self cells and tissues (206, 207). Regulatory mechanisms act at some key points and about half of the proteins of the complement pathways are regulatory proteins.

Regulation of C1

C1 inhibitor (C1-INH) regulates the activity of C1 by blocking proteolytic activity of C1r and C1s by mimicking their normal substrates, thus limiting cleavage of C4 and C2 and formation of the classical pathway C3 convertase.

C1-INH also binds free C1 in serum and inhibits spontaneous activation of C1.

Limitation of C3 convertases

The lifetime and activity of C3 convertase is regulated by several inhibitory proteins in all pathways.

- Factor I, a serine protease that cleaves C3b, C4b and their breakdown products inhibits formation of C3 and C5 convertase. Factor I is regulated by membrane bound and fluid-phase cofactors:
- Membrane bound cofactor protein (MCP) binds C3b and C4b monomers and facilitates factor I mediated proteolysis.
- C4 binding protein (C4BP) (acts on C4bC2a) and factor H (acts on C3bBb), which catalyze the permanent inactivation of C3b and C4b via proteolytic cleavage by factor I and Decay Accelerating Factor (DAF).
- CR1 promotes dissociation of the C3 convertases by binding to and displacing covalently bound C4b or C3b.

Inactivation of C3b and C4b

The reactive thioester bond in C4b and C3b must react with hydroxyl or amino groups to form covalent linkage to target surface. If this bond is not formed rapidly, C4b and C3b are cleaved by hydrolysis and inactivated.

Regulation of the terminal pathway MAC

Regulation of terminal pathway MAC and excessive cell lysis is mediated by membrane bound and plasma inhibitors.

- The membrane bound CD59 inhibits MAC formation by binding to C8 and C9.
- S-protein and clusterin bind soluble C5-7 complexes or C8 and C9 and prevent their insertion into host cells.

Restriction of complement activation to pathogens

Deposition of complement on normal cells is limited by several key inhibitors, but promoted on pathogen surfaces.

Complement activation is selectively inhibited on host cells, which express CR1, DAF, MCP and CD59 and favor binding of factor H, mediating the dissociation of C3 convertase. These regulatory proteins are not expressed on pathogen surfaces.

The complement system and disease

Deficiency of complement components has been associated with autoimmune and infectious diseases.

Complete deficiency of the early classical pathway components, C1, C4 and C2, is strongly associated with increased susceptibility to SLE (206, 215).

MBL deficiency or low serum MBL levels are associated with increased frequency of infections and have also been associated with SLE as a minor risk factor (170, 172, 174, 218-224).

Deficiency of C3 is associated with membranoproliferative glomerulonephritis and is also related to bacterial infections.

Deficiency of the terminal complement pathway components (C5-C9) is associated with bacterial infections due to lack of cell lysis.

When the regulatory mechanisms of complement activation fail, the complement system may have severe effects due to over-activation of complement. Defective regulation of C3 is typically associated with glomerulonephritis. Hereditary C1-INH deficiency is associated with angioedema.

Complement deficiency in SLE

Mouse models

Mice deficient for C1q or C4 develop autoimmune diseases similar to SLE, displaying autoantibodies and SLE-like clinical manifestations (216, 225).

C1q-/- mice exhibit glomerulonephritis characterized by multiple apoptotic cell bodies and immune deposits, suggesting that C1q deficiency causes autoimmunity by impaired clearance of apoptotic cells (226, 227).

C4-/- mice display impaired clearance of circulating ICs which has been suggested as a causal mechanism in the development of a lupus-like autoimmunity phenotype (228).

C3-/-C4-/- mice develop a SLE autoimmune phenotype, whereas C3-/- with C4+/+ mice do not. This may taken to suggest that it is the absence of C4 that predisposes to SLE (229).

Mice deficient for MBL display defective clearance of apoptotic cell, but no autoimmune phenotype (230).

Complement deficiency and SLE in humans

Complete (homozygous) deficiency of early classical pathway components, C1, C4, and C2 and MBL, has been associated with SLE.

A hierarchical association is seen between deficiency of classical pathway components and SLE depending on the position within the sequence of complement activation, with the earlier components being associated with a higher frequency of SLE (C1 > C4 > C2) (231) (232) (Figure 4).

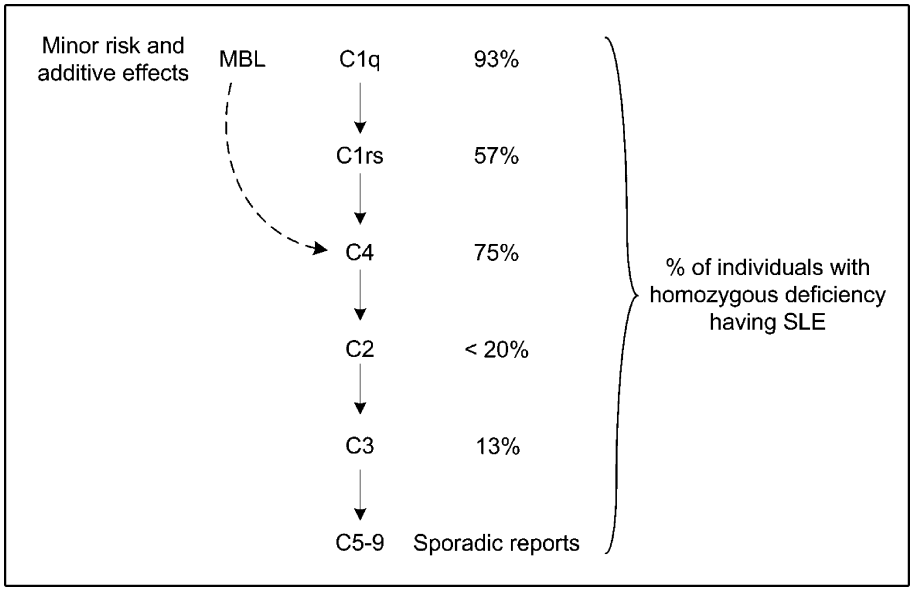

Figure 4. A hierarchical association of complete complement deficiency with SLE. Adapted from Lewis 2006 (232).

Complete complement deficiency is rare. However, partial deficiency of some complement components is more common, but whether partial deficiency predisposes to SLE in a similar or dose dependent way has been questioned.

In SLE, deficiency of the early classical complement components is paradoxical. On the one hand, deficiency leads to decreased clearance of ICs and apoptotic cell debris harboring auto-antigens. On the other hand, complement mediates inflammatory responses at sites of IC fallout causing tissue injury.

Papers I and II describe the association of C4AQ0 with SLE.

Complement mediated clearance of apoptotic cells and immune complexes

Complement mediated clearance of apoptotic cells

Apoptotic cells undergo morphological changes including nuclear chromatin condensation, DNA fragmentation, surface blebbing and formation of membrane bound debris (apoptotic debris). Under normal circumstance apoptotic cells are removed quickly by means of phagocytosis before they spill their intracellular content, which includes potential lupus antigens (233).

Complement mediated clearance of apoptotic cells is mediated mainly by C1q, but MBL is also able to bind to apoptotic cells. C1q and MBL are able to bind directly to apoptotic cells via their globular head domains. C4b and C3b are deposited on the apoptotic cell and mediate binding to phagocytes via complement receptors (207, 231). Apoptotic cells are engulfed and their contents degraded.

Deficiency of C1q may lead to decreased clearance of apoptotic cell debris, which has been suggested as the auto-antigen driving force in SLE (231, 234).

Complement mediated clearance of immune complexes

One of the imperative physiological roles of complement is to promote clearance of ICs. ICs are produced whenever there is an antibody response to soluble antigen. As the immune response progresses larger ICs are formed that are able to activate the complement system. Clearance is mediated mainly by the classical pathway via binding of C1q to the immunoglobulin portion of ICs with subsequent opsonization of ICs with C3b and C4b (216).

As ligands for CR1, C3b and C4b mediate binding of ICs to erythrocytes. ICs bound to erythrocytes are transported to the reticuloendothelial system where they are taken up by resident phagocytes via binding to CR3 and CR4 and FcR (Figure 5).

Complement furthermore inhibits precipitation of ICs by solubilizing immune aggregates. C1q binds to the antibody portion of ICs and interferes with antigen-antibody interaction thus decreasing the size of ICs, facilitating their clearance (235, 236).

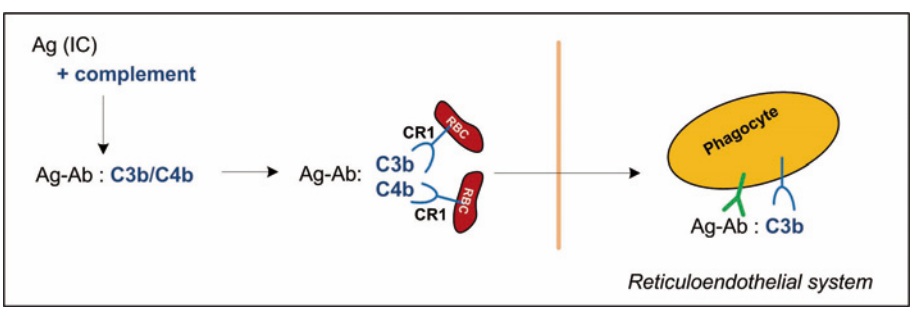

Figure 5. Complement mediated clearance of ICs.

Impaired clearance of immune complexes has been associated with complement deficiency, in particular with deficiency of C2 and C4, which are strong risk factors for SLE. C4 and C2 deficiency may lead to decreased production of the classical pathway C3 convertase, C2aC4b and decreased cleavage of C3 with decreased opsonization of ICs with C3b and C4b (in C4 deficiency) resulting in decreased binding of ICs to erythrocytes and insufficient transport of ICs to the reticuloendothelial system and subsequent accumulation of ICs in peripheral tissues.

