Clinical and Developmental Immunology

Autoimmune Disease Genetics

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Editorial Board

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Editorial
Autoimmune Disease Genetics

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Genetic risk factors play an important role in autoimmune disease susceptibility. Recent advances genotyping techniques, statistical methods, and the organization of large patient cohorts have facilitated explosive progress in this field, and our understanding of the genetic architecture of human autoimmunity is rapidly expanding. Current studies have demonstrated that some genetic risk factors for autoimmunity are shared between diseases [1, 2], and that others may be specific to a particular disease or ancestral background [3, 4]. Knowledge of the genetic basis of disease provides us with a unique window into human pathogenesis, which will facilitate improved diagnostic and therapeutic strategies and enable personalized medicine. It is clear that this field represents a major frontier in human disease research which we are just beginning to understand.

Given this background, we have assembled this special issue with a goal of highlighting important progress in a diverse range of topics in human autoimmune disease. The study designs and topics represented include everything from traditional family-based heritability and candidate analyses which are still clearly relevant and important today to eQTL methods and considerations of how genetic polymorphisms may impact the human immune system. A paper by J. L. Schmidt et al. uses a family-based design to examine the occurrence of autoimmune disease in families with an index case of Aicardi-Goutières Syndrome in the paper entitled "*Family history of autoimmune disease in patients with Aicardi-Goutières Syndrome.*" T. Carvalheiro et al. in the paper "*Tolerogenic versus inflammatory activity of peripheral blood monocytes and dendritic cells subpopulations in systemic lupus erythematosus*" explore the human immune system and find a decrease in tolerogenic dendritic cells in systemic lupus erythematosus, a disease in which dendritic cells play an important role in pathogenesis [5]. Y. Koldobskaya et al. use a novel eQTL technique to prioritize additional candidate gene loci from a prior genome-wide screen of systemic lupus erythematosus, supporting the idea that we can increase the yield of our genetic screens when we can apply our knowledge of disease biology in candidate selection in the paper "*Gene-expression-guided selection of candidate loci and molecular phenotype analyses enhance genetic discovery in systemic lupus erythematosus.*" M. Dolcino et al. profile gene expression in the autoimmune disease dermatitis herpetiformis, demonstrating patterns associated with lesional skin in the paper entitled "*Gene expression profiling in dermatitis herpetiformis skin lesions*." A candidate gene study by M. Pesmatzoglou et al. provides evidence for genetic associations with immune-mediated thrombocytopenia in the island population of Crete entitled "*DNA methyltransferase 3B gene promoter and interleukin-1 receptor antagonist polymorphisms in childhood immune thrombocytopenia*." Other studies support the association of a polymorphism in the human type 2 deiodinase gene with disease severity and rate of remission in patients with Grave's disease in the paper "*Thr92Ala polymorphism of human type 2 deiodinase gene (hd2) affects the development of graves' disease, treatment efficiency, and rate of remission*," and association of polymorphisms in the SPP1 locus with multiple sclerosis in the Italian population

in the paper "*The impact of osteopontin gene variations on multiple sclerosis development and progression*." Z. Zagoriti et al. explore genetic associations with myasthenia gravis in the Hellenic population in the paper entitled "*Genetics of myasthenia gravis: a case-control association study in the hellenic population*." A. Lev et al. examine the characteristics of residual circulating T cells in the genetic syndrome severe combined immunodeficiency disorder, an immunodeficiency syndrome that can also sometimes demonstrate autoimmune manifestations in the paper "*Characterizing T cells in SCID patients presenting with reactive or residual T lymphocytes*." A paper by I. A. Sobenin et al. is an example of the diversity of topics covered, as they demonstrate that mitochondrial inheritance influences atherosclerotic disease "*Mitochondrial mutations are associated with atherosclerotic lesions in the human aorta*," an important condition that clearly has an immune-mediated and inflammatory component.

Review articles cover diverse topics, such as the role of coinhibitory molecules in autoimmune disease in "*Coinhibitory molecules in autoimmune diseases*," expression of the autoimmune regulator gene and its impact on immune tolerance in "*Expression of the autoimmune regulator gene and its relevance to the mechanisms of central and peripheral tolerance,*" and genetic factors associated with immune-mediated bone marrow failure syndromes in "*Genetic associations in acquired immune-mediated bone marrow failure syndromes: insights in aplastic anemia and chronic idiopathic neutropenia.*" T. Besenyei et al. review the overlap between human rheumatoid arthritis risk alleles and the corresponding risk loci in animal models of arthritis in the paper "*Non-MHC risk alleles in rheumatoid arthritis and in syntenic chromosome regions of corresponding animal models.*" Two review articles address systemic lupus erythematosus—one is a synthesis of genetic loci associated with the disease that impact monocyte pathways which is "*Genetics of SLE: functional relevance for monocytes/macrophages in disease*," and the other is a review of the pathogenic influence of the IRF5 locus in systemic lupus erythematosus which is "*Interferon regulatory factor 5 in the pathogenesis of systemic lupus erythematosus*." While the topics covered in this issue are diverse, they still represent a relatively small portion of the work being done in the large and rapidly moving field of autoimmune disease genetics. It is exciting to observe the ways in which genetic studies are steadily unraveling human autoimmune disease pathogenesis, and the papers presented in this issue contribute to this goal.

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Review Article

Non-MHC Risk Alleles in Rheumatoid Arthritis and in the Syntenic Chromosome Regions of Corresponding Animal Models

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Rheumatoid arthritis (RA) is a polygenic autoimmune disease primarily affecting the synovial joints. Numerous animal models show similarities to RA in humans; some of them not only mimic the clinical phenotypes but also demonstrate the involvement of homologous genomic regions in RA. This paper compares corresponding non-MHC genomic regions identified in rodent and human genome-wide association studies (GWAS). To date, over 30 non-MHC RA-associated loci have been identified in humans, and over 100 arthritis-associated loci have been identified in rodent models of RA. The genomic regions associated with the disease are designated by the name(s) of the gene having the most frequent and consistent RA-associated SNPs or a function suggesting their involvement in inflammatory or autoimmune processes. Animal studies on rats and mice preferentially have used single sequence length polymorphism (SSLP) markers to identify disease-associated qualitative and quantitative trait loci (QTLs) in the genome of F2 hybrids of arthritis-susceptible and arthritis-resistant rodent strains. Mouse GWAS appear to be far ahead of rat studies, and significantly more mouse QTLs correspond to human RA risk alleles.

1. Introduction

Rheumatoid arthritis (RA) is a polygenic systemic autoimmune disease that mainly affects the synovial joints, causing chronic inflammation and profound tissue destruction in affected patients. The pathological features of RA include leukocyte infiltration of the synovial tissue (mainly T cells and macrophages), autoantibody production (e.g., against immunoglobulins, citrullinated peptides, or tissue-restricted antigens), the accumulation of inflammatory cells (mainly neutrophils) in the joint fluid, the proliferation of synovial fibroblasts, and the formation of pannus; collectively, these features result in the destruction of articular cartilage and bone erosion. The identification of genetic alterations and variations in RA (involving either the major histocompatibility complex (MHC) or non-MHC genes) and an understanding of their functional consequences may impact the diagnosis, therapy, and prevention of RA [1], an autoimmune disease that affects approximately 1% of the human population. No other autoimmune disease appears in so many different clinical forms or is characterised by such heterogeneous and diverse clinical symptoms and laboratory tests. As a consequence, there are many experimental animal models attempting to mimic the multiple clinical symptoms of RA.

Animal studies may help to fill the gaps in human genome-wide association studies (GWAS) by allowing for gene mapping and functional studies, which cannot be performed in human patients and may yield greater insights into the mechanisms of autoimmune T and B cell responses in RA [2–4]. While the various animal models are tremendously helpful for investigating certain aspects of the human disease, none of these models recreates the full spectrum of diseases collectively called RA. Notably, thousands of investigators

and pharmaceutical companies use animal models of RA, perhaps without understanding the differences among the different subtypes of this disease and the corresponding animal models [2–5]. Based upon the clinical, immunological, and genetic components, the most appropriate animal models for RA seem to be (i) those that use genetically controlled systemic autoimmune joint diseases, (ii) those in which the MHC (class II molecules) plays a crucial role, (iii) those in which both T and B cells are involved, and (iv) those that apply (auto)antigenic molecules of cartilage or joint tissues for provoking ("targeting") synovial joint inflammation.

Among the animal models of RA that fulfil the above listed criteria from a genetic point of view and that are characterised by the presence of the most valuable biomarkers, such as rheumatoid factor (RF) and anticitrullinated peptide antibodies (anti-CCP or ACPA), the closest genetic, and clinical models of RA appear to be cartilage proteoglycan (PG) aggrecan-induced arthritis (PGIA) [6, 7] and cartilage type II collagen- (CII-) induced arthritis (CIA) [3, 8–11].

2. Progresses and Limitations of Human and Animal GWAS

In addition to certain MHC (or human leukocyte antigen (HLA) in humans) class-II alleles on human chromosome 6 that are most commonly (over 40%) associated with a genetic risk for RA [1, 12–16], currently there are 31 non-MHC RA risk alleles that have been confirmed by GWAS and meta-analyses [17, 18]. Many of these risk alleles are weak and are frequently "specific" for different ethnic groups or subpopulations, but there are at least 25 strong RA risk alleles within 23 non-MHC loci in the human genome that control disease susceptibility or severity [19]. These human RA risk alleles were identified and confirmed using hundreds of thousands of single nucleotide polymorphisms (SNPs) and designated by the name of the gene in which the SNP occurred most frequently. However, except for very few cases, none of the genetic risk loci identified to date represent the disease-causing or disease-promoting gene, in which mutations have occurred. SNPs, similar to postal ZIP codes, define only certain regions where a number of genes or noncoding elements (streets in the analogy) are located, but they do not define exact addresses. These risk loci or alleles defined by various numbers and frequencies of SNPs indicate only a chromosome region (carrying dozens to hundreds of genes) expected to have one or a few functionally defective genes involved in the pathomechanism of RA [20]. In fact, these RA-associated SNP risk alleles may indicate a risk for RA or a number of other autoimmune diseases [1, 19, 21–29], or their combination may be used as "predictive" markers for effective therapy selection. Due to extreme heterogeneity in the human population, the highly motivated and exciting early-stage studies have led to the current frustration, and only confirmatory or treatment-related meta-analysis studies have been published during the past couple of years.

In contrast to human studies using heterogeneous populations, there is a chance to use the combination of various arthritis-susceptible and arthritis-resistant inbred strains for GWAS and to identify disease-associated QTLs. There are over a hundred non-MHC genetic risk alleles identified in the rat and mouse models of RA. However, a surprisingly small number of these rodent QTLs (especially in rat arthritis models) correspond to the RA risk alleles or corresponding area in the syntenic human genomic area. Many of these rodent QTLs are listed as new discoveries and were never coordinated as the human studies were, and thus, they are frequently represented by duplicate or triplicate names when described by different research groups. Another limitation of these animal studies is that the different QTLs may represent different, probably over a dozen, phenotypes (e.g., onset, susceptibility, severity, tissue destruction, etc.) in combination with the presence or level of various biomarkers, such as autoantibodies or cytokines either in sera or *in vitro* stimulated spleen or lymph node cultures. The PCR-based method (single sequence length polymorphism, SSLP) used for the identification of QTLs in either mice or rats is a different technique from SNP microarray-based screening of the human genome, but the principal of the final linkage analysis is based on the same concept. Therefore, as it happened in human SNP-based studies where different sizes and types of arrays, populations, clinical phenotypes, disease durations, environmental factors, and responsiveness to treatment types create a heterogeneous picture of risk alleles, similar heterogeneity in genotype, phenotype, and biomarker distribution exists in animal studies.

3. Significance of Animal Models of RA

Human genetic studies are expected to be fast but fairly less reliable because either the function of the SNP-identified gene or intergenic region is unknown or the consequence of the mutation found in a gene (e.g., transcription factor binding site) is very rarely known in humans. Animal studies are slow and laborious, but using appropriate genetic combinations (selected combinations of intercrosses and GWAS of F2 hybrids, congenic/subcongenic, and interval-specific congenic (IVSC) processes, and genomic sequences of the target inbred region) they can find disease-promoting genes, even with a relatively weak disease-modulating effect. Moreover, animal models allow us to investigate the role of a single gene and the mechanisms of the disease, allowing development of more effective and appropriate treatments. These animal studies, however, are valuable only if they focus on the disease-affecting/causing gene(s) in humans. Human genetics often arrives at a dead end because the disease-affecting genes are unknown [20]. Furthermore, due to the enormous heterogeneity of the human population, it is not feasible to sequence large genomic areas of thousands of people before careful selection of a relatively homogeneous subpopulation of RA patients. This selection requires extensive bioinformatics analysis comparing hundreds or thousands of disease-associated SNPs and RA patients to identify homogeneous (identical, or close to identical) SNP combinations and allele frequency for the selected RA-associated locus in affected patients. In a recent study, we compared a few hundred seropositive RA patients

(all carrying the PTPN22 risk allele) but found only a dozen patients with the same SNP combinations. We expect that after high-throughput sequencing, there may be only a few (2–4) RA patients who show high genomic similarity within a small genomic region using bioinformatics analysis, but the appropriate programs and appropriate functional tests are not available at the moment.

Although there are limitations surrounding both human and animal genome-wide screening studies, in the future, the two lines of research may support similar findings and be consolidated to provide additional insight. There are a few animal models of RA that have identified highly significant disease-associated loci. Induced autoimmune models of RA usually represent an accelerated form of RA. For example, both CIA and PGIA are known to involve MHC class-II-restricted antigen presentation and generation of T cells and autoantibodies that cross-react with self (mouse) antigens such as mouse CII or PG [3, 6, 8, 10, 30, 31]. In addition to MHC, which controls at least 40–50% of the genomic susceptibility to RA, both models require an arthritis-prone non-MHC genetic background. Nonobese diabetic (NOD) mice are resistant to both CIA and PGIA. However, when KRN T cell receptor (TCR) transgenic mice were intercrossed with NOD mice, it resulted in the K/BxN model, which develops spontaneous arthritis. The KRN TCR is specific for the bovine pancreas ribonuclease and apparently cross-reacts with glucose-6-phosphate isomerase (GPI) [32–34]. However, the spontaneous K/BxN model is irrelevant for genomic studies. It has no MHC linkage, a ubiquitous (auto)antigenic component exists (which is present in all mammalian cells [35]), and anti-GPI antibodies can rarely be detected in RA patients [36–38]. The sera of these spontaneously arthritic mice can transfer arthritis to any strain of mice (serum-transfer arthritis); thus, the genetic components of either the K/BxN or serum-transfer arthritis models are vague and unclear. However, a genome-wide screening of serum-transfer-induced arthritis in heterogeneous stock (HS) mice resulted in very interesting results [39]. QTLs identified on six chromosomes matched two human RA risk alleles (TRAF1/C5 and PADI4 loci), of which the Traf1/Hc locus on mouse chromosome 2 (mChr2) is a dominant QTL in both CIA (*mCia2 and mCia4*) and PGIA (*Pgia2*) (Table 1).

SKG mice develop arthritis due to a spontaneous mutation in the SH2 domain of Zap70 [40]. Because the Zap70-mutation causes defective TCR signalling, it has been postulated that autoreactive T cells escape thymic deletion and accumulate in the periphery of SKG mice [40]. Altered thymic selection in SKG mice leads to the survival of otherwise negatively selected T cell clones that then spontaneously differentiate into Th17 cells in the periphery and attack the joints. In contrast, interleukin 1 (IL-1) receptor antagonist protein (IRAP) knock-out mice develop spontaneous arthritis due to increased production of proinflammatory cytokines (IL-1β, IL-6, IL-17, and tumour necrosis factor-alpha, TNFα) and autoantibodies in the absence of negative regulation of IL-1 signalling [41, 42]. In addition, human TNFα-expressing transgenic mice develop spontaneous chronic erosive arthritis due to their continuous production of TNFα [43]. This arthritis appears to be a highly simplified proinflammatory cytokine-induced arthritis; thus, it is similar to the serum transfer-induced arthritis (using anti-GPI antibody-containing sera from arthritic K/BxN mice) [44] and the collagen monoclonal antibody cocktail or LPS-induced arthritis (CAIA) [45–47].

All of these models, directly or indirectly, have contributed insights into the complex mechanisms behind RA and have facilitated the development of current therapeutics and biologics. It is important to note that all the previously mentioned experimental animal models of arthritis develop at a relatively young age (beginning at ~4–6-weeks), except PGIA [48], and that arthritis develops in SKG and IRAP-deficient mice only in the BALB/c genetic background [40–42]. This arthritis-prone BALB/c genetic background has also been shown to predispose mice to PGIA [7], human G1 domain-induced arthritis (GIA) [49], link protein [50] or human cartilage HC-gp39 protein [51]. The incidence of spontaneous arthritis in retired, breeder, wild-type BALB/c females is estimated at 0.5–1.0% (TTG, unpublished data), which is close to the ratio observed in the human population. Additionally, BALB/c mice carrying the HLA-DR4 transgene [52] or expressing a PG (5/4E8 epitope)-specific TCR [53, 54] develop arthritis spontaneously but only at an advanced age [55]. Although there are a number of other animal models of RA, we have listed only those that may have conceptual relevance to this paper. However, except for a relatively few studies [39, 56–58], GWAS in mice has almost exclusively been performed in PGIA and CIA; thus, we compare QTLs identified mostly in these two models with human GWAS and their subsequent meta-analyses (Table 1). Therefore, we summarise only those genomic regions (QTLs) of animal studies that correspond to the human chromosome region where risk alleles were identified in RA, and thus, may help to accelerate human studies. Interval-specific congenic (IVSC) mice representing human RA-associated regions present a high potential for sequencing homogeneous genomic regions, and any genes with potentially pathogenic variants (either in exons, introns or intergenic regions and in disease-promoting or disease-suppressive areas) may guide future human studies in terms of selecting appropriate patient populations for more detailed genetic and epigenetic analysis.

4. Tissue-Restricted (Cartilage) Antigens Can Provoke Arthritis in Genetically Susceptible Mice and May Contribute to the Severity of RA

Cartilage is one of the few immune-privileged tissues in the body in that it is essentially avascular and therefore not subjected to close "internal" immunological surveillance [59]. An incomplete central tolerance is most likely the dominant component of this special immune condition, a tolerance that can be breached when transgenes are expressed in cartilage and the cartilage-specific overexpression is "leaky," especially in the embryo. Several lines of evidence support this hypothesis. For example, cartilage link protein [60] or otherwise arthritogenic human G1 domain

Table 1: Arthritis-associated QTLs of mice corresponding to human SNP-based GWAS.

mChr	Markers	Region (Mbp)	Locus name (mouse)	Peak marker name	Peak marker position	Mouse gene And its position (Mbp)		References (mouse)	Corresponding human Chr position (Mbp)		Human locus name
1	D1Mit188–D1Mit122	3.9–40.5	***mCia14***	D1Mit244	37.6	**Aff3**	**(38.2)**	[9, 40, 92, 93]	**2q11**	**(100.2)**	**AFF3**
1	D1Mit426–D1Mit209	90.4–193.3	***mCia9*** ***Pgia1***	D1Mit166	178.3	**Fcgr2b**	**(170.9)**	[57, 92, 93]	**1q23**	**(172.9)**	**FCGR2A**
			Pia(Chr1)	D1Mit36	171.1						
2	D2Mit79–D2Mit237	21.9–40.8	*mCia2* *mCia4*	D2Mit238	33.9	**Traf1/Hc**	**(34.8)**	[2, 40–44]	**9q33**	**(123.6)**	**TRAF1/C5**
			Pgia2 HS	D2Mit81	33.4						
3	D3Mit75–D3Mit284	100.5–103.6	*mCia21* *mCia22*	D2Mit75 D2Mit284	100.5 103.6	**Cd2** **Ptpn22**	**(100.5)** **(103.6)**	[45, 46]	**1p23** **1p13**	**(114.3)** **(117.3)**	**CD22** **PTPN22**
3	D3Mit141.1–D3Mit323	93.0–152.4	***Pgia26*** ***(+3 subloci)***	D3Mit158	109.1	**Cd2** **Ptpn22**	**(100.5)** **(103.6)**	[47, 47, 48, 57, 92, 93]	**1p13** **1p23**	**(114.3)** **(117.3)**	**CD22** **PTPN22**
			Pia-Chr3	D3Mit100	96.8						
5	D5Mit388–D5Mit256	33.8–63.3	***Pgia16*** *mCia13*	D5Mit233	53.0	**Rbjp**	**(53.9)**	[9, 44, 49]	**4p15**	**(25.8)**	**RBJP**
6	D6Mit86–D6Mit318	4.4–65.1	***Pgia19***	D6Mit267	29.2	**Irf5**	**(29.4)**	[40, 50, 93]	**7q32**	**(128.2)**	**IRF5**
10	D10Mit206–D10Mit130	13.8–65.9	***Pgia6*** ***Pgia6b***	D10Mit124	20.9	**Tnfaip3** **Prdm1**	**(18.7)** **(44.1)**	[9, 40, 43, 92, 93]	**6q23** **6q21**	**(138.2)** **(106.5)**	**TNFAIP3** **PRDM1**
10	D10Mit12–D10Mit269	98.9–128.4	*mCia8* (“locus 5”) *AIL(Chr10)*	D10Mit102 D10Mit261	120.3 85.0	**Kif5a** **Pip4k2c**	**(126.7)** **(126.6)**	[40, 50, 58, 58, 94]	**12q13** **12q13**	**(57.9)** **(57.9)**	**KIF5A** **PIP4K2C**
13	D13Mit258–D13Mit78	95.6–119.6	***Pgia15*** *mCia19*	D13Mit51 D13Mit53	105.3 113.1	**Ankrd55** **IL6st**	**(113.0)** **(113.2)**	[9, 44, 51, 92, 93]	**5q11** **5q11**	**(55.4)** **(55.2)**	**ANKRD55** **IL6ST**
15	D15Mit121–D15Mit242	57.9–90.2	***Pgia9***	D15Mit28	74.4	**IL2b** **Bik**	**(78.3)** **(83.4)**	[9, 40, 44, 62, 92, 93]	**22q12** **8p3**	**(37.5)** **(11.3)**	**IL2RB** **BIK**
15	D15Mit279–D15Mit192	74.0–92.7	*mCia35–mCia37*	D15Mit159	87.3	**IL2b** **Bik**	**(78.3)** **(83.4)**	[53]	**22q12** **8p3**	**(37.5)** **(11.3)**	**IL2RB** **BIK**
18	D18Mit51–D18Mit80	61.3–77.0	***Pgia11***	D18Mit81	66.7	**Ptpn2**	**(67.8)**	[9, 40, 43, 93]	**18p11.3–p11.2**	**(12.8)**	**PTPN2**

Human RA loci validated in Caucasian, African American, and Asian ancestry and compared via meta-analysis [17, 18, 54, 55, 59–61, 63].
Human RA loci validated in Caucasian, African American, and Asian ancestry and compared via meta-analysis [17, 18, 54, 55, 59–61, 63].

(unpublished data) expression in mice, driven by the rat type II collagen promoter and enhancer, may be detected in cartilage tissue, but the transcript and protein could also be detected in other embryonic tissues. Additionally, when cartilage PG (or CII) is degraded by various matrix metalloproteinases, the newly generated neoepitopes may provoke an autoimmune reaction [61]. Further evidence is provided by posttranslational events (e.g., citrullination), as molecules unrelated to cartilage (e.g., filaggrin [62–64]) are first citrullinated far before the onset of joint inflammation. Subsequently, additional molecules (e.g., fibrinogen, vimentin, type II collagen, PG aggrecan, α-enolase, and a few virus proteins) also undergo posttranslational modifications (citrullination), and the cumulative effect of (auto)immune reactions may breach the immune tolerance in genetically susceptible human individuals.

Although immunity to the cartilage PG aggrecan has been less extensively studied than immunity to type II collagen (CII), cartilage PG is also considered to be a causal factor in rheumatoid joint diseases [65–67]. Either humoral or cellular immunity, or both, to human cartilage PGs have been detected in patients with RA [65–79], and the two most recent studies reported that the citrullinated version of a dominant arthritogenic (5/4E8) peptide of human cartilage PG [80, 81] induced substantial cytokine (IL-17, IL-22, IL-6, TNFα, IFNγ) production by T cells from the majority of RA patients [78, 79]. T cells from the same RA patients responded poorly to the native (noncitrullinated) peptide in both studies, and T cells from healthy subjects did not respond [78] or responded only to the citrullinated peptide by producing IL-6 [79]. Although the majority of RA patients tested were positive for anti-citrullinated cyclic peptide (anti-CCP) antibodies (ACPA), T-cell response to the citrullinated PG peptide was also noted in some ACPA patients [78, 79].

5. Overlapping Genomic Loci of RA and Autoimmune Mouse Models of RA

In this paper, we collected results from GWAS in mice and rats (over 100 QTLs) and compared the QTL localisations to those identified in human studies (over 30 RA-associated loci). It is technically impossible and scientifically unnecessary to cite all these studies; rather, we tried to select those that represent syntenic regions in humans and mice (and rats if available). We cite the most appropriate publications in Table 1 or in the text rather than indicating SNP codes (rsXXXX). The levels of significant association between the same SNP and RA is variable in different papers, and for the novelty of a new meta-analysis, investigators may preferentially use a SNP in close proximity to those that have already been published. In brief, we selected data from RA risk allele groups that also have syntenic regions in rodent studies and show one of a few on-going animal/human studies (mouse Chr3 versus human Chr1) in which the combined information may be not only quantitative but also qualitative (Figures 1 and 2). In other words, two chromosome regions (Figure 1) have not only SSLPs (andSNPs) in the "candidate" target regions but also functional defects in the protein encoded by the mutated gene that may either suppress or promote the onset and severity of arthritis. Thus, these particular mouse studies aid in the discovery of functional defects in disease-associated genes in humans with RA.

As mentioned, over 100 rodent QTLs have been described to date, but relatively few are syntenic with any of the 30 human RA risk alleles. In our laboratory, over 5,000 inbred wild-type parents, approximately 500 F1 hybrids (all negative for PGIA, data not shown) and 3,200 F2 hybrids of six different genetic intercrosses were genotyped using a total of 240 SSLP markers. The goal was to identify genetic alterations responsible for individual and overlapping qualitative (binary) QTLs that are linked to PGIA or CIA in the mouse genome and then compare the results with loci identified in human autoimmune diseases, preferably RA. Many of the risk alleles in RA overlap with a number of risk alleles of other autoimmune diseases [19, 21–27, 29], and a number of *Pgia* and *Cia* loci [10, 82–87] overlap with chromosomal regions identified in GWAS studies of RA patients [17, 19, 88–91]. CIA was considered as a model of seronegative RA, whereas PGIA, which has both rheumatoid factors and ACPA [7, 49], was considered a seropositive RA model. The overall hypothesis was that genes associated with a QTL in one or more genetic combinations of murine autoimmune arthritis should correspond to genes involved in RA. (A total of 26 loci out of 31 confirmed non-MHC loci were screened for corresponding mouse QTLs. Only those that were found in comparative studies of mouse genome-wide association (GWAS) studies ($n = 17$) are listed under the "human locus name." These mouse GWAS studies include over a dozen intercrosses screened in different laboratories. Occasionally, the same (mouse) *Cia* locus-number appears on different chromosomes in different publications, thus the references corresponding to the appropriate mouse *Cia* (*mCia*) loci are listed here. QTL of *Pgia* ($n = 9$) and mCia ($n = 2$) identified in our laboratory are italic and bold faced. Each human locus is listed by the gene-name and chromosome location using the "standard" name of the given RA risk allele; the corresponding mouse region/gene is indicated by the same gene name and location in the mouse genome given by the mega-base pair (Mbp) position (bold-faced). Tissue samples (tails and kidney) of each F2 hybrid mouse are catalogued and stored at -80°C. Many of the F2 hybrids were retested with additional, new markers in confirmatory studies (9 *Pgia* and 2 *mCia* loci). The average marker density in these confirmatory studies was 8.2 Mbp. Some of these reference markers shifted slightly after confirmatory studies using high density marker screening. Two QTLs on mouse chromosomes 3 and 15 have overlapping regions; therefore, they are listed in the Table 1 twice due to the information from different studies.)

Although there are a number of weaknesses for both human and animal GWAS, they may supplement and support each other. During the past 15 years, we and others have identified 29 *Pgia* and 40 *Cia* loci in different genetic combinations of F2 hybrid mice [3, 10, 11, 57, 58, 82, 86, 95–99] and a couple of corresponding QTLs in rats [100–105]. With a strong confirmation in the literature, we selected QTLs from all (published) mouse genomic studies [10, 56–58, 82–84, 92–96, 98, 106–112] that also correspond to one

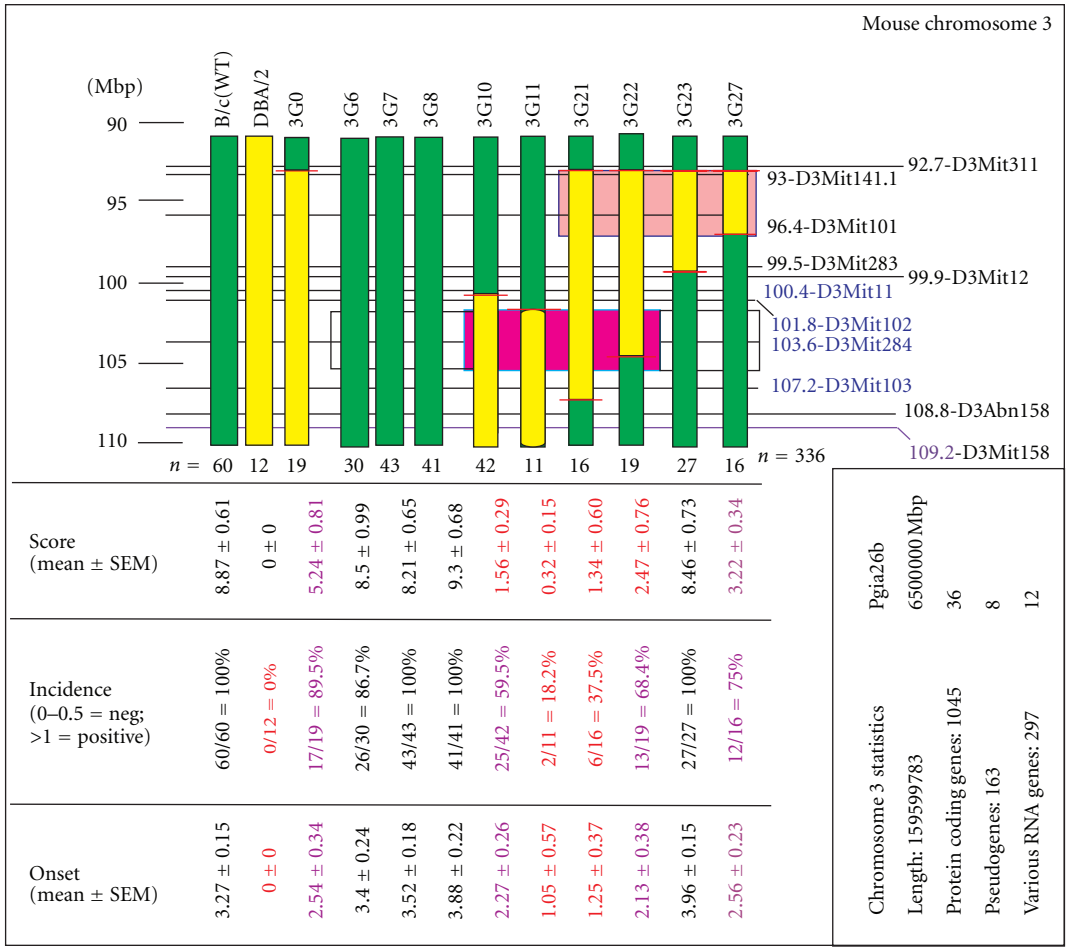

Figure 1: Summary of the genotypes and corresponding clinical phenotypes of parent stains and *Pgia26* (3G0) and *Pgia26* subloci that were identified in IVSC lines with overlapping chromosome intervals. The original mChr3 region (3G0: 90.4–156.5 Mbp in size) was reduced and separated into several subloci in 27 interval-specific subcongenic (IVSC) lines (3G1-3G27). For simplicity, only a 16.5 Mbp region is shown. Green columns represent BALB/c, and yellow columns represent the DBA/2 chromosome regions. Horizontal black lines with numbers at the right side (and with marker names) are shown. The short red lines crossing the IVSC chromosome region indicate the position between the two markers, where the DBA/2 allele continued as BALB/c [84]. The blue-framed red rectangular area indicates the position of the *Pgia26d* locus (between 101.4 and 107.2 Mbp); in the worst case, this region may include the entire flanking region between 99.9 and 108.8 Mbp where the disease-promoting gene(s) in BALB/c mice is located (or reciprocally, the suppressive genes in DBA/2). This area contains the most prominent *Ptpn22* (protein tyrosine phosphatase non-receptor-22) identified in human GWAS with SNPs, an allele that is associated with many autoimmune diseases. The mutation affecting R620W amino acid appears to affect both peripheral and central B-cell tolerance [120]. Under the worst scenario, this region contains 128 protein-coding genes, 19 miRNAs, 13 pseudogenes, and 9 non-protein-coding transcripts (http://www.ensembl.org/Mus_musculus/Info/Index). Other *Pgia26* subloci (with large scales) are presented in Figure 2 with the corresponding human, rat, and mouse RA risk alleles. Another disease-suppressive region (inherited from the DBA/2 strain), between 92.7 Mbp and 96.4/99.9 Mbp position (framed), is currently under sequencing and examination.

of the major risk loci of RA confirmed in a number of meta-analyses [19, 29, 90, 113–117]. Table 1 summarises the risk alleles selected that have corresponding genomic regions from human and mouse GWAS. Only QTLs that correspond to at least one major RA-associated locus in the human genome are listed; these QTLs were found on mouse chromosomes 1 (2x), 2, 3 (2x), 5, 6, 10 (2x), 13, 15 (2x), and 18 (a total of 13 QTLs). The list was organised in order of mouse chromosomes. At least one, and possibly two or three, QTLs from various animal studies covered the syntenic chromosome region of human RA-associated loci. Standard abbreviations of genes were used as they are listed in gene bank databases (e.g., http://www.informatics.jax.org/; http://www.ensembl.org/Mus_musculus/Info/Index or http://genome.ucsc.edu/cgi-bin/hgGateway), and many of their known functions are described in publications available from PubMed (http://www.ncbi.nlm.nih.gov/pubmed). Thus, we did not list the full names or discuss the function(s) of these genes used to identify RA susceptibility loci or the "most frequent" associated SNPs of meta-analyses. These "marker-specific" genes were usually located near the unknown genes that might carry the disease-causing genomic defect.