Deposition of ICs in peripheral tissues may lead to inflammation and tissue damage with increased release of autoantigens. This may initiate the cycle of enhanced autoantibody production, decreased clearance and deposition of ICs, inflammation and tissue damage (237).

Inflammatory damage may expose cryptic autoantigens and activate autoreactive T and B cells. In addition, abundant ICs may enhance antigen uptake and activation by APCs, promoting lymphocyte activation, release of inflammatory cytokines, and the abrogation of anergy (238).

The complement system and self-tolerance

Loss of self-tolerance - impaired complement mediated clearance of apoptotic cells

Normally in peripheral tissues, apoptotic cells are cleared very early by tissue macrophages, which produce anti-inflammatory cytokines and downregulate the antigen presentation function of DCs. It has also been suggested that immature DCs that have taken up apoptotic cells receive signals from the apoptotic cell, possibly via CR3, that inhibit development of antigen-presenting function of the DC (239, 240). The DC is thus not able to present self-antigens harboured in the apoptotic cell to autoreactive T cells and self-tolerance is preserved.

In complement deficiency states, where apoptotic cells and ICs are not cleared there is both an abundant amount of autoantigens for uptake by DCs and there is also an ongoing inflammatory response or danger milieu. Danger signals, such as pro-inflammatory cytokines, which stimulate DC maturation and activate macrophages, induce maturation of immature macrophages to DCs capable of antigen presentation to autoreactive T cells (232, 241).

Furthermore, it has been suggested that non-cleared apoptotic cells may undergo secondary necrosis. Necrotic cells are capable of stimulating secretion of pro-inflammatory cytokines by macrophages (239, 242). This is though controversial.

Loss of self tolerance in B cells

Self-tolerance in B cells is induced through negative selection of autoreactive B cells in the bone marrow, where autoreactive B cells encounter their cognate antigen leading to clonal deletion or anergy. Deficiency of C4 or CR1/CR2 has been hypothesized to lead to reduced negative selection of autoreactive B cells and impaired humoral responses (228).

The theory suggests that complement targets self-antigens to the bone marrow and peripheral lymphoid compartments, ensuring their localization and provision. Self-antigens opsonized with C3d are bound to the BCR via CR2 and autoreactive B cells are deleted or rendered anergic and self-tolerance is preserved (243).

Deficiency of C1, C4 and C2, would lead to decreased opsonization of target autoantigens with C3d and diminished localization of autoantigens in the bone marrow. Immature B cells recognizing self-antigens would not encounter their cognate antigen and would not be deleted or anergized. The same line of thinking would apply for deficiency of CR2, which would lead to impaired binding of the C3d-bound self-antigen to immature B cells via the BCR.

Complement component 4 (C4)

The C4 gene

The gene for C4 on chromosome 6 has been duplicated in humans and occurs as two genes, C4A and C4B that show 99% homology. The genes for C4A and C4B are arranged in tandem loci lying ~10 kb apart. Each human C4 gene consists of 41 exons.

Dichotomous size of C4 genes

The C4 genes are dichotomous in size and are either long (20.6 kb) or short (14.2 kb), depending on the presence of a 6.5 kb retroviral insert HERV-K (human endogeneous retrovirus) in intron 9. C4A genes are consistently long, while C4B genes can be either long or short and have the retroviral insert present in a 2:1 ratio (244, 245).

Duplication of C4 genes

Duplication of C4 genes occurs in modular fashion together with the genes RP1/RP2 (Ser/Thre nuclear protein kinase), CYP1/CYP2 (steroid 21 hydroxylase) and TNXA/TNXB (extracellular matrix protein X), which together with the C4 genes, constitute the RCCX genetic module.

RCCX is found in mono-, bi-, and trimodular form containing 1, 2 or 3 copies of C4 genes (80, 246) (Figure 6).

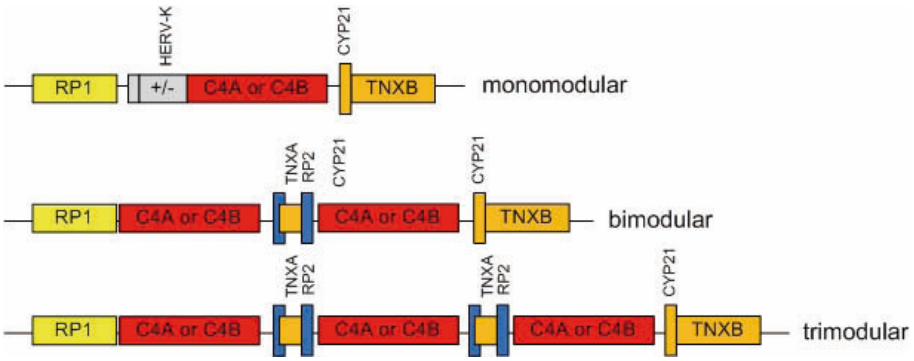

Figure 6. The RCCX genetic module with 1,2 and 3 copies of C4 genes.

The C4 protein

C4A and C4B protein allotypes

Human C4 is the most polymorphic protein of the complement system and >41 alleles have been identified including null alleles for both C4A (C4AQ0) and C4B (C4BQ0) (244, 247, 248).

Functional differences of C4A and C4B protein are attributed to differential activities of an internal thioester bond or thioester carbonyl group. The basis of the differing reactivities lies in four amino acid residues at positions 1101, 1102, 1105 and 1106, located in the C4d region of the C4 genes (249).

The C4A isotypic residues modulate activity of the reactivity of the thioester bond of activated C4A molecule to efficiently form a covalent amide bond. C4A binds more readily to amino groups, such as those found on ICs and amino-rich antigens (250).

The C4B isotypic residues catalyze the formation of a covalent ester bond between the thioester carbonyl of activated C4B and hydroxyl groups. C4B binds more readily to hydroxyl groups and therefore has more hemolytic activity (251).

In plasma, C4 consists of three polypeptide chains, α, β and γ and is encoded as a single-chain precursor with polypeptides in the order β, α, γ. The products (1725 amino acids) of the C4A and C4B genes differ by less than 1%.

Deficiency of C4A (C4AQ0)

Complete deficiency of C4 requires homozygote null alleles for both C4A (C4AQ0) and C4B (C4BQ0). Complete deficiency of C4 is strongly associated with SLE and lupus-like manifestations (232, 252, 253).

Partial deficiency of C4 due to C4AQ0 has been associated with SLE (6, 254-256) and at least one C4AQ0 allele has been found in up to 50% or more of SLE patients and homozygosity for C4AQ0 is reported in 11-13% of SLE patients (257). In Icelandic SLE patients the frequency of C4AQ0 is 50% compared to 25% in the population (254, 255).

C4AQ0 is primarily associated with SLE, but has also been reported in primary Sjögren´s syndrome (258), autoimmune hepatitis (259), type 1 diabetes (260, 261) and systemic sclerosis (SSc) (262).

C4AQ0 alleles have been identified in varying frequency in most populations; 20-25% in Caucasians (254, 263, 264), 25% in Koreans (265), 1,5% in Malaysians (266), but not found in a control group of Thais (267).

Molecular basis of C4AQ0

C4AQ0 defines C4A protein deficiency as determined by protein electrophoresis, irrespective of the underlying genetic change. The molecular basis for C4AQ0 differs from one racial group to another and even within the same ethnic groups.

Among known genetic mechanisms for C4AQ are deletion of the C4A gene (252), deleterious mutations in exon 20 and 29 (252, 267-269), gene conversion at the C4A locus rendering the C4A genes to encode a C4B-like protein (270) and copy number variation, which may be related to the mono-, bi- and trimodular structures of RCCX (205).

C4AQ0 and the MHC

The location of the C4A gene within the MHC and the linkage disequilibrium among MHC genes has made it difficult to verify association of C4AQ0 with SLE susceptibility.

Whether C4AQ0 is directly associated with SLE or is a marker reflecting another disease allele within the MHC has been debated. Family based studies identifying MCH haplotypes associated with C4AQ0 have helped in assessing the strength of the association of C4AQ0 with SLE. To deduce with greater certainty an independent association of C4AQ0 with SLE analysis of the genetic mechanisms underlying the null alleles is important.

Mannan-binding lectin (MBL)

The MBL2 gene

The MBL2 gene encoding MBL lies on chromosome 10. The gene consists of four exons interrupted by three introns. Exon 1 encodes the signal peptide, exon 3 encodes a neck region and exon 4 a CRD, the region that recognizes not only microbial carbohydrates, but also molecular structures on dying host cells, including nucleic acids. The promoter sequence of the MLB2

gene contains several consensus elements, which are believed to regulate the vast majority of the gene expression (271, 272).

The MBL protein

MBL is an oligomer C-type lectin made up of two to six triple helices with collagenous tails that are cross-linked and coiled together and globular heads that contain carbohydrate recognition domains (CRDs). MBL must be polymeric to be optimally functional and monomers are not able to activate the complement system. The affinity of a single CRD is quite low and high-affinity binding requires that several CRDs bind appropriate carbohydrate residues in a three dimensional pattern (273).

Deficiency of MBL

Polymorphisms in the *MBL2* gene (variant alleles), both in the regions encoding the protein itself as well as in the promoter region have been shown to affect serum MBL levels.

The variant MBL alleles are common worldwide, and around 3% of Caucasians are homozygous and a third heterozygous for one of the variant structural alleles (173, 274). In an Icelandic control cohort, the frequency of MBL variant alleles is 33% (171).

Exon 1 minor alleles

Three common polymorphisms at different loci in exon 1 of the *MBL2* gene are correlated with low MBL serum levels (<1000 μg/L); minor or variant alleles B (codon 54), C (codon 57) and D (codon 52).

The variant alleles B, C and D, commonly referred 0 alleles, result in an amino acid substitutions that can interfere with polymeric assembly of the MBL monomers.

All three variant alleles have a profound effect on MBL serum levels, with a decrease in the level of functional MBL by up to 90%, even in heterozygous individuals. The effect of the D allele in heterozygotes is less than that of the B or C alleles. Homozygosity of or combinations of 0/0 minor alleles results in almost complete deficiency of serum MBL (275, 276).