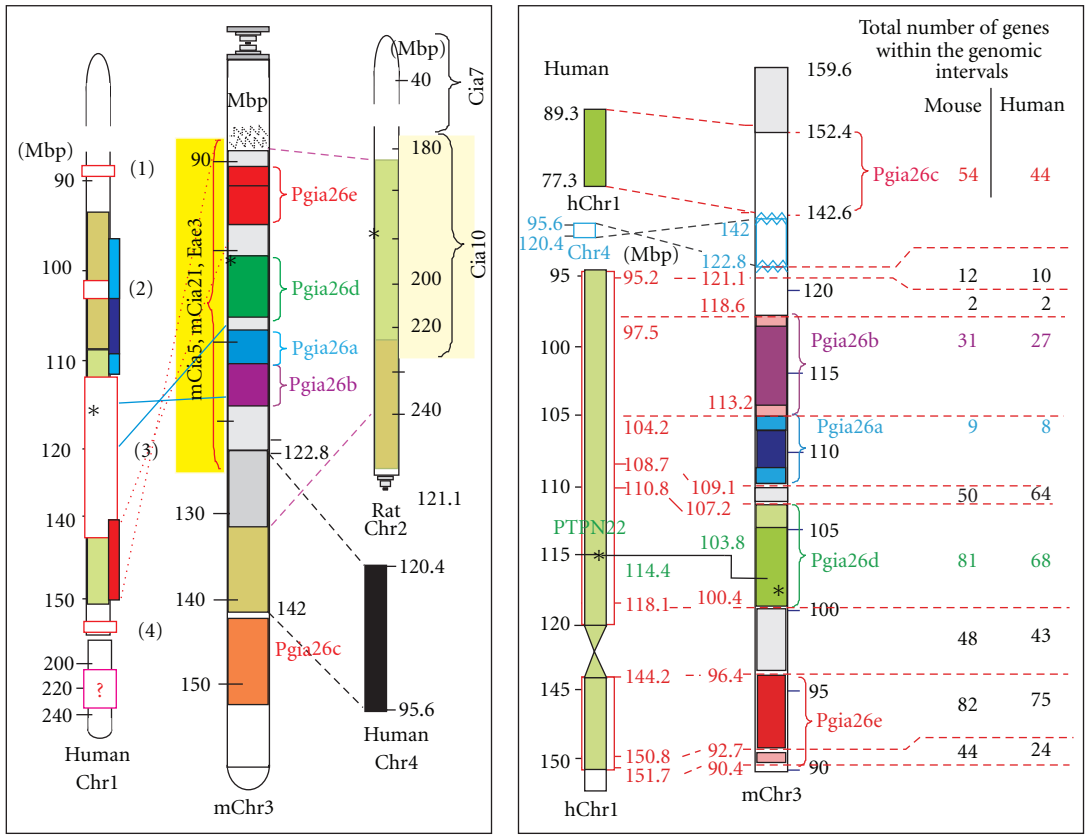

FIGURE 2: Mouse chromosome 3 (Chr3) with *Pgia26* subloci identified in IVSC mice (Figure 1) and corresponding human and rat chromosome regions with their corresponding risk alleles. Panel (a) summarises the location of five *Pgia26* subloci with corresponding mouse *mCia5* and *mCia21* (collagen-induced arthritis) [3, 108, 121], mouse Eae3 (experimental allergic encephalomyelitis) [122, 123] (between mChr1:84.3–126 Mbp), and the corresponding rat Chr2 region of rat *Cia10* [124, 125]. The left side of the panel identifies risk alleles on human Chr1 [126], with red-framed boxes and numbers in parentheses indicate the following regions: (1) between 87–89 Mbp [127], (2) 105.4 Mbp [128], (3) 113–142 (including the PTPN22 gene at 114.4) Mbp positions [129–131], and (4) the FCGR family between 158 and 159 Mbp [132, 133]. Panel (b) displays the syntenic risk alleles of human Chr1 and mouse Chr3 (*Pgia26a-e*) with the number of genes localized in the different chromosome regions.

For example, SNPs of two of the strongest RA risk alleles, TRAF1/C5 and TNFAIP3/OLIG3, are in the intergenic regions, making it difficult to establish causality of these regions at this moment [20, 88, 118]. Although both TRAF1 and TNFAIP3 are "preferential" gene candidates based on their function in TNF signalling, known to be important in RA [119], none of the genes having SNPs or genomic mutations evidently affect their function.

In the next section, we show an example of how we can integrate information from the human and mouse studies. This method may be one of the potential ways to identify causal variants that map to human RA-associated chromosome regions.

6. Benefits of MHC-Matched Susceptible and Resistant Mouse Strains: IVSC Strains Targeting Human RA Risk Alleles

To eliminate or reduce the dominant effect of MHC in cases where the association of a QTL with an arthritis phenotype has been sufficiently confirmed, one of the most successful alternative approaches is to use MHC-matched arthritis-susceptible and arthritis-resistant strains to establish congenic and subcongenic lines. Either a disease-promoting chromosome region can be "inserted" into a resistant strain, or reciprocally, the same region containing a disease-suppressive allele can be inserted into a fully susceptible genetic background. Either direction is acceptable, but from a practical point of view and based on many congenic experiments during the past decade, the latter solution appears to be more manageable. First, F1 males are selected, for example, from the intercross of a PGIA-susceptible BALB/c female and a resistant DBA/2 male (both MHC $H2^d$) carrying the DBA/2 genomic region of interest. These F1 males are backcrossed several times with wild-type BALB/c females, and the offspring are genotyped for each litter until the N_1-N_X generations have sufficient numbers of recombination events (and, if possible, overlapping areas) (Figure 1). These N_x males are intercrossed with wild-type BALB/c females, and the resulting heterozygous N_{x+1} males

and N_{x+1} females are intercrossed to establish a homozygous IVSC strain(s).

During the ongoing backcrossing process, fewer and fewer previously heterozygous loci need to be tested by PCR. If a gender effect is expected, it is necessary to replace the Y chromosome with a single reciprocal backcross, but it is both practical and sufficient to do this replacement near the final step.

Subsequently, the chromosome intervals from the resistant strain of a relatively (and usually) large QTL (several cM or Mbp in size) need to be tested for clinical phenotypes. For example (Figure 1), the "Chr3G0" ("3G0") subcongenic line contains an overlapping region ~66 Mbp in size that significantly affected all clinical phenotypes when compared to either susceptible BALB/c or resistant DBA/2 parental strains [84], a finding that needs to be further confirmed by *in vitro* tests (i.e., measuring biomarkers). In this case, males from the congenic 3G0 strain can be used to reduce the chromosome interval with new recombination events with matings into inbred BALB/c females. On the other hand, only the critical interval of mChr3 with high-density markers needs to be genotyped because the entire genome was previously genotyped for BALB/c (during the selection of 3G0 congenic line). Then, mice with the most appropriate recombination products are used as founders for fine mapping of chromosome intervals generating IVSC strains. Conceptually, the same backcrossing to the susceptible BALB/c strain and genotyping approach, as described above, are used for the selection of new congenic strains. However, investigators need to (i) focus on the new recombination events within selected chromosome interval using high marker density within the region of interest (e.g., *Pgia26*) and (ii) genotype both males and females. Depending on the volume of backcrossing (i.e., the number of breeding pairs and offspring) and the shortest chromosome interval achievable after a few generations, we are able to select a number of heterozygous males and females with identical recombination events at different positions (if possible with overlapping regions as shown in Figure 1: e.g., Chr3G0-Chr3G27) to establish homozygous IVSC strains for *in vivo* and *in vitro* tests.

To save time, it is practical to genotype both males and females for all new recombination products within the chromosome interval of interest, a locus that corresponds to the selected human RA risk allele. As shown in Figures 2(a) and 2(b) and Table 1, the *PTPN22/CD2* human risk locus most likely represents a complex trait on mChr3 (syntenic with hChr1) containing both disease-suppressive and disease-promoting alleles [84]. Distinct regions, alone or in combination, may result in clinically similar phenotypes (Figure 1), while the IVSC-associated biomarkers may show significant differences. Thus, a relatively small IVSC chromosome region may be separated for different genotypes representing similar clinical phenotypes (Figure 1, only the centromeric region of the mapped mChr3 is shown). However, while clinical phenotypes are comparable, fundamentally different genes in nearby chromosome regions may control disease susceptibility, onset and severity. Needless to say, fine mapping of chromosome regions and selecting narrow genomic regions with high probability for successful genomic high-throughput sequencing might be difficult, if not impossible, to complete using RA patients from the heterogeneous human population. Further, this highly specific and laborious animal study is valuable only if it represents human relevance, that is, if the corresponding region where the human risk allele was localised had already been identified.

Figure 2 shows simplified schematics comparing the previously outlined IVSC approach (*Pgia26* on mChr3) in combination with mouse (*mCia*) and rat CIA loci syntenic with the RA loci identified on human Chr1. Colours, numbers of genes, locations of syntenic genomic loci, and their flanking regions are indicated in Figure 1 and legend. With the advent of genome sequencing techniques, SureSelect Target Enrichment kit (Agilent, San Diego, CA, USA), library amplification and Illumina parallel sequencing methods made it realistic to oversequence 10–30 Mbp of homogeneous genomic regions from inbred IVSC strains and compare sequences with parent strains (susceptible versus resistant). It is also a reasonable approach to confirm the function of arthritis-susceptible or arthritis-resistant murine strains with transgenic methodologies. Today, the real challenge in human genetics is to find and select appropriate human patients with nearly identical genomic region(s) for high-throughput genomic sequencing due to the extreme heterogeneity of the human population. While SNP analyses using thousands of samples can give an extremely high statistical power, the same approach (SNP selection for genomic sequencing) is unsuccessful in the selection of human samples [20].

However, there are promising directions based on the combination of human-mouse GWAS. Selected homozygous regions of IVSC mice sequenced first with high-throughput sequencing method and affected genes and/or intergenic (relatively small) regions are genome-sequenced from selected humans with appropriate primers. In fact, a certain number of mutations/SNPs of the syntenic regions (identified in IVSC mouse and confirmed using conventional Sanger sequencing of human genomic DNA) may guide the selection of human RA patients for high-throughput sequencing of the region of interest (Figure 2). Alternatively, for example, if miRNA-related sequences are expected, the high-throughput sequencing of RNAs isolated by cross-linking immunoprecipitation (HITS-CLIP) with antibodies against the RNA-binding protein Argonaut (Ago HITS-CLIP) [134–138] may offer another solution.

7. Overall Summary and Perspectives

Overall, mouse studies, especially with congenic strains, appear to be a fundamental resource for the identification of candidate gene(s) in RA. During the past 15 years or so, almost concurrent with the first human genomic studies in RA, a number of rodent (mouse and rat) GWAS studies have been performed. At approximately the same time, both the human and mouse genome sequencing studies were completed and, simultaneously, unlimited numbers of new markers became available for both species. The number

of human studies expanded; tens of thousands of RA patients, along with controls, were genotyped; new and more reliable SNP arrays became available; more risk alleles became identified in RA and in almost all autoimmune diseases. However, after extensive progress in GWAS, the direction of RA research moved towards confirmatory studies of previously tested patients, examinations of different ethnic groups or comparisons of the therapeutic effects of different biologics. Briefly, human studies turned to mainly *in silico* and meta-analysis studies rather than aimed towards finding causative and functional (aetiological) reasons. The previously identified genomic regions were confirmed using a high marker density, but the large chromosome regions with tens of Mbps in size still remained unmanageable. Only a very few SNPs causing missense mutations proved to be associated with disease, and usually only in a narrow selection of the patient population. However, the number of risk alleles increased, and previously identified marker positions were confirmed.

Unfortunately, animal studies also slowed down, although due to completely different reasons from human studies. Increasing the number of new combinations of disease-susceptible and disease-resistant inbred strains revealed more and more QTLs, but not a disease-causing gene. Recognising the limitations as well as the potential of both human and mouse GWAS, approximately 10 years ago, a number of congenic strains carrying the most promising traits representing the strongest clinical phenotypes were established. These strains carry overlapping traits identified in different animal models and syntenic with genomic regions identified as RA risk alleles. In other words, at the time when the human GWAS explored the most critical RA risk alleles, congenic backcrossing had selected inbred IVSC strains with syntenic regions to the major human risk alleles. We selected two QTLs for more detailed analysis: *Pgia26/Cia5/mCia21/Eae3* on mChr3 and rat *Cia10,* corresponding to the *PTPN22/CD2* allele on human Chr1 (Figure 2); *Pgia2/Cia2/Cia3* on mChr2 (corresponding to the *TRAF1/C5* allele on hChr9). Then, we generated IVSC strains (Figures 1 and 2, *Pgia26* is shown). All other congenic and subcongenic strains were cryopreserved. The two major/dominant mouse QTLs were separated into narrow subtraits and simultaneously tested for arthritis susceptibility, for disease onset and severity, and for over 15 biomarkers that might have some potential relevance for RA [84]. Simultaneously, some of the IVSC genomic regions representing homogeneous regions of disease-susceptible and disease-resistant IVSC mice (and the corresponding parent genomic regions) were sequenced, and a few mutated genes were identified (with "known" or completely unknown function). Occasionally, these genes had not been previously associated with arthritis, but all of them had localised in close proximity to a gene used to name the human RA risk alleles. The analyses of these genes and a targeted selection of appropriate human genomic DNA samples used for high-throughput sequencing are currently in progress in a number of laboratories. The approaches and concepts outlined in this paper (especially in Sections 4 and 5) are not the only possible avenues for the identification of the RA (or other autoimmune disease)-related defects in the genome. However, these approaches may allow us to merge currently available results of human GWAS with findings of GWAS and IVSC studies in mice. Nonetheless, to confirm the role of these genes in RA, researchers must identify not only the genomic identity but also the corresponding functional defects in mice analogous with those present in patients with RA. Unfortunately, mechanistic and functional studies, manipulation of the genome, and pretesting of new therapeutic approaches cannot be applied in human patients, which underlines the relevance of and necessity for laborious genetic studies in animal models.

Abbreviations:

Anti-CCP:	Anti citrullinated peptide antibody
ACPA:	Anti-citrullinated protein antibody
CII:	Type II collagen
CAIA:	Collagen antibody-induced arthritis
CIA:	Type II collagen-induced arthritis
Chr:	Chromosome
cM:	Centi-Morgan
GIA:	Human cartilage PG's G1 domain-induced arthritis
GPI:	Glucose-6-phosphate isomerase
GWAS:	Genome-wide association studies
HLA:	Human leukocyte antigen
HS:	Heterogeneous stock
IFNγ:	Interferon gamma
IL:	Interleukin
IVSC:	Interval-specific congenic
mCia:	QTL of mouse CIA
Mbp:	Mega-base pair
MHC:	Major-histocompatibility complex
QTLs:	Quantitative trait loci
PG:	Cartilage proteoglycan aggrecan
PGIA:	PG aggrecan-induced arthritis
Pgia:	QTL of PGIA
Pia:	QTL of pristane-induced arthritis
RA:	Rheumatoid arthritis
RF:	Rheumatoid factor
SSLP:	Single sequence length polymorphism
TCR:	T cell receptor
TNFα:	Tumor necrosis factor alpha.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

T. Besenyei and A. Kadar wrote the first draft of the paper, and then all other authors wrote and corrected the final version of the paper, which was submitted by the corresponding author (Z. Szekanecz).

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Review Article

Genetics of SLE: Functional Relevance for Monocytes/Macrophages in Disease

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Genetic studies in the last 5 years have greatly facilitated our understanding of how the dysregulation of diverse components of the innate immune system contributes to pathophysiology of SLE. A role for macrophages in the pathogenesis of SLE was first proposed as early as the 1980s following the discovery that SLE macrophages were defective in their ability to clear apoptotic cell debris, thus prolonging exposure of potential autoantigens to the adaptive immune response. More recently, there is an emerging appreciation of the contribution both monocytes and macrophages play in orchestrating immune responses with perturbations in their activation or regulation leading to immune dysregulation. This paper will focus on understanding the relevance of genes identified as being associated with innate immune function of monocytes and macrophages and development of SLE, particularly with respect to their role in (1) immune complex (IC) recognition and clearance, (2) nucleic acid recognition via toll-like receptors (TLRs) and downstream signalling, and (3) interferon signalling. Particular attention will be paid to the functional consequences these genetic associations have for disease susceptibility or pathogenesis.