The wild type A allele is associated with high MBL serum levels (>1000 μg/L).

Promoter region polymorphisms

Low MBL serum levels can also be associated with the L and X polymorphism in the promoter region of the *MBL2* gene.

H and Y promoter polymorphisms are associated with higher MBL levels (275, 276).

P/Q promoter polymorphism does not affect serum MBL levels (273).

Co-stimulatory pathways of T cell activation and inhibition

According to the two-signal model, full activation of T cells requires two signals. The first signal is antigen-specific and is provided by the interaction of antigenic-peptide MHC molecules on APCs with the TCR. The second signal is antigen independent and is provided by the interaction of co-stimulatory molecules expressed on APCs and T cell co-receptors (277).

The B7 family of co-receptors

The B7 family of co-receptors are structurally related type I membrane proteins that regulate immune responses in balanced cooperation via three main pathways, the CD28:CTLA4 pathway, ICOS:LICOS interactions and the PD-1 pathway (277-279) (Figure 7).

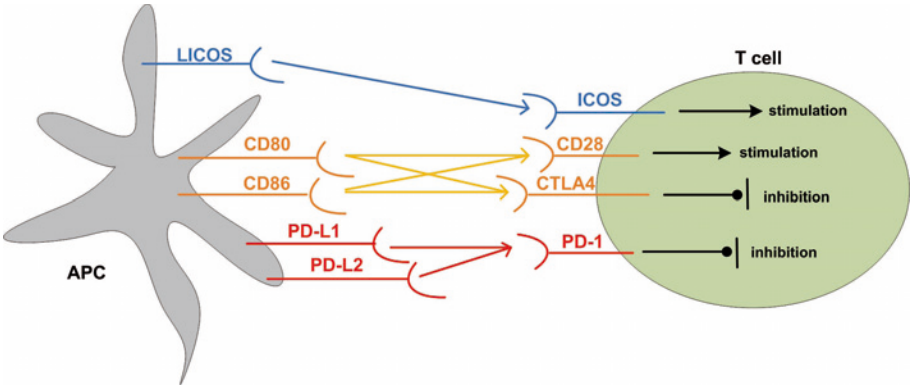

Figure 7. B7 family T cell co-receptors and their APC ligands.

The CD28:CTLA4 pathway

The best characterized of the B7 family co-stimulatory pathways, so far, is the CD28:CTLA4 pathway, which involves interaction of the CD80 and CD86 ligands system on APCs with CD28 and CTLA4 on T cells. Co-stimulatory signals for T cells activation are delivered via CD28 and co-inhibitory signals via CTLA4. CD28 and CTLA4 compete for binding to their APC ligands CD80 and CD86. CTLA4 has greater affinity for CD80 and CD86 than CD28.

CD28 is expressed on T cells and provides co-stimulation signals required for full T cell activation. CD28 is up-regulated on T cells after encounter with antigen and binds to CD80 and CD86, delivering the second signal necessary for T cell proliferation, cytokine production and prolonged T cell survival (280).

CTLA4 is rapidly expressed on T cells following activation and is highly upregulated by CD28 engagement. Ligation of CTLA4 antagonizes CD28 mediated T cell stimulation, leading to decreased IL-2 production, inhibition of cell cycle progression and modulation of TCR signaling (281, 282). CTLA4 plays an important role in preventing autoimmune diseases by promoting long-lived anergy (280).

The critical role of CTLA4 is evident in CTLA4-/- mice, which develop lymphoproliferative disease and die within 3-4 weeks after birth (283). In humans, polymorphisms in the CTLA4 gene have been implicated in susceptibility to several autoimmune diseases including SLE, RA, type 1 diabetes, myasthenia gravis, coeliac disease and SSc (277, 280, 284-286).

CTLA4-Ig, an antagonist of the CD28/B7 co-stimulatory interaction is now in clinical use (Abatacept) for RA patients. CTLA4-Ig is also a potential beneficial drug for SLE patients and has been shown to effectively prevent SLE onset in several murine models and to induce remission of active SLE nephritis (287).

ICOS:LICOS interaction

ICOS (Inducible co-stimulator) is expressed on activated T cells and shares several structural and functional similarities with CD28. Like CD28, ICOS has potent co-stimulatory effects on T cell proliferation and production of cytokines. ICOS is also important for germinal centers formation and clonal expansion of T cells (277, 288).

The PD-1 pathway

The PD-1 pathway consists of the PD-1 receptor and its APC ligands, PD-L1 and PD-L2. PD-1 mediates inhibitory signals which downregulate T and B cell activation (82).

PD-1 is expressed on T cells, B cells, natural killer cells, monocytes and myeloid DCs. PD-1 is not expressed on resting T cells, but is inducibly expressed after activation (289). PD-1 is inducibly expressed on APCs and monocytes, but its function on these cells in not clear (290).

PD-1 is structurally related to CTLA4. The widespread expression of PD-1 suggests that, compared to CTLA4, it may have a broader effect in regulating immune responses. Signalling through PD-1 limits T cells function including IFN-γ production, proliferation and increased apoptosis (291).

The PD-1 ligands differ in expression. PD-L1 is highly expressed on monocytes and found on plasmacytoid and myeloid DCs and T cells and is also expressed on a wide range of non-hematopoietic cells. PD-L1 is upregulated by type I and type II interferons (292). PD-L2 expression is more restricted and PD-L2 is inducibly expressed on DCs, macrophages, and bone marrow derived mast cells.

The CD28/CTLA4 ligand CD80 was recently identified as a binding partner for PD-L1 (293).

The PD-1 pathway

The *PDCD1* gene

The *PDCD1* gene on chromosome 2q37 consists of 5 exons, exon 1 encodes a short signal sequence, exon 2 an Ig domain, exon 3 the stalk and transmembrane region, exon 4 encodes the start of the cytoplasmic domain and exon 5 the C-terminal intracellular residues and a long 3´UTR.

Splice variants of the *PDCD1* gene have been cloned from activated T cells, yielding transcripts lacking exon 2, exon 3, exons 2 and 3 or exons 2, 3 and 4. All transcripts except for the splice variant lacking exon 3 are expressed as full length PD-1 in resting PBMCs and are all increased in expression upon activation of T cells with αCD3 and αCD28 (294).

The PD-1 receptor

The protein structure of PD-1 includes an extracellular Ig domain followed by a transmembrane region and an intracellular tail, which contains two phosphorylation sites. The most membrane proximal tyrosine is located in an immunoreceptor tyrosine-based inhibitory motif (ITIM) and the distal tyrosine is located in an immunoreceptor tyrosine-based switch motif (ITSM).

During cellular activation, the tyrosine residue of the ITIM motif is phosphorylated by the Src homology 2 protein tyrosine phosphatase 2. This leads to deactivation of downstream signaling molecules such as phosphatidylinositol 3-kinase, dephosphorylation of downstream signaling molecules and activation of the Ras/Raf/MEK/Erk pathway leading to G0/G1 cell cycle arrest, providing peripheral control of continued cellular activation (295, 296).

PDCD1 gene polymorphisms and autoimmune diseases

***Pdcd1-/-* mouse models**

The important inhibitory function of PD-1 was first appreciated by the autoimmune-like phenotype of pdcd1−/− mice, which provided the first evidence for PD-1 as a key player in autoimmunity (82, 297, 298).

Pdcd1-/- mice with different genetic backgrounds all developed autoimmune disease with differing phenotypes. C57BL/6 mice with PD-1 deficiency were found to have increased incidence of glomerulonephritis and exhibited arthritis, with synovial cell proliferation, lymphocyte infiltration, and pannus formation (297). The phenotypes resembled the clinical manifestations of SLE and RA in humans. In contrast, PD-1-deficient BALB/c mice developed autoimmune cardiomyopathy, with IgG deposition in the heart (298).

PD-1 gene polymorphisms in humans

To date over 30 polymorphisms have been identified in the human *PDCD1* gene, some of which have been associated with different autoimmune diseases, including SLE (91, 93, 299), RA (84, 87, 92), type 1 diabetes (89, 90, 300, 301), Graves´ disease (83, 88), ankylosing spondylitis (AS) (86), primary biliary cirrhosis (PBC) (302) and MS (85).

The PD-1.3A polymorphism

PD-1.3A was the first polymorphism in the *PDCD1* gene to be associated with an autoimmune disease in humans (81).

A genome scan in 8 Icelandic multicase SLE families identified five chromosomal regions with lod-scores indicative of possible linkage to SLE, among them 2q37. In 6 of the 8 families there was a high frequency of C4AQ0 (254) and the presence of C4AQ0 was the basis for stratification. The 2 families that do not have C4AQ0 were excluded from further analysis. This reduced heterogeneity for chromosome 6 alleles considerably and lod-scores for a number of regions increased, in particular at 2q37 (69).

The 2q37 region was replicated in Swedish multicase SLE families and a pooled analysis of the Icelandic and Swedish SLE family sets gave a combinational effect and a highly significant lod-score of 4.24. Fine mapping in the region indicated the location of the locus most likely to be associated with SLE. The locus was denoted as SLEB2 (303).

The PD-1.3 polymorphism is a G→A change. PD-1.3 is located in intron 4 of the of the PDCD1 gene and is thought to alter a binding site of the runt-related transcription factor (RUNX1/AML1), which modulates transcription of the PDCD1 gene, suggesting a mechanism through which it could contribute to the development of SLE in humans.

The association of PD-1.3A with SLE has been replicated in SLE cohorts of different ethnicity. The association is strongest in Caucasian populations, but also found in other populations, for instance Mexican and African-American SLE patients (77, 81, 93, 299, 304-306). PD-1.3A has, furthermore, been found to be associated with other autoimmune diseases, such as RA (84, 87, 92), type 1 diabetes (89, 90), MS (85), AS (86) and Graves´ disease (83, 88) (Table 4).

There are, however, reported non-associations of the PD-1.3A allele with SLE and other autoimmune disease. In a Spanish cohort, a lower frequency of PD1.3A was found in SLE patients compared to population controls (307) and PD-1.3A was not associated with SLE in African-American patients (81).

The discrepancies in association and non-association with autoimmune diseases may reflect ethnic differences. Geographical variation is, for instance, apparent in the frequency of the PD-1.3A allele across Europe, with decreasing frequency form north to south (77) demonstrating the importance of studying relatively homogeneous populations.

Table 3. *A list of studies reporting association and non-association of PDCD1 gene polymorphisms with different autoimmune diseases.*

Autoimmune disease	***PDCD1* gene polymorphism**	**Association**	**Population / Ethnicity**	**Ref. no**
SLE	PD-1.3A	Yes	Icelandic, Swedish	(81)
		Yes	Mexican	(81)
		Yes	European and African-American	(305)
		Yes	Swedish	(309)
		Yes	Caucasian	(304)
		Yes	Northeast Europe	(77)
		No	African-American	(81)
		No	Spanish	(307)
		No	Southwest Europe	(77)
	Other	Yes	Danish	(91).
		Yes	Taiwan	(93)
		Yes	Hispanic	(304)
		Yes	Polish	(299)
		Yes	Han Chinese	(310)
		No	Taiwan	(87)
		No	Asian	(304)
		No	African-American	(304)
		No	Polish	(299)
RA	PD-1.3A	Yes	Swedish	(92)
		No	Japanese	(311)
	Other	Yes	Taiwan	(87)
		Yes/No	Chinese	(84)
		No	Japanese	(311)
Type 1 diabetes	PD-1.3A	Yes	Danish	(90)
	Other	Yes/No	Japanese	(301)
		Yes	Japanese	(89).
		No	Swedish	(312).
		Unclear	UK	(313)
Graves' disease	Other	Yes/No	UK Caucasian	(88).
AS	Other	Yes	Korean	(86)
MS	PD-1.3A	Yes	German	(85)
PBC	PD-1.3 A	Yes	USA	(302)

Taken together, there is accumulated evidence supporting association of different *PDCD1* gene polymorphisms with SLE and other autoimmune diseases.

To date, there is, to our knowledge, only one published study on cellular expression of the PD-1 receptor in humans (308). Paper III describes PD-1 cellular expression on T cells in correlation to the PD-1.3A in Icelandic and Swedish SLE patients.

Polymorphisms in the PD-ligand genes

Studies on the association of polymorphism in the genes for PD-L1 and PD-L2 have shown controversial results, which may again reflect ethnic and population differences.

The genes encoding PD-L1 and L2 are located in close proximity on chromosome 9p24. A genome scan of Icelandic multicase SLE families showed suggestive linkage to a marker located close to the PD-ligand genes. However, analysis of SNPs within the PD-ligand genes on Swedish, Mexican and Argentinean trios as well as independent sets of patients and controls from Sweden did not support genetic association of PD-ligands to SLE (314). A study on SLE patients in Taiwan, on the other hand, found that a PD-L2 gene polymorphism may be associated with susceptibility to SLE, but no significant association with PD-L1 polymorphisms was found (315).

The present investigation

Aims

- To study the genetic basis of C4A protein deficiency (C4AQ0) and its associated MHC haplotypes in Icelandic multicase SLE families.
- To study the association of low MBL serum levels with SLE in Icelandic multicase SLE families having a C4AQ0 susceptibility background.
- To study cellular expression levels of the PD-1 receptor on T cells in SLE patients, relatives and controls and correlate with the PD-1.3A polymorphism.
- To study autoimmune diseases in Icelandic multicase SLE families and the association of three SLE susceptibility factors, C4AQ0, PD-1.3A and low MBL with SLE and other autoimmune diseases.

Study cohorts

The papers presented in this thesis are based on analysis of SLE patients and relatives from Icelandic multicase SLE families and Swedish SLE patients.

- Paper I: Nine Icelandic multicase SLE families, 26 SLE patients and 73 first- and 14 second-degree non-SLE relatives.
- Paper II: Nine Icelandic multicase SLE families, 24 SLE patients, 83 first- and 23 second-degree non-SLE relatives and as an ethnically and age matched control group, 24 unrelated family members (spouses and in-laws).
- Paper III: Icelandic and Swedish SLE patients, their relatives and unrelated healthy non-autoimmune controls. In total 14 SLE patients, 7 relatives and 16 controls.
- In paper IV: Eight Icelandic multicase SLE families, 23 SLE patients and 101 first- and second-degree relatives and population control cohorts.

The number of SLE patients and relatives studied in the papers varies depending on life samples and clinical information available at the time of study.

Icelandic multicase SLE families

A multicase family is defined as a family with two or more affected individuals of third degree relation or less (Figure 8). Families 7 and 9 include SLE patients of more distant relation. The patients with SLE fulfilled 4 or more of the ACR 1982 classification criteria for SLE.

The Icelandic multicase SLE families originate from different parts of the country and they vary in size. The two largest families originate from areas in Iceland that were geographically isolated up until the middle of the 20th century, in the North-West and East.

Data for both the SLE patients and their relatives has been systematically collected; clinical data from hospital records and detailed information on autoimmune diseases based on diagnosis of rheumatologists. These families have furthermore been analyzed for immunological parameters; autoantibody profiles and complement component C4.

Detailed genealogical information about Icelanders dating back as far as 250-300 years is accessible, making it easy to trace ancestry and relations back several generations. Relations of 250 SLE patients diagnosed in Iceland from 1975 have identified multicase SLE families. Nine of them have been analyzed extensively and are the basis for several studies.

The Icelandic population, of 320.000, is almost entirely Caucasian and is today still remarkably homogeneous. Most Icelanders are descendants of

Norwegian settlers and Celts from Ireland and Scotland brought over as slaves during the age of settlement (874-930). DNA analysis suggests that around 66% of the male settler-era population was of Norse ancestry, whereas the female population was 60% Celtic (316).

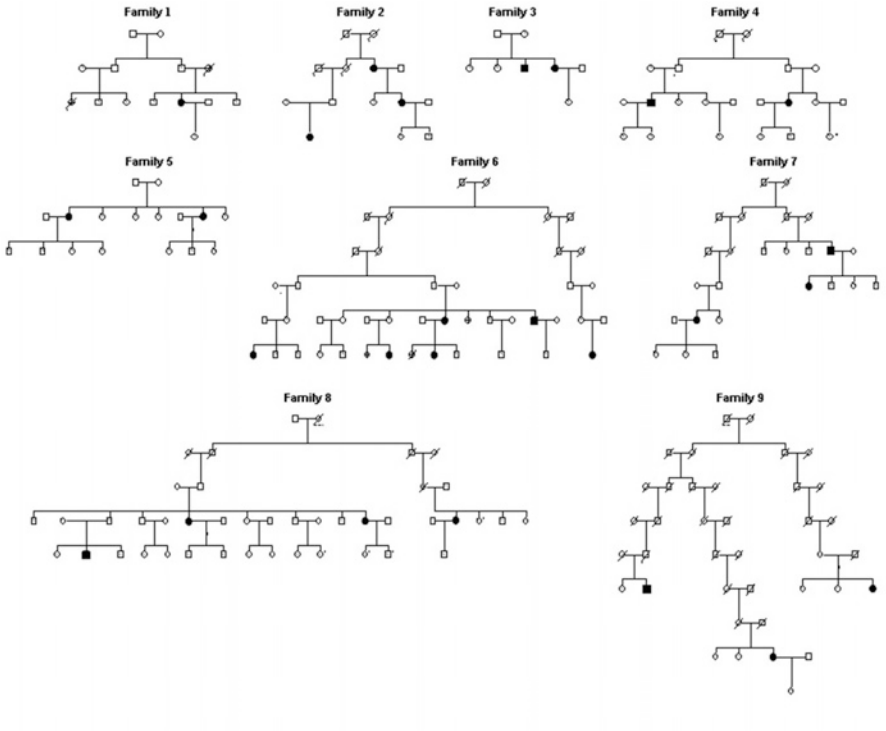

Figure 8. Pedigrees of Icelandic multicase SLE families. SLE patients are shown in black.

Materials and methods

Analysis of complement component C4

C4 allotyping

C4 allotypes were analyzed by high voltage agarose electrophoresis on caboxypeptidase (Sigma, Type I) and neuraminidase (Sigma, Type VIII) treated serum samples followed by immunofixation with monoclonal antibodies (Incstar).

C4A and C4B allotypes were determined by the relative intensities of C4A and C4B bands with visual inspection and comparison to control samples. Zygosity was corroborated using haplotype analysis.

C4A deletion genotyping by long range PCR

A novel long range PCR method was applied to detect deletion of the C4A gene. The method is based on two separate PCR assays, one deletion specific and one specific for non-deleted C4A genes as described by Grant, Kristjánsdóttir et al (317).

A universal forward primer was used for both assays. The primer is located in the RP1 (G11) gene, which is the closest unique sequence to the C4A gene.

A reverse primer in exon 10 of the C4A and C4B genes is specific for deleted C4A genes. The C4B1 gene, which is consistently found with C4A gene deletion is short and lacks the 6.5 HERV-K(C4) retroviral insert in intron 9. In case of a deleted C4A gene a 5.4kb PCR product is generated through amplification of the short C4B1 gene. In case of non-deleted C4A genes, which are long and contain the 6.5 viral insert, the expected 11.8 kb PCR product (C4A + C4B) would not be generated since it is too long for the reaction conditions.

C4A genes are long and contain the retroviral insert in intron 9. Deleted C4A genes lack the insert. A reverse primer specific for non-deleted C4A genes anneals at the junction between HERV-K(C4) and the unique sequence of intron 9 and specifically detects non-deleted C4A genes. Non-deleted C4A genes render a 5.2 kb PCR product.