1. Macrophages in Disease: SLE Candidate Genes and Functional Relevance

Systemic lupus erythematosus (SLE) is a multisystem chronic autoimmune disease, which affects approximately 0.1% of the population, with women being approximately nine times more likely to develop the disease than men [1]. SLE is a complex disease encompassing a broad spectrum of clinical symptoms, particular combinations of which can result in varying disease severity. To date the majority of work undertaken with respect to understanding the pathophysiology of this condition has focused on the autoreactive B and T lymphocytes [2]. However, recently attention has shifted to the role of the innate immune system and particularly myeloid cells in disease. Both monocytes and macrophages are phenotypically altered in SLE, with SLE macrophages demonstrated to have reduced uptake of apoptotic cells, enhanced activatory status, an altered skew of proinflammatory and anti-inflammatory macrophages and an overproduction of inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), and antiviral type I interferons (IFNs) (Figure 1) [3–5]. As such, SLE monocytes and macrophages present self-antigens to autoreactive T cells in an inflammatory context, rather than the immuno-silent presentation normally associated with material from apoptotic cells [3]. In addition to this, the overproduction of type I IFNs by myeloid cells (including dendritic cells) also contributes to the breaking of immune tolerance due to their ability to stimulate antibody production and class switching from B cells [4]. The inadequate regulation of these processes in myeloid cells may be as a result of the influence of variants within SLE susceptibility genes.

Genetic analysis in human and murine studies indicate that susceptibility to SLE is heritable and that a number of different genetic loci are associated with disease risk [5]. Both candidate gene studies and Genome-wide association studies (GWAS) have unearthed many genes whose function can

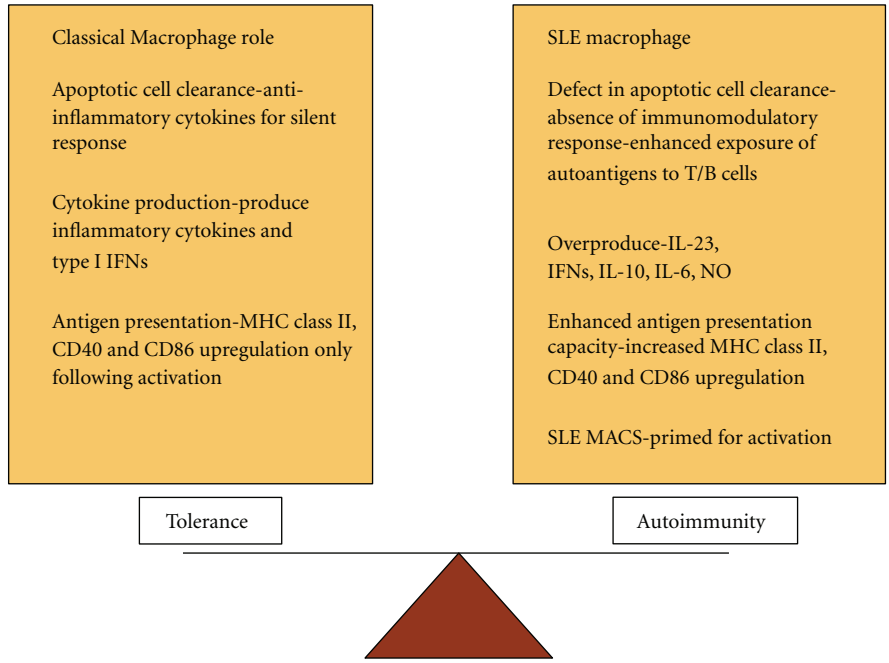

Figure 1: Dysregulation of macrophage function in SLE. The ability of the immune system to regulate macrophage function is altered in patients suffering from SLE. SLE macrophages have a defect in apoptotic cell clearance, overproduce IL-21, IFNs, IL-10, IL-6, and NO, have enhanced antigen presentation capacity and are primed for activation, leading to a skew towards autoimmunity.

be clustered into 3 different categories, each clearly rooted in innate immune cell signalling and function (Table 1): (1) immune complex (IC) recognition and clearance such as the complement components and the Fc gamma receptors [6–8]; (2) nucleic acid recognition via toll-like receptors (TLRs) [9–11] and downstream signalling components such as TNF receptor-associated factor-6 (TRAF6) [12] and interferon regulatory factors (IRFs) [13] and (3) interferon signalling [14]. Characterisation of the molecular involvement of many of these genes in the function of SLE monocytes and macrophages has placed these cells as key orchestrators of SLE pathogenesis. Whilst the focus of this paper is the involvement of these candidate genes in macrophage function and their contribution to SLE pathology, it must be stressed that many of the candidate genes discussed below, particularly those that regulate type I IFN production, also play an important role in dendritic cell-driven autoimmune pathology [15, 16].

2. Immune Complex Recognition and Uptake

2.1. The Complement System and Its Association with SLE. The principal function of activated components of the complement system include production of inflammatory and chemotactic proteins (C3a and C5a), cell lysis through the formation of the membrane attack complex (complex of C5b-9 proteins), and most importantly in the context of SLE, recognition and clearance of immune complexes and apoptotic cells (C1–C4) [6–8, 44]. Although genetic deficiencies in individual loci are rare, homozygous deficiency of each of the classical pathway components (C1q, C1r, C1s, C4, and C2) has been shown to be associated with SLE in humans [22]. A hierarchy of susceptibility and severity of disease is present where association is greatest with homozygous C1q deficiency followed by homozygous C4 and C2 deficiency [8]. Hereditary deficiencies of C1s and C1r are rarer than that of C1q and, in the majority of cases, deficiencies of both these components are inherited together [19, 20]. Both C1q and C4 are important in clearance of apoptotic cells and immune complexes, thereby preventing inappropriate activation of autoreactive B and T cells. Thus, reduced functioning of this important housekeeping function of complement proteins is strongly associated with increased risk of developing SLE [13–15, 19, 45].

C1q functions in facilitating clearance of immune complexes and apoptotic cells, thus protecting against autoimmunity. In addition, recent work has demonstrated that C1q can protect against SLE by preventing the production of type I IFN by dendritic cells [45, 46]. Individuals with a congenital deficiency of *C1q* gene (C1qD) develop SLE-like symptoms at more than 90% prevalence [47, 48]. Interestingly, the importance of ethnicity and the possible influence of haplotypes is highlighted by the observation that although C1q deficiency has been reported in Turkish [17] and Mexican [18] individuals affected by SLE, no association has been in Malaysian patients [49].

Homozygous deficiency of complement C4 is one of the strongest genetic risk factors for SLE and results in lupus-like disease in approximately 80% of the 28 known affected individuals [23, 24, 47]. To date, 28 individuals with complete C4 deficiency from 19 families have been reported, among these 15 individuals developed SLE, 7 developed lupus-like

Table 1: Polymorphisms of genes associated with SLE outlined in this review.

Category	Gene	SNP	Ethnicity
Immune complex recognition	C1q [8, 17, 18] C1r [19] C1s [20] C2 [22] C4 [8, 22–24]	Rs292001 [21]	Turkish [17], Mexican [18]
Nucleic acid recognition	TLR7 [25]	rs3853839 [25]	Chinese, Japanese [25]
		rs179019 [26]	Japanese [26]
		rs179010 [26]	
		rs179008 [27]	Brazilian [27]
	TLR9	rs5743836 [27]	Brazilian [27]
		rs352139 [28]	Japanese [28]
		rs352140 [29]	Chinese [29]
	IRF7	rs1131665 [30]	Asian [30], European American [30], African American [30]
	TRAF6	rs5030437 [12]	
		rs4755453 [12]	African American [12]
		rs540386 [12]	
	TNFAIP3	rs13192841 [31]	European [31]
		rs2230926 [31, 32]	European [31],
		rs6922466 [31]	Chinese Han [33]
		rs5029939 [34]	European [31]
	TNIP1	rs7708392 [35, 36]	Caucasian, Chinese [35, 36]
		rs10036748 [35, 36]	Japanese [26] Caucasian,Chinese [35, 36]
Interferon signalling	STAT4	rs7582694 [37]	Caucasian [37]
		rs7601754 [38]	Caucasian [38]
		rs7574865 [38]	Caucasian [38], Northern Han Chinese [39]
	TYK2	rs7582694 [40]	Caucasian [40]
		rs280519 [41]	UK, Swedish [41]
		rs2304256 [13]	Scandinavian [13]
	IRF5	rs12720270 [42]	UK [42]
		rs10488631 [43]	

disease and four of the remaining subjects had kidney disease [24]. Through a five nucleotide substitution in exon 26, the *C4* gene can encode either a C4A or a C4B protein [50], both of which have differential functions. C4A preferentially binds to amino groups in immune complexes and is the preferential ligand for complement receptor 1 (CR1) [51] whereas C4B is thought to be a more potent initiator of the complement activation cascade. The complement *C4* gene located in the class III region of the major histocompatibility complex (MHC) on chromosome 6p21.3 and exhibits significant interindividual copy number variation (CNV). Boteva et al. demonstrated low *C4A* genome copy number significantly predisposed to SLE in UK and Spanish populations ($P < 0.001$) however, high *C4A* genome copy number was not associated with disease in either case ($P = 0.63$ and $P = 0.76$, resp.) [52]. Interestingly, *C4B* genome copy number demonstrated no association in the UK SLE group but was significantly associated with the Spanish cohort ($P = 0.001$). The discrepancies reported across different patient populations with respect to *C4* copy number suggests that

partial *C4* deficiency states secondary to low *C4A* or *C4B* copy number are not independent genetic risk factors for susceptibility to disease [52, 53].

In addition to the rare inherited immunodeficiencies observed, many SLE patients have reduced levels of circulating C1q or C4 as a result of autoantibodies against these proteins, thus resulting in loss of their protective functions. Thus combined, mutations or decreased function of the early complement components has a profound effect on an individual's susceptibility to developing SLE.

2.2. Fc Gamma Receptors. Studies have investigated the contribution of the Fc-gamma family of receptors (FcγRs) to the pathogenesis of SLE given their role in the recognition of the Fc portion of IgG and subsequent responses to circulating and deposited immune complexes. Recent work in animal models indicates that the development of many human autoimmune diseases might be caused by impairment of the FcγR regulatory system (reviewed in [54]). FcγRs bind IgG, and can be further classified as activatory (FcγRI, IIA, IIIA, IIIB, and IV) or inhibitory (FcγRIIB) following IgG binding [55]. Additionally they can be subcategorised by relative affinity for IgG, with FcγRI having highest affinity, while FcγRII and III display lower affinity [56]. Currently there are no known polymorphisms in the *FcγRI* gene reported in humans and the rare individuals lacking this gene are healthy with no signs of autoimmune immune pathology [57]. However, polymorphisms in the activatory receptors *FcγRIIA* and *FcγRIIIA* have been identified [58–64].

FcγRII and III are encoded by two families of genes (*FCGR2, FCGR3*) clustered on chromosome 1q23-24, each containing multiple distinct genes [58]. FcγRIIA is a low-affinity receptor, comprised of multiple isoforms, which is expressed by B cells, monocytes, macrophages, and dendritic cells (DCs). It has two codominantly expressed alleles, R131 and H131, which differ in their affinity for IgG subclasses. Substitition of arginine to histidine at position 131 at the membrane proximal portion of the receptor results in enhanced affinity of FcγRIIA for binding of IgG2 and IgG3 by the H131 variant and increased levels of phagocytosis [59]. The allelic variant of FcγRIIA (R131) has been found to be strongly associated with lupus nephritis and renal failure in Brazilian lupus patients ($P = 0.06$) [60]. Interestingly meta analysis of European, African, and Asian populations demonstrated a significant association between the homozygous RR genotype and the development of SLE ($P = 0.0016$). This polymorphism was shown to increase the risk of devolving SLE 1.3-fold [61]. However analysis of this polymorphism in a Malaysian population found no significant association with disease [62].

FcγRIII encodes an activatory FcγR which is expressed on NK cells and monocytes, and has two isoforms: FcγRIIIA and FcγRIIIB. The wild-type sequence at position 176 encodes a phenylalanine (176-F) while the polymorphic variant is 176-valine (176-V) resulting in increased binding of IgG1 and IgG3 [63]. Recent studies in Japanese and Chinese patient cohorts found that positivity for the 176F allele was significantly increased in patients ($P = 0.02$ and $P = 0.05$ resp.), indicating a significant association of this allele with SLE [64, 65]. Additionally a significant association with this polymorphism and the development of lupus nephritis was observed among the Japanese patient cohort ($P = 0.03$) [64].

FcγRIIIB is an alternative membrane form of FcγRIII that is predominantly expressed on neutrophils and preferentially binds IgG1 and IgG3. The *FcγRIIIB* gene has three polymorphic forms known as HNA-1a, HNA-1b, and HNA-1c, encoded by the alleles *FCGR3B*01, FCGR3B*02*, and *FCGR3B**03 (also referred to NA1, NA2, and SH) [66]. These different isoforms of *FcγRIII* exhibit differential function with increased levels of phagocytosis reported for FCGR3B*01 homozygotes compared to cells from FCGR3B*02 homozygotes, despite similar levels of receptor expression [67]. Reduced function of the FCGR3B*02 allele has been associated with impaired IC clearance in Caucasian populations [68] and has been strongly associated with disease susceptibility in Japanese and Thai populations ($P = 0.008$ and $P = 0.02$, resp.) [69, 70] and significantly associated with the development of lupus nephritis among the Japanese patient cohort ($P = 0.007$), whereas as no association was found in other population studies [62, 71].

As an inhibitory FcγR, loss of FcγRIIB not surprisingly results in development of lupus-like symptoms in mice, with the development of autoantibodies and autoimmune glomerulonephritis, consistent with a lack of inhibitory mechanisms on the development of autoreactive B cells [72]. Subsequent studies have demonstrated that increasing the expression of FcγRIIB in B cells derived from autoimmune-prone mice restored tolerance and prevented autoimmune disease [73]. With respect to the role of FcγRIIB in human autoimmune disease, reduced expression of FcγRIIb has been reported for memory B cells and plasma cells from SLE patients [74]. Interestingly, a polymorphism of FcγRIIb which changes the threonine at position 232 to an isoleucine (I232T) was found to be associated with SLE as positivity for the 232I allele was significantly decreased in SLE patients suggesting a significant association of the 232T/T genotype with SLE [64]. This study also found that the odds ratios (ORs) for the development of SLE among individuals with the T/T and I/T genotypes versus the I/I genotype were 2.3 and 1.1, respectively. A further comparison of genotype frequencies with patient clinical data revealed that *FCGR2B* polymorphismsstrongly associated with lupus nephritis ($P = 0.01$). This amino acid is in the transmembrane domain of FcγRIIb, and the polymorphism reduces the signalling capability of FcγRIIb due to its exclusion from lipid rafts [75]. Thus balanced signalling through activatory and inhibitory FcγRs regulates the activity of various cells in the immune system and genetic evidence in both mice and humans strongly supports the role of this receptor family in preventing the development of autoimmunity.

2.3. CD11b/ITGAM. ITGAM encodes integrin alpha-M (also commonly known as CD11b or complement receptor 3), the alpha chain of $\alpha M\beta 2$ integrin which binds the cleavage fragment of complement component C3b, an opsonin and facilitates uptake of C3b-coated particles/pathogens into phagocytic cells (reviewed in [76]). Genetic association of ITGAM with SLE was found independently in 2 European

GWAS [77, 78], with a non-synonomous functional variant being identified in a subsequent study [79]. Functionally this variant encodes an arginine to histine mutation at amino acid 77 which alters both the structure and function of integrin αM, thus reducing its ability to clear immune complexes [80].

3. Toll-Like Receptor Signalling and IFN Induction in SLE

Our increased awareness of the role played by cells of the innate immune system in disease has stemmed from the discovery of families of innate immune receptors, such as the TLRs, which have evolved to recognise and discriminate between different classes of pathogenesis reviewed in [81]. A link between antiviral pathogen recognition receptors and SLE is now well established, thus giving credence perhaps to the long-held view point that viral infection plays an important role in either the etiology of SLE or in driving flares in affected individuals [82]. With respect to SLE, receptors that can recognise viral nucleic acids, such as the endosomally located antiviral TLRs (TLR3, 7/8, and 9) [9–11] the intracellular RIG-I-like receptors (RLRs) [83] and AIM2-like receptors (ALRs) receptor families [84], have been implicated in SLE. There now exists strong genetic and functional evidence that RNA/DNA immune-complexes found in lupus patients can drive IFN-α production through the activation of TLR7 or TLR9 [85], respectively, indicating that TLR7/9 activation may be an important primary trigger for the generation of autoimmune disease (reviewed in [5, 86]). Plasmacytoid dendritic cells have been identified as the primary interferon-producing cells [87], however immature monocytes have also been demonstrated to produce significant levels of IFN-α in a mouse model of lupus and also in human SLE monocytes in response to immune complex activation [88, 89]. In addition to the viral TLRs themselves playing a role in the pathogenesis of this condition, downstream signalling components of these and their products may also contribute to the progression of this condition. Firstly, it is well documented that roughly half of all SLE patients overexpress IFN-α, thus giving rise to changes of gene expression that can be detected in peripheral blood monocytes, termed the IFN gene signature [90–93]. More recently, the activity and expression of certain members of the IRF family of transcription factors which regulate IFN production and mediate its effects, specifically IRF3 and IRF5, have been shown to be enhanced in SLE monocytes, resulting in increased expression of a subset of IRF-dependent genes [89, 94, 95]. For example, recent studies have shown that the levels of IRF3 bound to the promoter of a key pathogenic cytokine in SLE, IL-23, are enhanced in monocytes from SLE patients, thus resulting in increased basal production of this cytokine in SLE monocytes [95]. Likewise, monocytes from SLE patients present increased basal levels of nuclear IRF5 thus potentially contributing to enhanced production of the cytokines IFN-α, TNF-α, and IL-6 [96]. Thus not only are the triggers for activating SLE monocytes or macrophages in abundance due to impaired apoptotic cell clearance but also key downstream transcription factors such as the IRF family appear to be hyperresponsive in SLE monocytes [96], a finding inspired by genetic evidence linking IRF5 to disease [97].

3.1. Genetic Association of Antiviral Toll-Like Receptors TLR7 and TLR9 with SLE. With respect to the initial recognition of self-RNA and self-DNA by the antiviral TLRs, there have been several genetic studies in both human and murine models that further implicate these receptors in the pathogenesis of this condition in particular TLRs 7 and 9 [25–27, 88–96, 98–102].

3.1.1. Toll Like Receptor 7 (TLR7). Mice lacking the *TLR7* gene (located at Xp22.2) exhibit ameliorated disease, decreased lymphocyte activation and a marked reduction in the levels of RNA-containing antigens [98]. Interestingly, BBXSB/MpJ (BXSB) mice bearing the *Yaa* gene (Y chromosome-linked autoimmune acceleration gene) spontaneously develop a lupus-like autoimmunity, with males being affected much earlier and to a greater extent than their female counterparts. These *Yaa* containing mice were found to have increased expression of TLR7 due to the translocation of approximately 17 genes, including TLR7, onto the pseudoautosomal region of the Y chromosome [99, 100]. Deane et al. (2007) demonstrated that this duplication of the *TLR7* gene and as a result, increased TLR7 expression, promoted the production of RNA-containing autoantibodies and development of lupus nephritis [101]. Although murine studies have indicated associations between *TLR7* gene variations and SLE, there is controversy regarding human association studies. Using candidate gene approaches, Shen et al. (2010) investigated a role for TLR7 in SLE in Eastern Asian populations in which they identified a functional polymorphism in 3′ UTR of the *TLR7* gene. This common variant (rs3853839G/C) was found to be robustly associated with SLE ($P = 0.016$), with a stronger effect seen in male subjects compared to their female counterparts [25]. The elevated levels of *TLR7* transcripts and as a result, the enhanced IFN signature in patients with the G-allele of this single nucleotide polymorphism (SNP), have supported a functional role for this polymorphism in SLE. However when this SNP was studied in a non-Asian population, there was no evidence for this SNP as a risk factor for SLE in males with only females of non-Asian descent showing this association [102]. Following on from this multicentre study, Kawasaki et al. (2011), observed two additional variants located within the intron (rs179019A/C and rs179010T/C) that were also associated with SLE in a Japanese cohort ($P = 0.016$ and 0.018, resp.) thus further supporting the role of TLR7 as a risk factor for the development of this autoimmune condition [26]. Further studies into TLR7 polymorphisms in a Brazilian population also suggested the *TLR7* SNP rs179008A/T as an SLE susceptibility factor in women of European descent ($P = 0.020$); however, this was not replicated in a Spanish population [27, 103]. Moreover an additional study into the role of copy number variants of *TLR7* in SLE identified that increased *TLR7* copy number was also a risk factor for the onset of juvenile SLE [104].

3.1.2. Toll-Like Receptor 9 (TLR9). In addition to enhanced TLR7 expression, TLR9 has also been demonstrated to be upregulated in SLE B cells [105], further implicating a role for these viral TLRs in B cell tolerance and as result in the progression of SLE. In murine models, a role for TLR9 in disease susceptibility has also been examined with varying results. Christensen et al. (2005) demonstrated that TLR9 knockout mice crossed with lupus-prone mice exhibit decreased levels of anti-DNA antibodies implicating this gene as important in the progression of this condition [11]. However, in contrast the genetic ablation study carried out by Wu and Peng investigating the role of TLR9 in SLE, demonstrated that MRL mice lacking TLR9 developed more severe lupus than MRL controls, demonstrating a protective role for this gene in the pathogenesis of this condition [106]. Although numerous SNPs have been identified in the *TLR9* locus (chromosome 3p21.3), which falls into the SLE susceptibility region, there is very little correlation between these variants and the onset of SLE and again this is an area of major controversy within the literature. A number of these common SNPs (rs187084, rs5743836, rs352139, and rs352140) were investigated in a Hong Kong Chinese population [107], however although overrepresented in SLE showed no significant association. When the rs5743836 SNP was further analysed in Caucasian American individuals, again no functional association was identified with this polymorphism [108]. In contrast, studies in Brazilian patients replicated these results reporting this SNP as an SLE susceptibility factor ($P = 0.045$) [27]. Consistent with results seen by Tao et al. investigating TLR9, the exonic region rs352139A/G SNP has been mildly associated with SLE ($P = 0.040$), with genetic analysis in a Japanese population indicating that carrying the G allele of this polymorphism predisposes individuals to an increased risk of SLE through the downregulation of TLR9 expression levels in reporter gene assays [28]. In addition to this, when the rs352140C/T in exon 2, was examined using a family-based association in China it was also reported that this SNP was also mildly associated with disease susceptibility ($P = 0.045$) [29].

The divergent roles played by TLR7 and 9 in autoimmunity are reflected in the variation seen in *TLR7/9* polymorphisms and their subsequent effect on disease progression. TLR7 polymorphisms appear to increase expression of this gene leading to the enhanced recognition of autoantibodies culminating in an enhanced IFN signature thus predisposing these individuals to SLE [99]. On the other hand, TLR9 polymorphism associations, whilst controversial, particularly with respect to the different genetic backgrounds of the populations examined, suggest that *TLR9* SNPs downregulate its expression and in doing so increase disease susceptibility [107]. Although the exact mechanism for this is not yet known, it has been suggested that lower levels of TLR9 expression lead to defective T regulatory cell activation which contributes to the decrease in number and immunosuppressive function of these cells in the MRL model of murine lupus [106]. Despite some controversy surrounding individual *TLR7/9* SNPs in SLE, there is a growing body of evidence emerging to suggest that polymorphisms in these receptors play a role in genetic predisposition to this condition.

3.2. TLR Signalling Components. Activation of TLR7 and TLR9 by self nucleic acids and immune complexes has been demonstrated to contribute to the pathogenic production of IFN-α and proinflammatory cytokines such as TNF-α, IL-6 and IL-12 [109, 110]. A number of genes involved in type I IFN production and signalling have been linked to SLE [111–113]. Proteins directly activated downstream of TLR7 and TLR9, such as TRAF6 [12] and the IRF family of transcription factors [13], have known genetic association with SLE. In addition, proteins that negatively regulate TLR-induced activation of transcription factors IRF7 and NF-κB such as A20, have also been shown to contribute to lupus susceptibility in a combination of either GWAS or candidate gene approaches [31, 33, 114].

3.2.1. TRAF6. TNF-receptor-associated factor 6 (TRAF6) plays an important role in many signalling pathways that are important for immune regulation. TRAF6 was firstly identified in 1996 as a signal transducer in the NF-κB pathway which associates with interleukin-1 receptor-associated kinase (IRAK) [115]. Recent studies have suggested that polymorphisms within *TRAF6* may be associated with the development of SLE, with SNPs in the *TRAF6* gene giving nominal signals of association with SLE in an extended family Swedish cohort [116]. A more recent study showed a direct correlation between *TRAF6* SNPs and SLE, supporting the notion that TRAF6 is potentially involved in the pathogenesis of autoimmune conditions [12]. In this study, fifteen SNPs across *TRAF6* were evaluated in 7,490 SLE patients and 6,780 control subjects from different ancestries. Evidence of associations was detected in multiple SNPs, with rs5030437 and rs4755453 showing the strongest association [12].

3.2.2. TREX1. *TREX1* encodes the most abundant 3′-5′ exonuclease in mammalian cells and has also been implicated in the cell death process, recognising and degrading genomic DNA and endogenous retroviral elements to minimize potential immune activation by persistent immunostimulatory DNA in the cytoplasm [117] (reviewed in [118]). Various genetic studies have identified a number of loss-of-function mutations in *TREX1* that give rise to SLE, familial chilblain lupus (FCL), or Aicardi-Gautieres syndrome (AGS), an autoimmune disorder that presents as early onset encephalopathy resulting in severe intellectual and physical handicap [119–122]. Functional studies into these loss-of-function mutants of *TREX1* demonstrate that they result in enhanced levels of immunostimulatory DNA resulting in enahnced type I IFN production. For example, *TREX1D18N* and *TREX1D200N* heterozygous mutants have been identified in FCL and AGS, respectively, and functionally are completely deficient at degrading dsDNA and demonstrate a lower rate of degradation of ssDNA than wild-type TREX1. The *TREX1R114H* homozygous mutation identified in AGS patients is found as a heterozygous mutation in SLE. As a homodimer *TREX1R114H* shows defects in its

ability to degrade both ds- and ss-DNA, indicating that loss of function of TREX1 results in enhanced levels of immunostimulatory DNA which in turn results in enhanced levels of type I IFNs observed in both SLE and AGS [123, 124]. These findings clearly implicate TREX1 as an important endogenous DNA sensor that works to prevent inappropriate immune activation.

3.2.3. IRF5. Association of IRF5 genetic variants with SLE susceptibility has been first reported following a screening of genes involved in type I IFN production and response in Swedish, Icelandic, and Finnish patients with SLE [13]. Since then, the evidence of a link between IRF5 and SLE has been replicated in a number of case-control linkage studies in different populations [125–128] and GWAS analyses (reviewed in [129]). Association of IRF5 with SLE is complex, and a number of genetic studies have allowed defining risk, neutral, and protective haplotypes. Initially, 3 common polymorphisms in the *IRF5* gene (SNPs rs2004640 in the 5′ UTR and rs10954213 in the 3′ UTR and a 30 nucleotides insertion in exon 6) [97] were proposed to alter the function or levels of IRF5 mRNA and proteins, thus explaining the association of risk alleles of these polymorphisms with SLE. A subsequent study by Sigurdsson et al. [130] identified two *IRF5* polymorphisms independently and strongly associated with SLE: a 5 bp CGGGG insertion located 64 base pairs upstream of IRF5 exon 1a ($P = 4.6 \times 10^{-9}$) and a SNP (rs10488631) downstream of the *IRF5* gene ($P = 9.4 \times 10^{-10}$). The presence of the insertion creates an additional binding site for the transcription factor Sp1, leading to increased transcription of IRF5 [130]. Interestingly, the CGGGG insertion is in linkage disequilibrium with SNPs rs2004640 and rs10954213, thus accounting for the association previously observed between these two SNPs and SLE. Interestingly, the CGGGG insertion in *IRF5* promoter has been associated with a number of autoimmune conditions, such as primary Sjögren's syndrome [131], Multiple sclerosis [132], inflammatory bowel disease and Crohn's disease [133], while the haplotype tagged by rs10488631 seems to be specific in conferring SLE susceptibility [130]. A recent study by Hedl and Abraham [134] has found that monocyte-derived cells from healthy individuals carrying the risk alleles of SNP rs2004640 and the CGGGG insertion secreted elevated levels of proinflammatory cytokines following stimulation with Nod2 and TLRs ligands, thus suggesting a correlation between IRF5 genetic variants and transcriptional activity. In keeping with this, it has been shown that patients carrying IRF5-risk haplotypes have increased levels of circulating IFNα in the serum compared to patients carrying neutral or protective haplotypes. Of note, such correlation was observed only in patients positive for either anti-dsDNA or anti-RBP autoantibodies [135], and the study was sub sequentially expanded to show that different classes of autoantibodies are linked to different *IRF5* haplotypes. Since autoantibodies can deliver self nucleic acids to endosomal TLRs [136], thus activating IRF5, the authors proposed that distinct classes of autoantibodies could activate specific IRF5 variants, leading to dysregulation of IFNα production and increased transcription of interferon-stimulated genes [137].

3.2.4. IRF7. IRF7 is considered the master regulator of IFNα production downstream the antiviral TLRs [138], and polymorphisms in this gene could therefore be an ideal candidate for genetic susceptibility to SLE. Together with IRF5, IRF7 has been shown to be necessary for murine DCs-mediated production of IL-6 and IFNα induced by immune complexes isolated from SLE patients' sera, again indicating a central role for these transcription factors in the disease context [85]. SNPs in the genetic region spanning the *IRF7* gene (adjacent to the PHRF1 locus, also known as KIAA1542) have been identified, and different groups have attempted to associate common genetic variants at this site with SLE susceptibility. A GWAS in women affected by SLE has found a correlation between SNP rs4963128 in *KIAA1542* and lupus ($P = 3 \times 10^{-10}$). Since this SNP is in strong linkage disequilibrium with SNP rs702966 located within 0.6 kb of *IRF7*, it was thought that variability at this site could represent the signal deriving from *IRF7* [78]. Association of these two SNPs with lupus susceptibility has been replicated in populations of different ancestries by Salloum et al. [139]. Interestingly, this study demonstrated a correlation between the risk alleles of these SNPs and increased levels of IFNα, but only in patients with autoantibodies. Similar to what has been suggested for IRF5, potential autoantibodies might cooperate with SLE-associated IRF7 variants through TLR activation, resulting in increased type I IFN production which leads to breaking of tolerance and the onset of disease. In keeping with this, SNP rs4963128 was correlated with nephritis and anti-Ro/anti-La autoantibodies in a Chinese population, although no association of this SNP with lupus susceptibility was observed in this genetic background [39]. To date, the only known functional polymorphism in *IRF7* is the nonsynonymous SNP rs1131665 which encodes a protein carrying a Q to R mutation at position 412 [30]. This variant has been shown to be associated with SLE patients of Asian and European American ancestry ($P = 6.18 \times 10^{-6}$), and functional analysis of the mutated protein revealed its enhanced transcriptional activation of an ISRE-dependent promoter. This is in keeping with the hypothesis that SLE-associated *IRF7* polymorphisms may lead to the expression of proteins with increased activity downstream of the TLRs, thus leading to overproduction of type I IFN characteristic of the disease.

3.2.5. TNFAIP3. Tumour necrosis factor α-induced protein 3 (TNFAIP3), the gene product of which is the ubiquitin editing protein A20, is an essential negative regulator of pathways regulating NF-κB [140–142]. Recently TNFAIP3/A20 has been shown to interact with and negatively regulate IRF7, thus potentially explaining its molecular involvement in SLE [143]. Polymorphisms within the TNFAIP3 genomic locus, located at 6q23, have been associated with autoimmune disorders such as SLE [31, 35, 114, 144, 145] in Caucasian, Asian and Japanese populations. In particular, three independent SNPs in the TNFAIP3 gene (rs13192841, rs2230926 and rs6922466) are thought to be associated with SLE patients of European ancestry [31]. More recently the results of a meta-analysis of genome-wide association scans and replication in independent sets for *TNFAIP3* polymorphism

and SLE showed another *TNFAIP3* SNP (rs2230926) to have an association with SLE [32]. This sample set contained 12,416 subjects with SLE from multiple ethnic groups and so suggested that this particular SNP may be conserved throughout diverse populations. In order to fully characterise the TNFAIP3 risk haplotype, fine mapping and genomic resequencing in ethnically diverse populations were carried out [146]. Results suggested a TT>A variant to be the most likely functional polymorphism responsible for the association between *TNFAIP3* and SLE in subjects of both European and Korean ancestry [146]. This variant displayed a reduced binding avidity for NF-κB subunits. In addition, the haplotype carrying this variant resulted in reduced TNFAIP3 mRNA and A20 protein expression [146]. These findings underscore the crucial role of NF-κB regulation in the pathogenesis of SLE.

The TNFAIP3 interacting protein 1, (TNIP1), also known as ABIN1, interacts with TNFAIP3/A20 and promotes inhibition of NF-κB activity [147, 148]. TNIP1 has also been shown to be associated with SLE in a wide range of ethnic groups. Two individual GWAS revealed association of TNIP1 intronic SNPs, rs7708392, and rs10036748, with SLE in both Caucasian and Chinese populations [35, 36]. Subsequently a study was carried out in a Japanese population which confirmed the association of TNIP1 rs7708392 with SLE [148]. Interestingly in this study, this SNP showed a tendency of stronger association with SLE patients with renal disorder than in all SLE patients. Overall these studies highlight the important role that both TNFAIP3 and TNIP1 play in genetic predisposition to autoimmune disorders such as SLE.