Each PCR reaction was carried out on 100 ng of genomic DNA in a total volume of 20 µl with 0.4 µM concentration of each primer, 200 µM dNTPs and components of the rTth DNA polymerase XL (Perkin Elmer), 1 mM Mg(OAc)2 universal and the associated buffer. Thermal cycling conditions were as follows: 94°C for 30s, followed by 35 cycles of 90°C for 10s, 55°C for 10s and 65°C for 10 min. The rTth polymerase mix has exonuclease activity and therefore the reaction was initiated immediately after its addition.

The products underwent electrophoresis (in the presence of 0.25% bromophenol blue and 40% (w/v) sucrose) on a 0.8% agarose gel (Sigma) in Tris-borate-EDTA (TBE) buffer for 100 V/h.

Screening for mutations in exons 20 and 29 of the C4A gene

PCR methods were applied according to protocol to screen for a point mutation in exon 20 (318) and exon 29 (268) of the C4A gene.

Analysis of HLA alleles and construction of MHC haplotypes

Typing of MHC class I and II alleles

Typing of MCH class I HLA-B allotypes was performed on serum samples using the lymphocytotoxicity test (319) and typing of MHC class II HLA-DR alleles by PCR with sequence specific primers (PCR-SSP) (Dynal) (320). Performed at the Blood Bank of Iceland at Landspítali-University Hospital, Reykjavík, Iceland.

Construction of MHC haplotypes

MHC haplotypes (HLA-B – C4A-C4B – HLA-DR) were constructed using pedigree information to verify typing results. For individuals with incomplete HLA or C4 typing, haplotypes were deduced form first-degree relatives.

Analysis of MBL variant genotypes and serum levels

Genotyping of MBL variant alleles

A real time polymerase chain reaction (RT-PCR) was carried out in the LightCycler (Roche Diagnostics, Mannheim, Germany) (274). In brief, using temperature curve analysis the three mutant structural alleles B, C and D in exon 1 were detected in one reaction. The promoter polymorphisms H→L and Y→X were detected in two reactions: H/L polymorphism by single-color detection and the X/Y and P/Q polymorphisms using dual color probes in one capillary. Performed at the Department of Immunology, Landspítali-University Hospital, Reykjavík, Iceland.

Measurement of serum MBL levels

A sandwich-ELISA was used to measure serum MBL levels (321). Microtiter wells were coated overnight at 4°C with monoclonal mouse anti-human MBL antibody (SSI). Two dilutions of the test sera (1/25 and 1/200) were then incubated for 1 h at room temperature together with a serial dilution of a standard calibrated with highly purified MBL (SSI). Sera that were low in MBL were retested at 1/3 and 1/9 dilutions. Biotinylated Mab (1:8000) was then added for 1 h at room temperature followed by horseradish peroxidase labeled streptavidin (1:8000) (Sigma) for a further 1 h at room temperature. After 5 min incubation with tetramethyl benzidine the reaction was stopped with 0.18 M H_2SO_4 and the absorbance read at 450 nm. The microtiter wells were washed 3 times with PBS/0.5 M NaCl/0.5% Triton-X 100 after each step. Three control sera with low, medium and high MBL concentration were included in each test run to monitor assay variability. The lower detection limit of the assay was 20 µg/L. Serum MBL levels below 1000 µg/L were defined as low MBL levels. Performed at the

Department of Immunology, Landspítali-University Hospital, Reykjavík, Iceland.

Analysis of PD-1

Genotyping of PD-1.3 G/A

PD-1.3 G and A alleles were distinguished using PCR followed by restriction fragment length polymorphism using P*st*I restriction enzyme.

100ng genomic DNA was amplified in 50 µl reaction mixture (2-5µl DNA, 4µl dNTP, 10pmol each primer, 5µl 10X PCR Gold Buffer, 1.5mM MgCl2, 5U AmpliTaq Gold Polymerase (Applied Biosystems). The sequences of primers were: forward primer 5´ CCC CAG GCA GCA AGG TCA AT3´ and reverse primer: 5´ GAC CGC AGG CAG GCA CAT AT3´).

The PCR conditions were: 10 min. at 95°C, followed by 45 cycles at 95°C for 15s, 60°C for 30s and 72°C for 15s and with a final extension for 5 min. at 72°C. PCR product was digested overnight at 37°C with 4U P*st*I restriction enzyme (New England Biolabs) and resolved on a 4% agarose gel.

*Pst*I cuts at GA sites leaving wild-type G alleles uncut resulting in a 180 bp fragment and mutation A alleles cut resulting in 2 fragments of 150 and 30 bp.

Cellular expression of the PD-1 receptor

Isolation, freezing and thawing of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA whole blood by standard density-gradient method using Ficoll-Paque (GE Healthcare Bio-Sciences AB) and suspended in RPMI-1640 media with 10% FCS.

PBMCs were then cryopreserved in DMSO media containing 40% FCS and 20% DMSO. At the time of culture cells were thawed gently in 37°C water bath and transferred to culture media containing RPM1 1640 10% FCS. Cells were centrifuged and washed 2 times to remove DMSO and resuspended in culture media RPM1 1640 10% FCS and cell viability assessed by trypan blue count.

Stimulation and culture of peripheral blood mononuclear cells

PBMCs were stimulated with αCD3 and αCD28 antibodies (R&DSystems) using the following protocol: Culture plates were coated with 10µg/ml αCD3 antibodies and incubated for 3 hours at 37°C and then washed with PBS. 500 µl of 20µg/ml αCD28 in RPMI-1640 10% FCS media were added to each well along with 500µl of $1x10^6$ cells/ml. Final concentration in culture wells was 10µg/ml αCD28 and 0.5x106 cells/ml. Cells were cultured at 37°C and 5% CO_2 for 48 hours.

Flow cytometry

Cell surface expression of markers was analyzed by flow cytometry. At t0 and after 48 hours of culture cells were stained with fluorescent monoclonal antibodies against PD-1, CD3, CD4, CD8, CD25 and IgG isotype controls (BDPharmingen). Briefly, 100 µl of cells were added to tubes containing antibody cocktails and incubated on ice for 30 min. in the dark. The cells were then washed twice with PBS and finally resuspended in 0,5% paraformaldehyde.

FoxP3 expression was analyzed by intracellular staining according to the manufacturers' protocol using FoxP3 antibody and FoxP3 staining buffer set (Fixation/Permeabilization diluent and 10X Permeabilization buffer) (eBioscience). Briefly, surface-stained cells were resuspended in 1 ml of FoxP3 fixation/permeabilization buffer and incubated at room temperature in the dark for 30 min.. Following centrifugation cells were washed twice and then incubated for 20 min in FoxP3 permeabilization buffer. FoxP3 antibody or isotype control was added to the cells and incubated at 4°C for 30 min. in the dark. Cells were then washed twice in FoxP3 permeabilization buffer and finally resuspended in flow cytometry staining buffer (eBioscience).

Cells were harvested on a FACSCalibur machine and 10000 cells collected per staining. FACS data was analyzed using FlowJo® software. Data is presented as the frequency or percentage of cells within analysis gate and geometric mean of fluorescence intensity (MFI) on a histogram.

Evaluation of autoimmune diseases and autoantibody profiles

Protocol for evaluation of autoimmune diseases

Following a predefined protocol, involving interviews and physical examination and review of medical records by two rheumatologists, all family members were evaluated for the presence of SLE and autoimmune diseases.

All SLE patients fulfilled 4 or more of the 1982 ACR classification criteria for SLE. Autoimmune disease refers to the diagnosis of autoimmune diseases other than SLE and does not include family members having only autoimmune manifestations not fulfilling criteria for an autoimmune disease or having only autoantibodies.

Autoantibody profiles

The following panel of autoantibodies was measured: ANA, ENA (RNP, Sm, SSA (Ro), SSB (La), Scl-70, centromere, Jo-1), anti-dsDNA, rheumatoid factor by agglutination and by an isotype specific ELISA (IgA, IgG, IgM), in addition to antibodies against striated and smooth muscle (SMA), mitochondria, thyroglobulin (TG), thyroid peroxidase (TPO), and

gastric parietal cells. Performed at the Department of Immunology, Landspítali-University Hospital, Reykjavík, Iceland.

Statistical analysis

- Paper II: For statistical comparison of the frequency of C4AQ0 and low MBL in groups of SLE patients, autoimmune and non-autoimmune relatives and controls, p-values and odds ratios were calculated using Fisher´s exact test. Non-parametric tests were used for analysis of MBL serum levels as these do not fulfill normal distribution, Mann-Whitney Rank Sum test was used to compare two groups and Kruskal-Wallis Analysis of Variance on Ranks for comparison between three groups. All tests were two-sided and the level of significance was set at $p < 0.05$.
- Paper III: The mean frequency of cells expressing PD-1, CD4, CD8 and CD25 and the geometric mean of MFI was calculated for each SLE patient, relative and control. The mean value for the groups of SLE, relatives and controls were compared using paired t-test. Fisher's exact test with two-tailed probability was used for comparison of groups and paired t-test for intra-sample comparison. Significance level was set at 0.05.
- Paper IV: For statistical comparison of the frequency of PD-1.3A, C4AQ0 and low MBL in groups of SLE patients, autoimmune and non-autoimmune relatives and controls, p-values and odds ratios were calculated using Fisher´s exact test with two-tailed probability and significance level set at 0.05.

Paper I: A study of the genetic basis of C4A protein deficiency. Detection of C4A gene deletion by long range PCR and its associated haplotypes

Background and aims

C4AQ0 has been found in varying frequency on different MHC haplotypes in several ethnic groups. In Caucasians the most common C4AQ0 haplotype is the conserved HLA-B8-C4AQ0-C4B1-HLA-DR3 haplotype, which has been associated with a deletion of the C4A gene (254, 322-324).