3.3. Interferon Signalling Components. Serum levels of type I IFN correlate with disease activity and clinical manifestation [14], and interestingly lupus-like disorders can be induced during type I IFN therapy, again highlighting the pivotal role of these cytokines in disease development [149, 150]. Secreted type I IFN can then signal through the type I IFN receptor and kinases; tyrosine kinase 2 (TYK2) and janus kinase 1 (JAK1) [151]. Activation of the type I IFN receptor triggers phosphorylation of the transcription factors signal transducer and activator of transcription 1 and 2 (STAT1 and STAT2) and assembly of the interferon stimulated gene factor 3 (ISGF3) complex, which then translocates to the nucleus where it regulates production of IFN-stimulated genes necessary to establish the antiviral state (Figure 2) [152]. Polymorphisms in genes such as TYK2 and STAT4, involved in signalling downstream of the type I IFN receptor and a number of other cytokines, have been identified that might instead alter responses to type I IFN in SLE [37, 38, 41, 42, 153–159].

3.3.1. STAT4. *STAT4*, the signal transducer and activator of transcription 4 gene, encodes a transcription factor that mediates the effect of several cytokines, including IL-12, the type I interferons, and IL-23 in T cells and monocytes [153]. Thus, STAT4 has a role in T-cell differentiation, monocyte activation, and IFN-γ production. STAT4 was confirmed in 2003 by Jacob et al. to play a key role in the pathogenesis of a lupus-like disease in mice [154]. They showed that loss of STAT4 led to accelerated renal disease and increased mortality. A number of genetic studies have identified *STAT4* SNPs with links to SLE in Caucasian populations for example, rs7582694 [37], rs7601754 and rs7574865 [38], and rs7582694 [155], in addition to rs7574865 and SLE in a Northern Han Chinese population [39]. Using transmission disequilibrium test analysis the rs7582694 SNP was found to have a strong association with SLE ($P = 0.002$, OR = 2.57) in a Finnish family cohort [37]. Using meta-analysis the SNPs rs7601754 and rs7574865 were found to have a significant association with SLE ($P < 0.001$) in populations of European and African origin [38]. Sigurdsson et al. (2008), in using a candidate gene study, also identified the SNP rs758294 as part of a common-risk haplotype for SLE ($P = 1.7 \times 10^{-5}$) in Swedish patients with SLE [155]. Li et al. (2011), using a candidate gene study in a Northern Han Chinese population, found a strong association between the SNP rs7574865 and SLE ($P = 1.57 \times 10^{-6}$) [39]. These SNPs are located within introns and are therefore suggested to play a role in the regulation of the expression level or splicing of the gene [155].

3.3.2. TYK2. TYK2 binds to the type I IFN receptor (IFNAR), thus initiating the JAK-STAT signalling cascade, culminating in the transcription of further type I IFN and IFN inducible genes [156]. A number of SNPs in *TYK2* have been recently reported to be associated with SLE in Caucasian populations, namely, rs280519, rs2304256, and rs12720270 [13, 41, 42]. The *TYK2* SNP rs280519 was found to be associated with SLE across a genome-wide association combined between a UK and Swedish cohort ($P = 3.88 \times 10^{-8}$) [41]. The *TYK2* SNP rs2304256, was found to be associated with SLE in a Scandinavian cohort ($P = 5.6 \times 10^{-6}$) [13], but not associated with SLE in a UK cohort [42], however this same UK study also found another *TYK2* SNP, rs12720270, associated with SLE that was not found within the Scandinavian cohort ($P = 0.004$). This SNP, rs12720270, however, was not found to be associated with SLE by Lee et al., when conducting meta-analysis on associations between SLE susceptibility and this SNP of *TYK2* [157]. The rs2304256 is located in exon 8, and the rare A allele of the SNP causes a substitution of Val to Phe at residue 362 in the Jak-homology 4 (JH4) region of TYK2. This region is important for the interaction of TYK2 with IFNAR1, its function [158], as well as for maintaining the expression of IFNAR1 on the cell surface [159], suggesting that this SNP may reduce the function of TYK2 and thus susceptibility to autoimmune diseases.

4. Conclusion

Evidence from GWAS and candidate gene approaches have uncovered an array of genes that have functional consequences for how monocytes and macrophages respond to immune challenge during the course of disease. Many of these genes regulate either phagocytic, TLR, or IFN

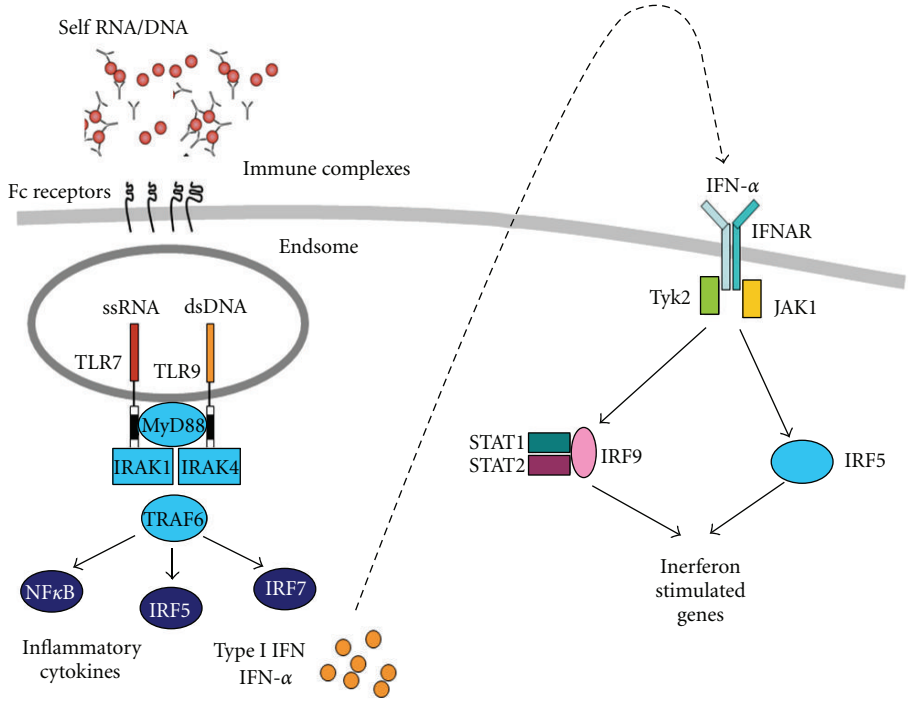

Figure 2: TLR induced IFN production and signalling in SLE. A brief outline of the signalling pathways involved in the production of type I IFNs in SLE. Activation of the transcription factors downstream of endosomal TLRs and Fc Receptors leads to the production of type I IFNs. These IFNs are secreted and further detected by IFN receptors, further activating interferon stimulated genes.

systems—three areas now well recognised to contribute to disease pathology. And as we become increasingly aware of the growing role of macrophages in disease pathology, it is interesting to note that cross-regulation of dendritic cells, the other major innate immune cell player in SLE pathology, by macrophages has an important role in driving disease. For example, *C1q* deficiency not only results in reduced uptake of immune complexes by macrophages and dendritic cells but it also is a negative regulator of IFN production by dendritic cells, thus its loss negatively impacts both macrophage and dendritic cell function in the context of disease pathology—exacerbating type I IFN production and contributing to a vicious cycle of reduced immune tolerance [160]. With respect to many of the genes discussed above, the functional relevance of their genetic variation has yet to be determined—do they contribute to pathogenic splice variants, altered transcript, or protein stability, or indeed introduce functional mutations that contribute to either over- or underactivation of the gene product? For others however, such as Trex1, not only is the molecular involvement of these variants in disease known, but research into their involvement in disease has uncovered novel functions for these proteins in innate immunity. However, where genetic associations uncovered have yet to conclusively demonstrate functional relevance for immune function in the context of SLE, we must be aware that many of the SNPs uncovered in SLE susceptibility regions may in fact have no true role in genetic susceptibility but instead, through linkage disequilibrium, act as a tag or marker for the real susceptibility gene. As researchers continue unravelling the functionality of genetic variability within SLE and translating these findings functionally to their contribution to immune dysregulation in SLE then we can undoubtedly expect this knowledge to contribute to greater insight into the molecular workings of disease. Already there are indications that certain SNPs appear to stratify with different disease manifestations and autoantibody profiles in SLE [161, 162], indicating the utility of screening to better inform and manage disease.

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Research Article

Characterizing T Cells in SCID Patients Presenting with Reactive or Residual T Lymphocytes

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Introduction. Patients with severe combined immunodeficiency (SCID) may present with residual circulating T cells. While all cells are functionally deficient, resulting in high susceptibility to infections, only some of these cells are causing autoimmune symptoms. *Methods*. Here we compared T-cell functions including the number of circulating $CD3^+$ T cells, *in vitro* responses to mitogens, T-cell receptor (TCR) repertoire, TCR excision circles (TREC) levels, and regulatory T cells (Tregs) enumeration in several immunodeficinecy subtypes, clinically presenting with nonreactive residual cells (MHC-II deficiency) or reactive cells. The latter includes patients with autoreactive clonal expanded T cell and patients with alloreactive transplacentally maternal T cells. *Results*. MHC-II deficient patients had slightly reduced T-cell function, normal TRECs, TCR repertoires, and normal Tregs enumeration. In contrast, patients with reactive T cells exhibited poor T-cell differentiation and activity. While the autoreactive cells displayed significantly reduced Tregs numbers, the alloreactive transplacentally acquired maternal lymphocytes had high functional Tregs. *Conclusion*. SCID patients presenting with circulating T cells show different patterns of T-cell activity and regulatory T cells enumeration that dictates the immunodeficient and autoimmune manifestations. We suggest that a high-tolerance capacity of the alloreactive transplacentally acquired maternal lymphocytes represents a toleration advantage, yet still associated with severe immunodeficiency.

1. Introduction

Severe combined immunodeficiency (SCID) is typically characterized by significantly low number and/or defective function of T and B cells. In some cases, T cells may present, as a result of residual autologous cells or transplacentally acquired maternal lymphocytes [1]. Residual autologous T cells are usually emerging from partial thymic maturation impairment such as in the case of Major histocompatibility complex class II (MHC-II) deficiency. MHC-II molecules drive the development, activation, and homeostasis of $CD4^+$ T-helper cells. It is thus not surprising that the absence of MHC-II expression results in a severe primary immunodeficiency disease. Yet, the residual cells in MHC-II deficient patients are considered as nonreactive; therefore patients typically do not display significant autoimmune phenomena. Although immunity is extensively impaired in such cases, regulatory tolerance mechanisms are not known to be affected [2]. Moreover, while the mainstay of the diagnosis of MHC-II deficiency is the absence of constitutive and inducible expression of MHC-II molecules on all cell types, other tests for T-cell function are less informative in such patients. In contrast, patients with self-reactive cells have significant autoimmune features in addition to their clinical and molecular immunodeficient state. The origin of the reactive cells in such patients are either thymic release of T-cells that expand at the periphery or transplacentally transfers of maternal T lymphocytes. These cells expand in the periphery, causing tissue infiltration and damage due to breakdown of both central (e.g., autoimmune regulator, AIRE protein

dysfunction) and peripheral ($FOXP3^+$ deficiency) tolerance mechanisms [3]. For example, Omenn syndrome, a typical case of impaired T-cell differentiation with abnormal self-reactive cells, is invariably characterized by autoimmune features such as generalized scaly exudative erythroderma, enlarged lymphoid tissues, and peripheral expansion of oligoclonal T-cells, in addition to increased susceptibility for severe infections [3, 4]. The suggested mechanism for this phenomenon is the possible inability of the thymus to delete these abnormal clones due to compromise of both central and peripheral tolerance mechanisms [5]. A distinctive feature of SCID patients, which sometimes can clinically resemble Omenn, [6] is the presence of alloreactive cells originated from transplacentally maternal T lymphocytes. The maternal placenta, an incomplete bidirectional barrier, allows transfer of maternal cells to occur in healthy neonates. Immunocompetent newborns can rapidly reject the HLA-mismatched maternal cells by effective T-cell immunity. In contrast, SCID patients fail to eliminate these cells and T-cell engraftment was reported in as many as 40% of them [7]. Immunologic characterization of these cells and their advantage of passing the placenta and surviving, compared to other maternal T cells, have not been investigated in depth. In the minority of cases these cells were found to have a normal phenotype with some degree of *in vivo* activation, as shown by the expression of MHC class II molecules and/or the IL-2 receptor [8]. Moreover, maternal engraftment provided the required immune competence and resulted in prolonged survival in rare cases of SCID [9]. In most cases, however, maternal T cells have been described as clonal cells [10], suggestive of either transplacental passage of a very small selected number of T cells or secondary expansion of alloreactive clones in the host. Transplacentally acquired maternal T lymphocytes and the autoreactive cells seen in Omenn phenotype have many clinical and laboratory features in common, including atypical skin eruption, hepatosplenomegaly, eosinophilia, elevated IgE levels, pattern of TH2 cytokines, lack of T-cell activity, and a restricted repertoire of the T cell receptor [11]. These cells do not provide enough immunity and may clinically be symptomatic, attacking the patient's organs. However, in contrast to Omenn patients where symptoms are typically severe, clinical findings associated with the transplacentally acquired maternal T lymphocytes are usually mild, with up to 60% being asymptomatic or mild symptomatic graft-versus-host disease (GVHD) [12]. The reason for this discrepancy is not clearly understood. In the current study, we have analyzed T-cell function, thymic capacity, and regulatory T cells (Tregs) enumerations in various severe immunodeficiency patients presenting with different origins of their T cells, hypothesizing that different severe immunodeficiency subtypes have different characterization of these cells, in correlation with the clinical features in each distinct subtype.

2. Materials and Methods

2.1. Patients. Six patients with clinical phenotypes suggestive of severe immunodeficiency, with or without Omenn features, were studied. The Institutional Review Board (Sheba Medical Center, Tel Hashomer) approved this study and a written informed consent was obtained from all parents of study's participants.

2.2. Immune Work Up. Cells surface markers of peripheral blood mononuclear cells (PBMCs), lymphocyte proliferative response to mitogens, T-cell receptor variable β (TCR Vβ) expression and the amount of signal joint (sj) T-cell receptor excision circles (TRECs) were determined as previously described [13]. To estimate TREC copies, we compared the amplification Ct value in a given sample with a standard curve obtained from PCRs performed with 10-fold serial dilutions of an internal standard. In 40 healthy age-matched control samples where immunodeficiency was excluded, TREC copies were >400.

2.3. Cell Isolation and Analysis of Treg Cells. PBMCs were obtained by density gradient centrifugation on Histopaque 1077 (Sigma). The mouse mAbs against various human-cell surface markers used were as follows: CD3-FITC, CD4-FITC, CD4-PE, CD4-APC, CD25-APC (all obtained from BD Pharmingen), CD25-PE (Miltenyi Biotec), and the 236A/E7 mouse anti-hFOXP3-APC mAbs (eBioscience). The isotype-matched control mAbs were all purchased from BD Pharmingen. For detection of forkhead box P3 (FOXP3), the cells were fixed/permeabilized using the eBioscience FOXP3 staining buffer set, according to the manufacturer's protocol (eBioscience). Cell samples were analyzed on a FACSCalibur using the Cellquest software. CD4 or CD8 positive T cells were isolated from PBMCs by positive selection with CD4 or CD8 microbeads (Milteny Biotec). IFNγ and IL-2 cytokine detections were used to verify the presence of Tregs. Briefly, T cells were reactivated with 20 ng/mL PMA and 0.8 μM ionomycin (Sigma) in the presence of monensin 2 μg/mL for 5 h (GolgiStop from BD Biosciences). Thereafter, the cells were fixed, permeabilized and stained for FOXP3 (236A/E7-APC) with the eBioscience Kit. In addition, the cells were stained with CD4-FITC and for cytokines with anti-IFNγ-PE, IL-2-PE (from BD Biosciences).

2.4. Visualization of Engrafted Maternal T Cells. The patients' lymphocytes were visualized by a multiparametric cell-scanning system (Duet, BioView Ltd., Rehovot, Israel) for detecting the presence of transplacentally acquired maternal T lymphocytes as previously described [14]. The system combines morphological and fluorescence *in situ* hybridization (FISH) analyses of the same cell, thereby enhancing the specificity of pathological cell detection.

3. Results

3.1. Patients. Six patients, all presented during infancy, were included in this study. The clinical, immunologic, and molecular features of the patients are listed in Table 1 and were consisted with a phenotype of classical SCID with maternal-fetal transfusion (Pt1, Pt2), SCID-Omenn (Pt3, Pt4) or the combined immunodeficiency (CID) MHC II deficiency (Pt5, Pt6). Patients 1, 3, and 4 were found to have

Table 1: Clinical and immunological findings in 6 patients diagnosed with SCID.

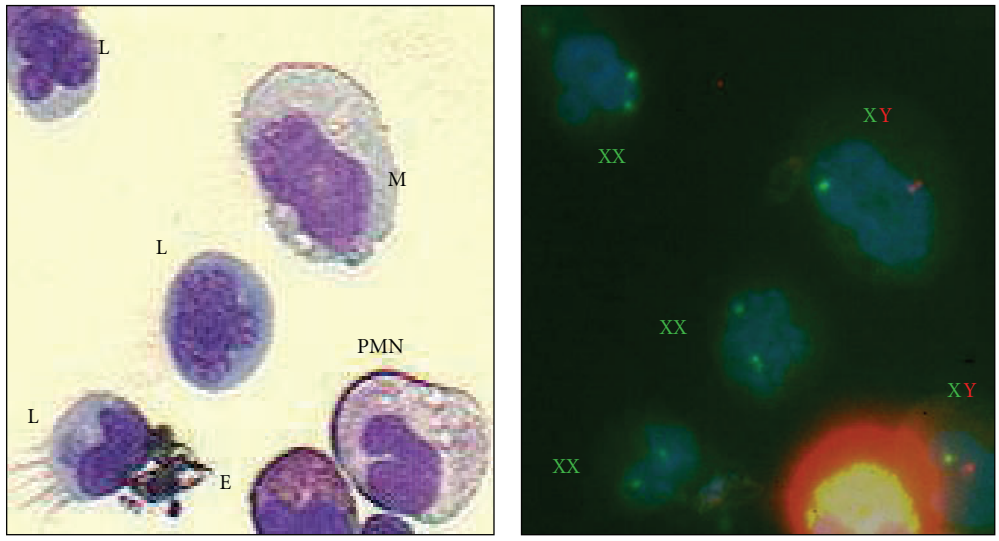
	Pt-1	Pt-2	Pt-3	Pt-4	Pt-5	Pt-6
Diagnosis, genetic defect	SCID-RAG2	SCID-γ_c	SCID-RAG2	SCID-RAG2	CID MHC-II	CID MHC-II
Age at diagnosis (months)	5/12	7/12	4/12	3/12	6/12	6/12
Maternal cells	100%	100%	3.5%	2%	0%	0%
Autoimmune features	Mild	Mild	Severe	Severe	No	No
Infections	+	+	+	+	+	+
Lymphocyte count/mm^3	5600	4500	1320	10686	4900	3416
Eosinophil/mm^3	1700	600	2960	4030	500	70
CD3/mm^3	4612	1500	488	2871	3552	1162
CD3$^+$CD4$^+$/mm^3	2855	360	244	2351	543	137
CD3$^+$CD8$^+$/mm^3	1757	1080	224	855	3305	1009
CD19$^+$/mm^3	1	3015	0	0	543	2186
CD3$^-$CD56$^+$/mm^3	504	0	500	3800	490	0
HLADR$^+$ (in total lymph)	47%	87%	30%	53%	0%	0%
IGM (IU/mL)	UD	UD	UD	110	UD	UD
IGG (IU/mL)	433	UD	UD	UD	253	UD
IGA (IU/mL)	79	UD	UD	UD	UD	UD
IGE (IU/mL)	UD	UD	UD	UD	UD	UD
PHA mitogenic response*	3.8%	2.4%	6%	6.5%	46.9%	94.8
aCD3 mitogenic response*	1.9%	ND	6.7%	31.5%	41.3%	32.8%
TRECs/0.5 mcg DNA	UD	UD	UD	UD	2769	4384

UD: undetectable, *percentage, CPM patient/CPM control, γ_c: common gamma chain, RAG: recombination activating gene.

mutations in the RAG2 gene including G156V, G35V, and G95V+E480X protein substitutions, respectively. Patient 2 was found to have the common gamma chain (γ_c) deficiency due to G68L mutation. In addition to the classical immunodeficiency clinical phenotypes (e.g., failure to thrive, recurrent infections) patients 3 and 4 had severe Omenn symptoms, including diffuse erythrodermia, alopecia, lymphadenopathy, and enlarged liver and spleen. In contrast, patients 1 and 2 had only mild diffuse skin eruption and initially were misdiagnosed as having mild Omenn phenotype. Patients 5 and 6 had no symptoms suggestive of Omenn and their cell HLA-DR expression was undetectable, suggestive of MHC-II deficiency.

3.2. Visualization of Maternal Engraftment. Combined morphological and FISH studies were used in all patients to examine the presence of transplacentally acquired maternal lymphocytes (Table 1). In patients 1 and 2, all lymphocytes were of maternal origin while other hematopoietic cells were of the patient's origin (representation of patient 1 is given in Figure 1), thereby excluding the possibility of the presence of autoreactive or residual endogenous T cells. Based on this finding the patients' symptoms were suspected to be secondary to GVHD. In contrast, maternal engraftment was undetectable in patients 5 and 6, or negligible in patients 3 and 4, suggesting the presence of either residual cells (Pt5 and Pt6) or autoreactive cells (Pt3 and Pt4).

3.3. Immunologic Studies. All patients had peripheral CD3$^+$ T lymphocytes. Four of them (Pt1, Pt2, Pt3, and Pt4) had skin erythrodermia and remarkable eosinophilia (Table 1). While 3 patients (Pt1, Pt3, and Pt4) were found to have no B lymphocytes, as could be expected in patients with the RAG2 deficiency, only patient 2 had no NK lymphocytes due to a genetic defect in the common γ_c. Patients 5 and 6 had reduced CD4$^+$ T lymphocytes with inverted CD4/CD8 ratio and subsequent measurement of HLA-DR revealed no expression at all. These findings were consistent with MHC-II deficiency. *In vitro* T-lymphocyte responses were significantly reduced in the patients with reactive T cells following phytohemagglutinin and anti-CD3 stimulations (3.8%–6.5% and 1.9%–31.5% of controls, resp.) and only slightly reduced in the MHC-II patients 5 and 6 (46.9%–94.8% and 32.8%–41.3% of controls, resp., Table 1). Similarly, the amount of recent thymic emigrant cells as determined by RQ-PCR analyses of TRECs were undetectable in patients 1, 2, 3, and 4 and normal in patients 5 and 6 (Table 1). Examination of T-cell receptor V beta region (TCR-Vβ) using FACS (Figures 2(a)–2(g)) revealed a clonal pattern in patients with autoreactive cells (patients 3 and 4, Figures 2(c) and 2(d), resp.). These patients had a clonal pattern with one dominant population (Vβ20) and markedly reduced 20 CD3$^+$ Vβs (patient 3) or two dominant populations (Vβ17 and Vβ7.2), and markedly reduced 17 CD3$^+$ Vβs (patient 4), indicating T-cell clonality. In contrast, both patients with transplacentally acquired maternal lymphocytes displayed skewed oligoclonal patterns in their TCRs (patients 1 and 2, Figures 2(a) and 2(b), resp.). These patients had a restricted pattern with one dominant population (Vβ17) and markedly reduced 8 CD3$^+$ Vβs (patient 1) or two dominant populations (Vβ3 and Vβ12), and markedly reduced 9 CD3$^+$ Vβs (patient 2), indicating T-cell restriction. Since in patient 1 cells were of maternal origin we also examined the Vβ repertoire of this patient's mother



(a) (b)

Figure 1: Visualization of the transplacentally acquired maternal cells. Combined morphological and FISH analysis confirmed the presence of transplacentally acquired maternal lymphocytes in patient 1 using the X and Y chromosomes probes. On the left (a), cells stained with Giemsa, and on the right (b) the same cells with FISH using dual-color XY DNA probe. XY genotype shows one green and one red FISH signals; XX genotype—two green signals. L = lymphocyte, YL = young lymphocyte, and PMN = polymorphonuclear cell.

and found normal peripheral blood repertoire (Figure 2(g)). TCR-Vβ of both patients with MHC-II deficiency who displayed residual T cells (patients 5 and 6) showed normal polyclonal patterns (Figures 2(e) and 2(f), resp.).

3.4. Regulatory T Cells Enumeration and Function. In order to quantify Tregs, unstimulated freshly isolated patients' peripheral blood mononuclear cells (PBMCs) were stained with CD25 and FOXP3 antibodies on live $CD4^+$ T cells. Patients 1 and 2 with the alloreactive cells displayed significantly high levels of circulating Tregs (25.4% and 12%, Figures 3(a) and 3(b), resp.). In contrast, low or near normal levels of circulating Tregs were found in patients 3 and 4, containing autoreactive cells (0.46% and 3.41%, Figures 3(c) and 3(d), resp.). Normal levels of Tregs were found in patient 5 who had nonreactive cells (6.05% of total gated cells, Figure 3(e)), compared to age-matched healthy control (4.19%, Figure 3(f)) and to the mother of patient 1 (4.52% of total gated cells, Figure 3(g)). In order to exclude the possibility that the high amount of circulating Tregs in patient 1 overlaps with the cell population showing expended clonality of Vβ17 receptor in this patient (Figure 2(a)), we examined the patient's $CD3^+$ Vβs for CD4 or CD8 expression. The $CD3^+$ Vβ17 receptor was composed mainly of $CD8^+$ cells (Figure 4) suggesting that the clonal expansion is not composed of Tregs. To examine if the transplacentally acquired Tregs lymphocytes detected in patient 2 are indeed functional, lack of IFNγ and IL-2 secretion from these cells was examined. As can be shown in Figure 5, while most of the FOXP3 negative cells produced IFNγ and IL-2 cytokines following T-cell stimulation with PMA and ionomycin (80.8% and 44.5% of total $CD4^+$ cells, resp.), $FOXP3^+$ cells obtained from patient 2 did not secrete IFNγ and IL-2 under the same condition, suggesting them as functional Tregs.

4. Discussion

Diagnosis of SCID is usually straightforward when patients present with the typical clinical features and a suggestive family history, supported by the results of general immunological tests. The latter includes reduced numbers of the lymphocyte subsets, depressed response of T cells to mitogen or antigen stimulation, and abnormal thymic activity. Immunodeficiency is the hallmark of SCID even in atypical cases where residual or reactive T cells are present. In some of these patients autoimmunity is present as a result of different tolerance mechanisms breakdown. Here we showed that severe immune-deficient patients with circulating T cells display different T-cell functions and regulatory patterns which are in correlation with their T-cell reactivity and the severity of their immunodeficiency. We speculate that some of these immunological parameters can be used to distinct immune deficient patients presenting with residual T lymphocytes of different origins (Table 2). It has been shown that patients with autoreactive cells have profound abnormalities of thymic epithelial cell differentiation and severe reduction of thymic dendritic cells and virtual absence of thymic $FOXP3^+$ Tregs [15]. In addition, low thymic and peripheral expression of AIRE and dysfunctional regulatory T cells was demonstrated [13, 16]. Even in cases where individual variability in the fraction of these circulating cells was observed, reduced thymic and lymph node expression of FOXP3 was found. Furthermore, in cases where peripheral FOXP3 expression was demonstrated, it did not identify a *bona fide* natural Treg cell and rather was consistent with an *in vivo* T-cell

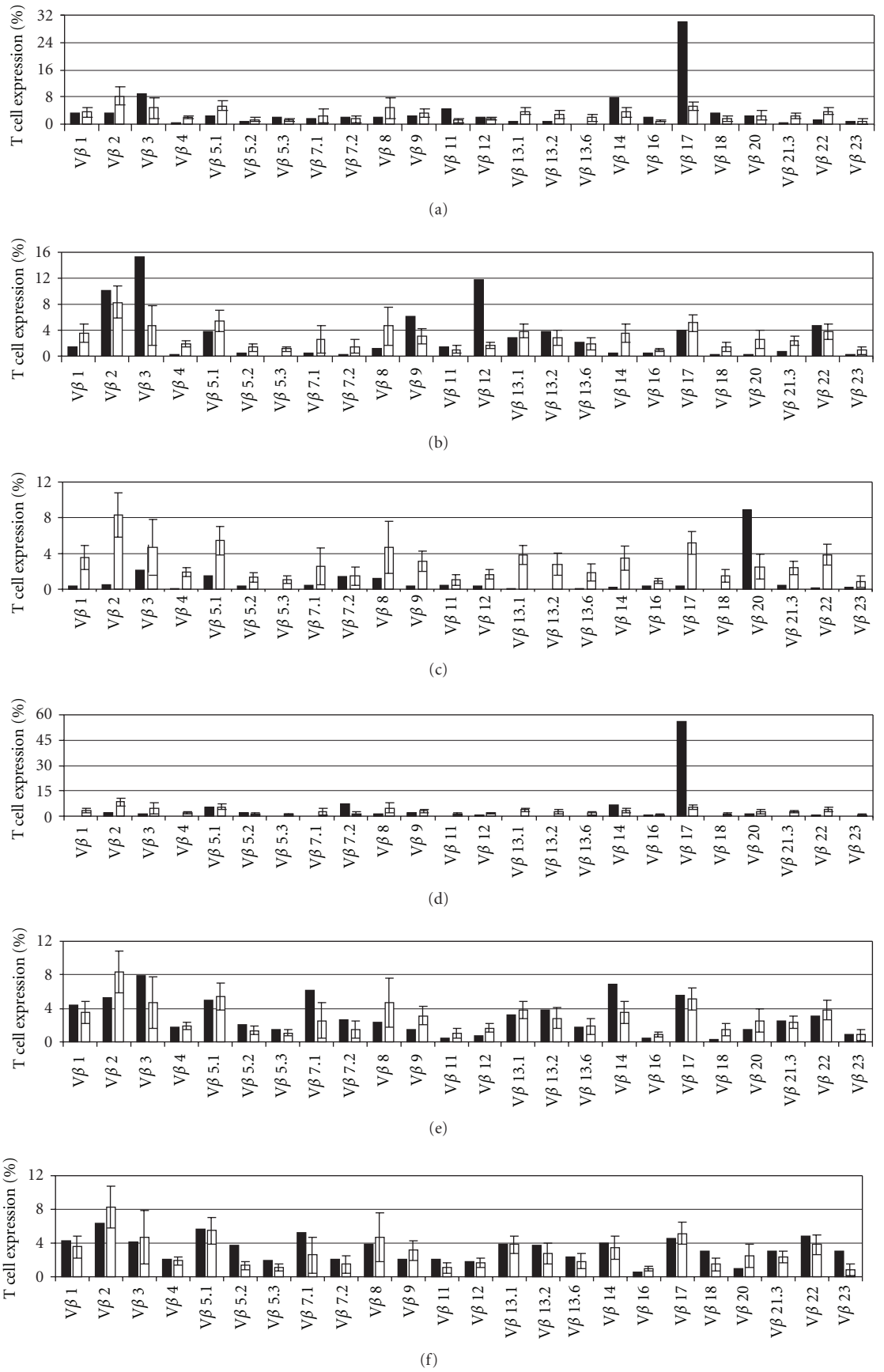
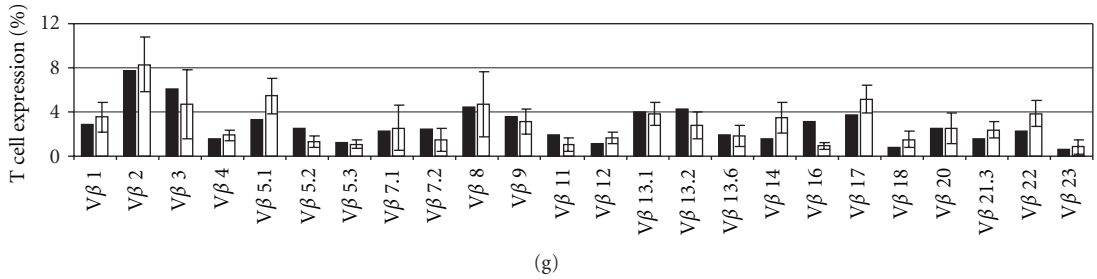


Figure 2: Continued.



(g)

Figure 2: T-cell receptor (TCR) Vβ repertoire. Relative expression levels of 24 different TCR Vβ families in CD3$^+$ cells (black bars) of pt1 (a), pt2 (b), pt3 (c), pt4 (d), pt5 (e), pt6 (f), and the mother of pt1 (g) compared with the relative expression of normal healthy controls (white bars) were obtained by FACS analyses. Normal control values were obtained using the IOTest Beta Mark TCR Vβ Repertoire Kit (Beckman Coulter).

Table 2: Suggested immunological distinctions between SCID patients presenting with residual T lymphocytes of different origins.