An earlier study on Icelandic multicase SLE families established an association of C4AQ0 with SLE. In this initial study, the frequency of C4AQ0 (homo- and heterozygote) was found to be significantly increased in SLE patients (50%) compared to a population cohort (25%). In the same study, analysis of MHC haplotypes identified 12 C4AQ0 bearing haplotypes revealing weaker linkage of C4AQ0 with HLA-DR3 than reported in other studies (254).

These results pointed to a heterogeneous genetic background for C4AQ0. In follow-up, the present study was undertaken with the aim to analyze the genetic basis of C4AQ0 with emphasis on determining the frequency of C4A gene deletion. Furthermore, to identify MHC haplotypes associated with the C4A deletion.

C4 “null alleles” are defined by protein electrophoresis (247), relying on visual inspection of protein band intensities to identify heterozygous ”null alleles”. Homozygous “null alleles” are identified by the lack of a protein band. Detection of heterozygous “null alleles” may be inaccurate when relying only on protein band intensity and heterozygous “null alleles” may be missed and the frequency of C4AQ0 underestimated. In this study, a novel long range PCR (LR-PCR) method was applied to verify deletion of the C4A gene (317) (see materials and methods).

Results

Frequency of C4A protein deficiency (C4AQ0)

Analyzed by protein electrophoresis the frequency of C4AQ0 was found to be 53,8% in the SLE patients and 47,9% in first- and 28,6% in second-degree relatives. C4AQ0 homozygosity was found in 10,6% of the family members, 11,5% of the SLE patients and 12,3% of first-degree relatives.

Frequency of C4A gene deletion

LR-PCR analysis verified a C4A gene deletion as the genetic basis for C4AQ0 in 60,4% of the family members having C4AQ0, accounting for 64,3% of C4AQ0 alleles in the SLE patients, 60,0% in first-degree and 50,0% in second-degree relatives.

Based on LR-PCR results, discrepancies were revealed in our initial C4 protein electrophoresis analysis of C4AQ0, which had not detected some heterozygous C4A 'null alleles'.

C4A gene deletion haplotypes

Analysis of MHC haplotypes confirmed C4A gene deletion in association with the classical B8-C4AQ0-C4B1-DR3 haplotype, which was found in 6 of the 9 families. The haplotype accounted for C4A deletion in 87,5% of family members; 88,9% in the SLE patients, 90,5% in first-degree and 50,0% in the second degree relatives having C4AQ0.

Furthermore, two variant forms of the classical C4A deletion haplotype were found to carry C4A deletion, one having DR7 instead of DR3 and one having B7 instead of B8.

The DR7 variant haplotype was found in 4 family members in one family and accounted for 12,5% of C4A deletion.

The B7 variant haplotype was found in 1 second-degree relative. The haplotype penetrated the family through marriage.

Discussion

A C4A gene deletion was found to be the genetic mechanism of 60% C4AQ0 alleles. Other, as yet undefined, genetic changes thus account for one-third of C4AQ0. Point mutations in exons 20 or 29 of the C4A gene were not found to account for C4AQ0.

C4A gene deletion was found on 3 MHC haplotypes, further suggesting that C4AQ0 is independently associated with SLE and not a marker in linkage disequilibrium with other MHC alleles, such as HLA-DR3. In addition to the classical deletion haplotypes, two other haplotypes carry a C4A gene deletion.

In summary, the data from our initial (254) and present studies confirm a heterogeneic background for C4AQ0 in association with different MHC haplotypes with varying HLA class I and II alleles. This is despite a strong disequilibrium among genes within the MHC.

Original studies identifying association of C4AQ0 with SLE were criticized on the basis that the association was secondary to segregation of C4AQ0 with the HLA-B8-DR3 haplotype. However, C4AQ0 on varying MHC haplotypes has been found to be associated with SLE in different ethnic groups suggesting that the association is genuine (232).

Taken together, our results give support for the hypothesis that C4AQ0 may be an independent susceptibility factor for SLE, irrespective of the genetic basis for C4AQ0 and other MHC alleles. C4A protein deficiency may be one of several genetic factors contributing in unison with other genetic factors to the pathogenesis of SLE.

Paper II: Mannan-binding lectin and deficiency of complement C4A in Icelandic multicase families with systemic lupus erythematosus

Background and aims

Low MBL serum levels have been associated with increased susceptibility to infections (176, 325) and to some extent with autoimmune diseases (326-328). With two exceptions (329, 330), previous studies on different populations have shown association of low MBL with SLE (170, 172, 174, 218-224).

MBL deficiency is largely genetically determined and low MBL serum levels are correlated with polymorphisms in the *MBL2* gene. Three common polymorphisms in exon 1, i.e. variant alleles B, C and D (designated 0) are correlated with low MBL serum levels (<1000μg/L). The wild type allele A is correlated with serum MBL levels >1000μg/L. Low MBL serum levels can also be associated with the L and X promoter region polymorphism, while the H and Y promoter polymorphisms are associated with higher MBL levels (275, 276).

The aim of the study was to analyze the frequency of MBL variant alleles and serum MBL levels in the Icelandic multicase SLE families and determine whether low MBL confers increased risk of SLE independently or in the setting of C4AQ0.

This is, to our knowledge, the first study analyzing whether low MBL confers increased risk for SLE in families that are predisposed to the disease.

Results

Correlation of MBL genotypes with MBL serum levels

Low MBL genotypes, were strongly associated with low MBL serum levels (<1000 μg/l) and there was no evidence of MBL consumption in the SLE patients. MBL concentration of 1000 μg/l clearly distinguished between family members with and without low MBL genotypes.

Frequency and distribution of low MBL genotypes and C4AQ0 in the families

In the nine multicase families, low MBL genotypes were found in 38% of the SLE patients, 26% of first-degree and 13% of second-degree relatives and in 33% of the non-consanguineous family members compared to 33% in the population cohort. Thus, low MBL showed only a marginally significant trend for SLE in the nine families pooled.

However, C4AQ0 was significantly increased in frequency in seven of the nine families compared to population controls and was found in 50% of the SLE patients, 47% of first-degree and 39% of second-degree relatives compared to 25% in the population cohort.

Analysis of "low MBL" families

In the course of analyzing the data, it became apparent that the distribution of low MBL genotypes and low serum MBL levels was uneven and was restricted to five of the families. These five families are designated "low MBL families".

Analysis of the "low MBL families" showed a significantly higher frequency of low MBL genotypes in SLE patients compared to the relatives groups; 64% of the SLE patients, 38% of first-degree relatives, but none of the second-degree relatives had low MBL genotypes.

In accordance with the increased frequency of low MBL genotypes in the SLE patients, serum MBL levels were also much lower in the SLE patients compared to first-degree and second-degree relatives.

Frequency of the combination of C4AQ0 and low MBL

The combination of C4AQ0 and low MBL was present in 36% of the SLE patients, 18% of first-degree relatives and no second degree relative and was significantly increased in frequency in the SLE patients compared to the relatives.

Discussion

When this paper was published, it was the first study on MBL in multicase SLE families. Our results for all of the nine families pooled, which showed that low MBL only tended to be associated with SLE, are in accordance with previous studies showing a weak association between low MBL and SLE. SLE patients had an increased frequency of variant MBL structural alleles in the *MBL2* gene compared to their close relatives, and a trend of stepwise decrease from the patients to first-degree to second-degree relatives was significant.

The families showed heterogeneity with regards to distribution of low MBL and when we stratified on the basis of low MBL, only five of the families were found to carry the low MBL genotypes and have low MBL serum levels.

Stratification of the families for the presence of variant MBL alleles, however, gave results indicating that variant MBL alleles are a risk factor for SLE in predisposed families. In the "low MBL families", low MBL was found to be significantly associated with SLE.

Our conclusion is that MBL deficiency is not sufficient to cause SLE, but adds to SLE susceptibility in genetically susceptible individuals. An additive

effect of a combination of C4AQ0 and low MBL (219, 221) or low MBL and FcγR polymorphisms (113) has been reported.

The Icelandic families have a predisposing genetic background of C4AQ0 (254, 256) and the combination of C4AQ0 and low MBL genotypes was found to confer an additive effect in SLE susceptibility. C4AQ0 may be part of a predisposing background, on which low MBL or other susceptibility factors can trigger disease expression.

The additive effects of C4AQ0 and low MBL support the hypothesis that multiple defects in the complement system can lead to accumulation of apoptotic debris and ICs, mediating autoimmune triggering, inflammation and disease expression.

Paper III: Lower expression levels of the PD1 receptor on CD4+CD25+ T-cells in SLE patients and correlation to the PD-1.3A genotype

Background and aims

The PDCD1 gene codes for the co-inhibitory immunoreceptor PD-1, suggested to play an important role in maintenance of peripheral self-tolerance in T and B cells.

PD-1.3A was the first PDCD1 gene polymorphism to be associated with autoimmune disease in humans. It was initially found to be associated with SLE in Icelandic and Swedish multicase SLE families and replicated in Mexican SLE families (81). More recently, these findings have been replicated in different populations (77, 93, 299, 304-306, 309). Moreover, other polymorphisms in the *PDCD1* gene have been identified in SLE and other autoimmune diseases (81, 83, 86-88, 90, 92, 93, 307, 309).

Pdcd1 knockout mice have been shown to develop an SLE-like disease (82, 297, 298) and hypotheses regarding to role of PD-1 in SLE and other autoimmune diseases in humans have, so far, been based on mouse models.

The PD-1.3A allele is thought to disrupt the predicted DNA-binding site for the RUNX1 transcription factor and may lead to aberrant expression of the PDCD1 gene (81).

The aim of the present study was to analyze cellular expression of the PD-1 receptor in SLE patients, relatives and non-related healthy controls and correlate with PD-1.3A.

Results

PD-1 expression on PBMCs

At t0 and on unstimulated PBMCs PD-1 receptor expression was low, but was significantly increased following activation with αCD3 and αCD28. PD-1 expression was significantly lower in the SLE patients and marginally lower in the relatives compared to controls.

PD-1 expression was increased 2.1-fold in the SLE patients, 3.1-fold in the relatives and 5.7-fold in the controls. The frequency of PD-1 expressing cells and PD-1 expression levels were significantly lower in the SLE patients compared to controls. The relatives, however, showed normal numbers of PD-1 expressing cells, but significantly lower PD-1 expression levels compared to controls.

PD-1 expression on CD4+ and CD8+ T cells

PD-1 expression was induced on both CD4+ and CD8+ T cells after stimulation and was significantly lower on CD8+ T cells compared to CD4+ T cells in the SLE patients and controls.

PD-1 expression on CD4+ was significantly lower in both the SLE patients and relatives compared to controls, but not significantly different in the SLE patients and relatives.

PD-1 mean MFI on CD8+ cells was significantly lower in the SLE patients compared to the relatives and was non-significantly lower in the relatives compared to controls.

PD-1 expression on CD4+CD25+ T cells

CD4+CD25+ T cells expressed high levels of PD-1, while CD4+CD25- T cells did not and there was an increasing gradient of PD-1 expression directly correlated to increased CD25 expression.

PD-1 expression on CD4+CD25+ T cells was significantly lower in both the SLE patients and the relatives compared to controls.

PD-1 was expressed on both FoxP3+ and FoxP3- cells and significant differences were not seen in the SLE patient, relatives and controls.

Correlation of PD-1 expression to PD-1.3A

For genotype correlation of PD-1 receptor expression, the SLE patients and relatives were pooled and A/G SLE and relatives, G/G SLE and relatives and G/G controls were compared.

Lower PD-1 expression levels were seen in A/G SLE and relatives compared to G/G SLE and relatives.

PD-1 MFI on CD4+ and CD8+ T cells was markedly lower in A/G SLE and relatives compared to SLE patients G/G SLE and relatives, who in turn showed lower PD-1 MFI than G/G controls.

On CD4 + CD25+ T cells, significantly lower PD-1 expression was correlated with the PD-1.3A/G genotype. PD-1 MFI on CD4+CD25+ T cells from A/G SLE and relatives was significantly lower compared to G/G SLE and relatives and G/G controls.

A significant difference in PD-1 expression levels was not seen between G/G SLE and relatives and G/G controls.

Discussion

Analysis of PBMCs revealed significantly lower PD-1 expression in SLE patients and relatives, indicating a general effect on different cell populations, possibly CD4+ and CD8+ T cells, B cells and monocytes. The study, however, focused on CD4+ T cells, but inappropriate activation or non-inhibition of autoreactive T cells may lead to loss of self-tolerance.

Higher PD-1 levels were correlated with increased expression of CD25 on CD4+ T cells, with significantly higher PD-1 MFI levels on CD4+CD25 ^{high} T cells. PD-1 expression was significantly lower in the SLE patients and the relatives compared to controls regardless of CD25 expression levels.

The frequency of PD-1 expressing cells and PD-1 expression levels were significantly lower in the SLE patients compared to controls, while the relatives showed normal numbers of PD-1 expressing cells, but significantly lower PD-1 expression levels compared to controls. Although there is heterogeneity in cell surface receptor expression, the observed differences in distribution between SLE patients and relatives compared to controls were statistically significant and quite clear with regards to PD-1 expression.

High PD-1 expression levels on CD4+CD25^{high} cells are consistent with prior studies of PD-1 expression on T-regs (331, 332). Therefore, we addressed the question of whether the CD4+CD25+ T cells expressing PD-1 are effector T cells or T-regs or both. T-regs may be distinguished as CD4+CD25^{high} cells and some studies use only this marker to identify them. Expression of the intracellular FoxP3 transcription factor is, however, a more reliable marker for T-regs. We studied PD-1 expression and FoxP3 expression on CD4+CD25+ cells and found that both FoxP3- T effector cells and FoxP3+ T-regs express PD-1.

CD4+CD25+ T cells expressed high levels of PD-1, while CD4+CD25- T cells did not. PD-1 expression on CD4+CD25+ T cells was significantly lower in both SLE patients and relatives compared to controls. This observation could not be explained by lower numbers of CD4+CD25+ T cells and strengthened our belief that PD-1 expression was irregular in SLE patients and relatives, and possibly associated with the PD-1.3A allele.

The final part of the study was a genotype-correlated analysis of PD-1 expression. Here we were faced with a problem, a lack of individuals homozygous for PD-1.3A and a lack of PD-1.3A/G controls. This fact is, however, also an interesting point and displays the significant difference in PD-1.3 A and G allele distribution in SLE patients and relatives compared to non-related healthy controls.

Our data shows that lower PD-1 expression is correlated to PD-1.3A, indicated in lower PD-1 expression levels in A/G SLE and relatives compared to G/G SLE and relatives and a non-significant difference between G/G SLE and relatives and G/G controls.

The data points to PD-1.3A having an affect on cellular expression of the PD-1 receptor and that PD-1.3A may contribute to loss of self-tolerance and in unison with other genetic and/or environmental factors lead to autoimmunity. In theory, both SLE patients and relatives having the PD-1.3A/G genotype may be genetically programmed for low PD-1 expression, but in the absence of other genetic or environmental factors, autoimmunity will not ensue. In individuals with SLE or other autoimmune disease, however, other genetic factors may be present leading to defects in other immunological pathways.

Paper IV: Association of three systemic lupus erythematosus susceptibility factors, PD-1.3A, C4AQ0, and low levels of mannan-binding lectin, with autoimmune manifestations in Icelandic multicase systemic lupus erythematosus families.

Background and aims

The focus of genetic studies of complex diseases, such as autoimmune diseases, has been on identifying single susceptibility factors in relation to a specific disease or disease phenotype. Susceptibility genes and loci have been identified, many of which show ethnic and/or population variability.

There is increasing evidence for the clustering of multiple autoimmune diseases in families and for the overlapping of disease susceptibility loci between different autoimmune diseases (16, 78, 333-336). This may be taken to suggest common genetic factors for autoimmune diseases (18) where an assortment of susceptibility genes, which overlap in different autoimmune diseases, may lead to different disease phenotypes depending on the genetic composition.

In our studies on Icelandic multicase SLE families we have identified a heterogenetic susceptibility background composed of three susceptibility factors; PD-1.3A (69, 81), C4AQ0 (4, 254) and low MBL (171). Additionally, there have been indications of increased frequency of autoimmune diseases in relatives of the SLE patients in these families.

This study was undertaken to study further autoimmune diseases other than SLE in these families and analyze the relationship of the three susceptibility factors and their possible interplay in susceptibility to autoimmune diseases.

The strength of the studies lies in extended, well-defined multicase SLE families and a high participation rate, enabling a detailed study on autoimmune diseases and autoantibody profiles in the multicase SLE families and their association with PD-1.3A, C4AQ0 and low MBL.

Results

Autoimmune diseases in the multicase SLE families

A high frequency of autoimmune diseases other than SLE was seen in the families. Of all 124 family members, 18.5% had SLE. Autoimmune diseases other than SLE were found in 13.7% of all family members or 16.8% of the relatives. Thus, in total, 32.2% of all family members had SLE or other autoimmune diseases.

The frequency of SLE and autoimmune diseases in the families ranged from 14.3% to 30% for SLE and from 14.3% to 50% for the other autoimmune diseases.

Various other autoimmune diseases, one or more, were found in 16.8% of the relatives and in 13,7% of the SLE patients themselves.

ANA and/or other autoantibodies were found in 53.2% of all family members, 42.6% of the relatives, and 100% of the patients with SLE. In total, 59.7% of family members had SLE, other autoimmune diseases, ANA, and/or other autoantibodies.

Frequency of PD-1.3A, C4AQ0 and low MBL in the families

The families show heterogeneity for the frequency of PD-1.3A, C4AQ0 and low MBL, which ranged from 0-85% in the families.

C4AQ0 was found in all eight families, in a frequency of 13-85% and was significantly increased in five of the families compared to a frequency of 25% in controls.

PD-1.3A was found in 6 of the families, in a frequency of 25-70% and was significantly increased in five of them compared to a frequency of 10% in controls.

Low MBL was found in seven of the families, in a frequency of 16-60% and was marginally increased in frequency in two of the families compared to a frequency of 33% in controls.

All three susceptibility factors were found in five of the families and the other three families had two factors present.

Frequency of PD-1.3A, C4AQ0 and low MBL in the SLE patients and the relatives

The frequency of both PD-1.3A and C4AQ0 was significantly increased in the SLE patients, the autoimmune- and non-autoimmune relatives compared to controls. The frequency of low MBL was not different in the SLE patients, the autoimmune- or non-autoimmune relatives compared to controls, in all the eight families pooled.

However, analysis of “low MBL families” showed a significantly higher frequency of low MBL in the SLE patients compared to the autoimmune- and non-autoimmune relatives and controls. The most significant difference was seen between the SLE patients and the autoimmune relatives, but the frequency of low MBL was decreased in the autoimmune relatives.

Additive effects of the susceptibility factors were analyzed. After subdivision of the relatives into autoimmune and non-autoimmune, the groups were too small for analyzing potential additive effects of combinations of the three susceptibility factors. We did, however, find that 91% of the SLE patients, 78% of the autoimmune and 75% of the non-autoimmune relatives carry at least one of the factors.

Association of PD-1.3A, C4AQ0 and low MBL with autoantibodies

Association of PD-1.3A, C4AQ0 and low MBL with autoantibody profiles was analyzed by comparison of family members having autoantibodies, either only ANA or ANA and other autoantibodies, to family members with no autoantibodies. No significant difference was found between the groups.

Discussion

Familial clustering of SLE is well documented (4-6) and there are also reports of familial aggregation for other autoimmune diseases, such as RA (7, 337), SSc (338) psoriatic arthritis (339) and MS (340).

There is, furthermore, evidence for familial clustering of different autoimmune diseases and there are a number of reports of coexistence of several autoimmune diseases within the same individual (10). Familial clustering of autoimmune diseases in SLE families has mainly been studied in single case cohorts, for instance, the study on the GLADEL cohort, which demonstrated convincingly familial aggregation of SLE, RA and other autoimmune diseases (11).

The present study, is to our knowledge, the first on autoimmune diseases in multicase SLE families. Autoimmune diseases and autoantibodies were searched for in a systematic way and a high frequency of autoimmune diseases and autoantibodies was found both in the relatives as well as in the SLE patients themselves. Compared to an estimated population frequency of 3-8% (1, 2), there was a significantly increased incidence of autoimmune diseases in the families, providing further evidence for familial clustering of multiple autoimmune diseases.

The three susceptibility factors, PD-1.3A, C4AQ0 and low MBL, function within immune pathways and are independently associated with SLE in the families. C4AQ0 is a unifying susceptibility factor for SLE in all the families. PD-1.3A is found in 6 of the 8 families. Low MBL is a minor risk factor for SLE, but shows strong association with SLE in those families carrying the defect.

Both C4AQ0 and PD-1.3A are overall increased in frequency in the families, both in the group of SLE patients and relatives and may be part of a primary genetic susceptibility background for autoimmunity. C4AQ0 is a strong risk factor for SLE, while PD-1.3A has shown broader association with autoimmunity and has been associated with SLE (91, 93, 299), RA (84, 87, 92), type 1 diabetes (89, 90), MS (85), AS (86) and Graves´ disease (83, 88).

This collective analysis of the multicase SLE families shows that they are characterized by a high frequency of autoimmune diseases and autoantibodies and by a strong combined genetic susceptibility background.

General discussion

Most human diseases are now recognized to be genetically complex, that is there are at least several disease predisposing genetic loci, as well as an important role for non-genetic factors (61).

Susceptibility genes are believed to act either in an additive fashion or by way of complex interactions, until a clinical expression threshold is reached. The genetic polymorphisms found to be associated with autoimmune diseases are usually relatively common and may not affect disease susceptibility as long as the combination with other genes and/or environmental factors does not pose a risk (16).

The major genetic factors that have been associated with SLE may be broadly classified, with considerable overlap, based on their function into genes affecting particular arms of the immune response. 1) Genes affecting the innate immune response, such as the genes for complement components and FcγR, 2) genes affecting apoptotis, such as genes for Fas and Bcl-2 and 3) genes affecting the adaptive immune response, such as MHC class II genes, cytokine genes and genes for co-inhibitory receptors, for example PD-1.

The impact of environmental factors in disease development is still unclear, although several hormonal, occupational and life-style factors have been proposed. The fast evolving life-style of humans over the past decades may provide new environmental triggers that unleash the effect of “silent” susceptibility genes.

Complement deficiency in SLE

Complement may be friend or foe, depending on the circumstances. Under physiologic conditions, complement promotes the clearance of ICs. If, however, ICs are not eliminated, then complement becomes chronically activated and can mediate inflammation at sites of IC fallout causing tissue injury (341).

The importance of an intact complement system in the handling of circulating ICs has previously been demonstrated in an Icelandic SLE patient with complete C2 deficiency (C2Q0) and has directly implicated C2 deficiency in disease pathogenesis. Infusion of plasma containing C2 restored binding of IC to CR1 on red blood cells and transport to the

reticuloendothelial system (237). It may be speculated that complete C4 deficiency could predispose to SLE in a similar manner. Complete deficiency is, however rare, but partial deficiency or C4AQ0 is common and associated with SLE (6, 254-256).

C4AQ0 is significantly increased in frequency in SLE patients and their relatives from Icelandic multicase families. The genetic basis of C4AQ0 varies and C4AQ0 is found on different MHC haplotypes (254). A C4A gene deletion accounts for 2/3 of C4AQ0 alleles and the deletion is found on 3 different MHC haplotypes (Paper I).

This points to C4AQ0 as an independent risk factor in SLE. Providing further support is the reported association of C4AQ0 with SLE in different populations and ethnic groups and the varying genetic mechanisms and MHC haplotypes associated with C4AQ0 in these cohorts. For example, in Japanese SLE patients, C4AQ0 is associated with disease, but the genetic background of C4AQ0 differs from that seen in Caucasians. In Japanese cohorts the C4A gene deletion is not found (342). C4AQ0 may thus contribute to the pathogenesis of SLE beyond the ethnical differences.

Yet another point, supporting C4AQ0 as an independent risk factor is the association of other complement deficiency states with SLE, namely deficiency of C1 and low MBL, but the structural genes for C1 and MBL are not located within the MHC.

Low MBL genotypes, which are correlated with low serum MBL levels, are also found to be associated with SLE in the Icelandic families. Unlike C4AQ0, which is found in a high background in most of the families, low MBL is restricted to five of the families. In these “low MBL families”, low MBL is associated with SLE and presents additive risk in combination with C4AQ0 (Paper II).

Prior studies have suggested that low MBL may be a minor risk factor for SLE, but stratifying the families for this particular genetic trait showed a significant association with SLE. This may again reflect the effect of genetic heterogeneity, not only when studying different populations or ethnic groups, but also when studying different families within the same population.

Deficiency of complement components may lead to impaired clearance of apoptotic cells and immune complexes and affect maintenance of self-tolerance.

Deposition of ICs in peripheral tissues may lead to inflammation and tissue damage with increased release of autoantigens initiating a cycle of enhanced autoantibody production, decreased clearance and deposition of ICs, inflammation and tissue damage (237).

Deficiency of classical complement components may further affect self-tolerance through decreased opsonization of target autoantigens with C3d and diminished localization of autoantigens in the bone marrow.

Impaired complement mediated clearance of apoptotic cells may also lead to loss of self-tolerance. Normally in peripheral tissues, apoptotic cells are cleared very early by tissue macrophages, which produce anti-inflammatory cytokines and downregulate the antigen presentation function of DCs. It has also been suggested that immature DCs that have taken up apoptotic cells receive signals from the apoptotic cell, possibly via CR3, that inhibit development of antigen-presenting function of the DC (239, 240). The DC is thus not able to present self-antigens harbored in the apoptotic cell to autoreactive T cells and self-tolerance is preserved.

In complement deficiency states, where apoptotic cells and ICs are not cleared there is both an abundant amount of autoantigens for uptake by DCs and there is also an ongoing inflammatory response or danger milieu. Danger signals, such as pro-inflammatory cytokines, which stimulate DC maturation and activate macrophages, induce maturation of immature macrophages to DCs capable of antigen presentation to autoreactive T cells (232, 241).

The PD-1 pathway in SLE

Autoimmune diseases are the result of loss of self-tolerance, which may be a consequence of aberrancies in different immune pathways. Among the regulators of self-tolerance are T cell co-inhibitory receptors CTLA4 and PD-1 (82, 291, 343).

Polymorphisms in the *PDCD1* gene have now been associated with SLE and other autoimmune diseases in different populations. The most widely studied polymorphism is PD-1.3A. PD-1.3A shows strongest association with SLE in Caucasian populations, suggesting that the mutation is recent and affects mostly Europeans and has a lesser effect in populations admixed with them (81, 307). Interestingly, other PDCD1 gene polymorphisms have been associated with SLE in non-Caucasian populations (93, 304, 310).

There is limited information on cellular expression of the PD-1 receptor in humans and our analysis is a step towards clearing that picture. Our data show lower expression of the PD-1 receptor in SLE patients and relatives correlated with PD-1.3A (Paper III). PD-1 expression on activated CD4+CD25+ T cells, both T effector cells and T-regs, was significantly lower in the SLE patients and their relatives, compared to controls.

Aberrant PD-1 expression may affect CD4+CD25+ effector T cell function by altering activation thresholds leading to activation of autoreactive T cells. The PD-1 pathway has been reported to be important in regulating T-regulatory cell functions, but whether the effect is direct is not clear (82, 344, 345). Regulatory cell function has been shown to persist in the absence of the PD-1/PD-L1 or CTLA4/B7 pathways, but higher ratios of

$CD4+CD25^{high}$ T cells have also been shown to be required to suppress proliferation if PD-L1 receptor is blocked (346).

It will be important to study the phenotype of both CD4+CD25+ effector and regulatory T cells in relation to disease activity. Moreover, to study proliferative responses, cytokine profiles, and interactions with antigen presenting cells, autoantibody production and PD-1 expression on B cells.

Familial clustering of autoimmune diseases

Paper IV is a compiled analysis of available data on the Icelandic multicase SLE families, including PD-1.3A, C4AQ0, low MBL, other autoimmune diseases and autoantibody profiles.

The study demonstrates a high frequency and clustering of different autoimmune diseases and autoantibodies in families that are heterogeneous regarding the genetic susceptibility factors, PD-1.3A, C4AQ0 and low MBL.

PD-1.3A and C4AQ0 may be part of a genetic background predisposing family members to autoimmune diseases. Such a "predisposing" genetic background may partially explain the apparent clustering of different autoimmune diseases in the families, but as demonstrated by an increased frequency of PD-1.3A and C4AQ0 in the non-autoimmune relatives, other genetic and/or environmental factors may be necessary for disease expression. Interestingly, other genetic factors have been identified in these families; low MBL, increased numbers of IL-10 producing cells (51) and increased apoptosis of T cells (188) and *IRF5* gene polymorphism (117).

Concluding remarks

Familial aggregation of different autoimmune diseases may have several explanations including genetic polymorphisms that impair immune pathways that maintain self-tolerance. As maintenance of self-tolerance is dependent on complex interactions of many components, different compilations of genetic polymorphisms affecting regulation of the immune response at different levels, may be responsible for different autoimmune diseases in different families. Still other disease specific genetic and/or environmental factors may subsequently determine the organ or tissues targeted by the autoimmune response, thereby determining the autoimmune phenotype (18).

Analyzing independent susceptibility factors may be the first step in creating a unifying picture of intertwining factors and pathways that can ultimately lead to loss of self-tolerance and autoimmunity.

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