Origin of patient's cells	Allo-reactive T cells	Auto-reactive T cells	Residual T cells
SCID phenotype	Transplacentally acquired maternal lymphocytes	Omenn	MHC-II deficiency
Autoimmunity	+	+++	−
Eosinophilia	±	+	−
Lymphocyte count	Normal	Normal	Normal
Lymphocyte subset	Inconsistent, based on the TCR clonality	Inconsistent, based on the TCR clonality	Usually CD4/CD8 reverse ratio
Immunoglobulin levels	Low	Low	Low
Lymphocytes response to mitogens	Low	Low	Normal
TREC	UD	UD	Normal
TCR-Vβ repertoire	Skewed, restricted	Monoclonal	Polyclonal
Treg cells	High	Inconsistent	Normal

UD: undetectable, TCR: T cell receptor, TREC: TCR excision circles, Treg: regulatory T cells.

activation process [17]. Moreover, the expression of FOXP3 does not entirely characterize Tregs in humans and it has also been reported in non-Treg cells. Clinically, these patients will present with autoimmune-like features (e.g., Omenn phenotype). Omenn phenotype was reported in some but not all genetic SCID defects. For example, the defect of MHC class II that leads to combined immunodeficiency with defective CD4$^+$ T-cell development and a lack of T-helper-cell-dependent antibody production by B cells, was not reported to cause Omenn phenotype so far [18]. This is probably due to the specific late partial arrest in T-cell maturation that is not necessarily affecting any of the tolerance-regulating mechanisms. In addition, MHC-II-deficient patients are known to have residual T cells with some degree of selected immunity as can be seen in our patients. Interestingly, we found that MHC-II deficient patients have near-normal lymphocyte function and detectable TREC levels. A possible explanation for this finding is the partial T-cell development arrest, and the ability of some residual cells to fully mature in such a deficiency. In addition, no peripheral expansions of T cells are known to occur in these patients that can dilute TREC levels and produce autoimmune features. Partial T-cell development is found also in other SCID variants, such as the common γ_c-R222C hypomorphic mutation, enabling thymic epithelial cell maturation, thymic AIRE expression, and development of FOXP3$^+$ T cells [15]. In contrast, as we showed here, patients presenting with reactive T cells (auto- or allo) were found to have severely depressed lymphocyte function and undetectable levels of TRECS. The latter is explained by either because of inability to reach the final stage of T cell maturation or because of a peripheral dilution, secondary to the expansion of T cells that bear no episomal TRECs. Patients with reactive cells, as we showed here, were already been shown to have a restricted TCR repertoire with clonal expansion and autoimmunity [4, 13]. Interestingly, patients with transplacentally acquired maternal T lymphocytes who displayed alloreactive cells present less severe clonal expansion and cell restriction in their circulating CD3$^+$ cells compared to the "true Omenn" patients. In addition, they had a high fraction of functional circulating Tregs. Moreover, these cells did not secrete either IFNγ or IL-2 cytokines following T cell stimulation, suggesting their ability to suppress autoimmunity. Yet, other assays of Treg function should be used to clarify if indeed these cells are active. The "true" Omenn patients with the autoreactive cells, had low or normal levels of circulating Tregs, as already been shown [17], therefore these patients were suggested to display severe clinical autoimmune phenotype. We speculate that these immunological parameters are able to distinguish between SCID patients presenting with reactive T cells of

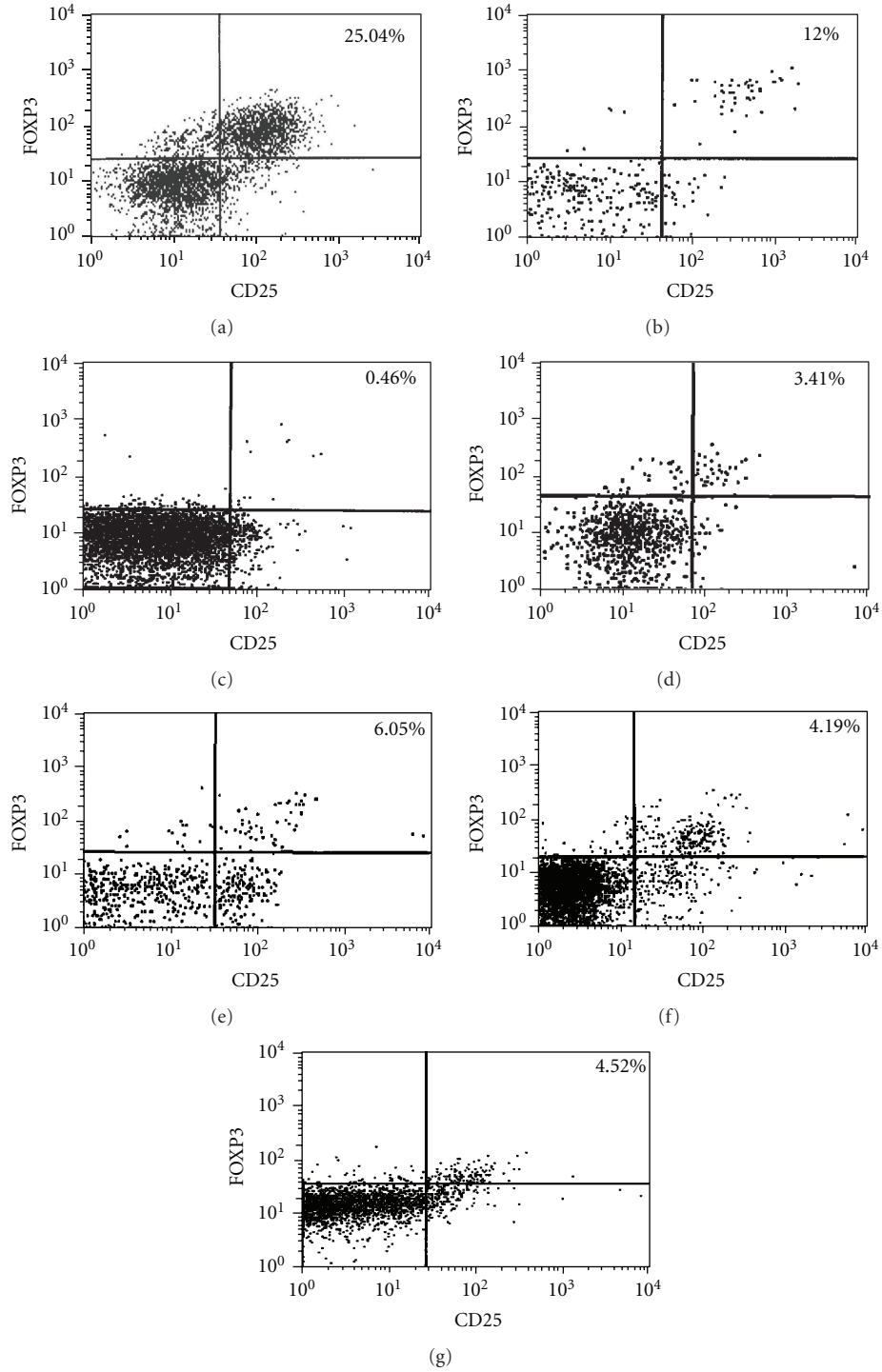


Figure 3: FOXP3 Treg cells in SCID patients. CD25 and FOXP3 expression levels in $CD4^+$ T cells of pt1 (a), pt2 (b), pt3 (c), pt4 (d), pt5 (e), age-matched healthy control (f), and the mother of patient 1 (g) were detected using FACS analyses. Quadrants were set up based on staining with isotype control. Boxed numbers indicate the percentage of Treg cells within the $CD4^+$ population.

different origins. While the breakdown of tolerance mechanisms in Omenn may occur simultaneously with the development of autoimmune manifestations, the high tolerance inducing function in some maternal cells allows some, but not all, cells to cross the placenta, survive in the recipient's circulation, and cause mild autoimmunity.

It is well accepted that maternal regulatory T cells mediate maternal tolerance to the fetus in addition to localized mechanisms. Expansion of maternal $CD25^+$ T cells with dominant regulatory T-cell activity during pregnancy was observed [19]. We show that these cells continue to express high fractions of the Treg phenotype that might enable better

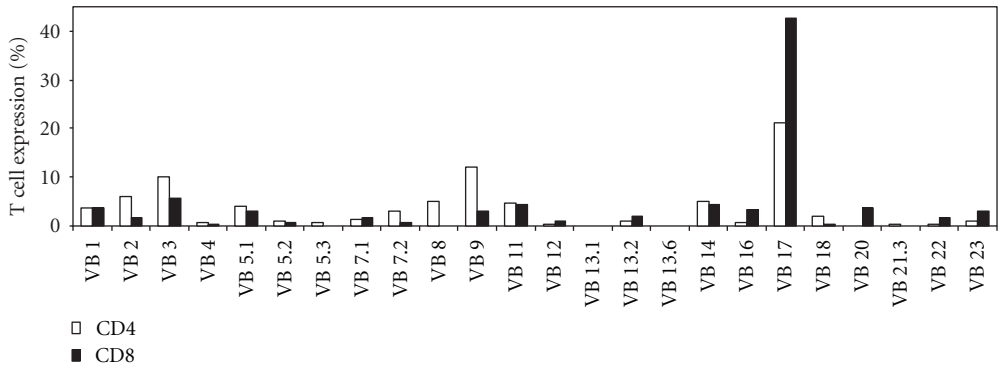


Figure 4: T-cell receptor (TCR) Vβ repertoire. FACS analysis of the relative expression levels of 24 different TCR Vβ families in patient 1 $CD3^+CD4^+$ cells (white bars) and $CD3^+CD8^+$ cells (black bars).

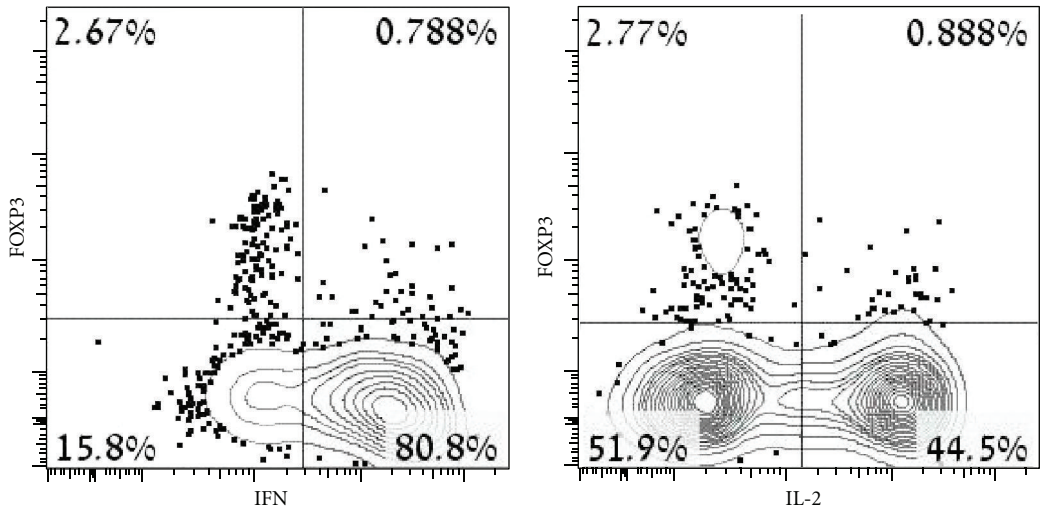


Figure 5: IFNγ and IL-2 cytokines secretion following T-cell stimulation. PBMCs obtained from patient 2 were stimulated with PMA and ionomycin, than stained with CD4, FOXP3, and IFNγ or IL-2 for the identification of functional Tregs. Detection was performed using FACS analyses. Quadrants were set up based on staining with isotype control. Boxed numbers indicate the percentage of cells within the $CD4^+$ population that secrete IFNγ or IL-2.

selection and survival. Since these cells are considered to be anergic, secondary expansion of alloreactive clones in patient 1 is unlikely. Moreover, careful analysis of the predominant TCR in our patient (Vβ17, Figure 1) revealed that this clone was composed mainly of CD8-positive cells, and therefore not responsible for the high fraction of the detected Tregs. The maternal cells detected in patients 1 and 2 caused only mild GVHD symptoms, although were HLA mismatched and likely to react with the recipient's organs. Since Tregs are thought to protect against GVHD by inducing and maintaining allogeneic tolerance [20], we then can speculate that the high fraction of circulating Tregs served to balance the immune reaction mediated by the maternal-host dissimilarities, thus protecting against severe GVHD. Indeed, trans-placentally acquired maternal T-lymphocytes cells are known to cause only few clinical manifestations, with most cases being entirely asymptomatic, possibly because of the oligoclonal repertoire of the maternal T cells with lack of alloreactivity toward the child's antigens [21]. Yet, the fact that these cells were completely dysfunctional, as evidenced by a lack of response to mitogenic stimulation and the absence of TREC copies, is an indication of poor T-cell differentiation in the thymus that resulted in a severe immunodeficient state. Our study attempts to explain why some patients with SCID and residual T cells present with autoimmunity, and others do not. We provide our data as a speculation since only two patients in each group were studied. Yet, only a small number of patients is expected because of the rarity of these conditions. In summary, our data show that SCID phenotypes with circulating T cells have distinct T cell function, thymic capacity and Treg enumerations which determine their T-cell reactivity and TCR repertoire patterns. Interestingly, transplacentally acquired maternal T lymphocytes in SCID patients have high fraction of functional circulating Tregs but poor T cell differentiation. We speculate that this represents a possible advantage mechanism for their

selection over other maternal cells and allows their tolerance by the patient's immune system while still causing a severe immunodeficient state.

Abbreviations

FISH: Fluorescence *in situ* hybridization
FOXP3: Forkhead box P3
GVHD: Graft-versus-host disease
PBMCs: Peripheral blood mononuclear cells
SCID: Severe combined immunodeficiency
TCR Vβ: T-cell receptor variable β
TREC: T-cell receptor excision circle
Tregs: Regulatory T cells.

Conflict of Interests

The authors declare no competing financial interests.

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Research Article

Thr92Ala Polymorphism of Human Type 2 Deiodinase Gene (hD2) Affects the Development of Graves' Disease, Treatment Efficiency, and Rate of Remission

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Clinical symptoms vary in thyrotoxicosis, and severity of these depends on many factors. Over the last years, impact of genetic factors upon the development and clinical significance of thyrotoxic symptoms became evident. It is known that a production of T3 in various tissues is limited by deiodinase 2 (D2). Recent studies revealed that certain single nucleotide polymorphisms (including threonine (Thr) to alanine (Ala) replacement in D2 gene codon 92, D2 Thr92Ala) affect T3 levels in tissues and in serum. Individuals with Ala92Ala genotype have lower D2 activity in tissues, compared with that in individuals with other genotypes. In our study, we have assessed an association of D2 Thr92Ala polymorphism with (1) frequency of disease development, (2) severity of clinical symptoms of thyrotoxicosis, and (3) rate of remissions, in Graves' disease patients.

1. Introduction

Over the last years, much attention has been paid to the emerging concept of a "personalized therapy." This recently developed approach, in particular, presumes using the information on patient's genotypes for the optimization of one's therapy. Analysis of genetic predisposition to sulfonylurea drug response in diabetes patients (Ser1369Ala variant in ABCC8 gene) serves as an example of the personalized therapy [1]. Ever increased introduction of genetic tests to the clinical laboratory promises developing a new therapeutic strategy—the personalized therapy of various diseases. In the current study, we have evaluated human type 2 deiodinase gene (hD2) polymorphism Thr92Ala as a potential genetic predictor of response to thyrostatic therapy in Graves' disease (GD).

Deiodinases are the selenoenzymes regulating the transformation of thyroxin (T4) into triiodothyronine (T3) [2–4]. Type 1 deiodinase (D1) is expressed and synthesized in liver, kidney, and thyroid gland [2, 3] and is responsible for the levels of circulating T3 hormone [2–6]. Type 2 deiodinase (D2) enables T3 production in central nervous system, pituitary gland, brown adipose tissue, cardiac and skeletal muscle, and placenta [2–4]; it is expressed on lower levels in liver and kidney [2, 3]. Thus, D2 plays the key role in local tissue T3 production [6–11]. According to the published data, type 2 deiodinase activities increase manyfold in some tissues in Graves' disease patients [10].

Recent studies showed that polymorphisms of some deiodinase genes affect the production of thyroid hormones: human D2 gene, threonine (Thr) to alanine (Ala) replacement in codon 92 (D2 Thr92Ala) among them [2, 5, 12]. Ala92Ala homozygous subjects demonstrate lower D2 tissue activity compared to Ala/Thr heterozygous and Thr/Thr homozygous subjects [12, 13]. Thus, Ala/Ala homozygous subjects have lower T3 effects in tissues with high D2

gene expression [2–8]. As Ala92Ala genotype association with insulin resistance and arterial hypertensions is well established [12, 14, 15], this polymorphism is suggested to influence clinical manifestations and the severity of heart damage in patients with thyrotoxicosis.

Our previous study aimed investigating the impact of D2 Thr92Ala polymorphism on the clinical course, laboratory, and EchoCG parameters in patients with Graves' disease [16]. We have identified negative correlation between Ala92Ala genotype and thyroid volume, and between the former and T3/T4 ratio. Thr92Thr genotype was associated with a risk of development of eccentric left ventricular hypertrophy [16]. In the current study, we have investigated (1) frequency of disease development, (2) severity of clinical symptoms of thyrotoxicosis, and (3) rate of remissions, in Graves' disease patients with various genotypes of type 2 deiodinase Thr92Ala polymorphism.

2. Patients and Methods

2.1. Patients. All patients with Graves' disease, either hospitalized or from outpatient department of the Almazov Federal Centre during year 2005–2010, were assessed for the following inclusion/exclusion criteria.

Inclusion criteria:

(1) age 20–55;

(2) established thyrotoxicosis associated with Graves' disease at the primary examination;

(3) consent of patient for participation in this study;

(4) the high quality of EchoCG images was required for better evaluation of heart structure and function.

Exclusion criteria:

(1) concomitant cardiovascular diseases that can result in fixed abnormal changes of EchoCG parameters (heart ischemic disease, hypertension, valvular disease, nonthyrotoxic cardiomiopathy, heart failure, diabetes mellitus, obstructive lung disease, and nonthyreotoxic arrhythmias);

(2) diseases have contraindications for long thyrostatic therapy (increase ALT or AST more 5-point normal range, hepatic or renal failure, intolerance thioamides);

(3) intoxication (alcohol, toxicomania);

(4) pregnancy or plane of pregnancy.

Among 250 screened patients 180 patients met the inclusion/exclusion criteria with 1- to 15-year-long history of Graves' disease; aged 18 to 54 years, without concomitant diseases, were included in the study (Table 1). The diagnosis of GD was confirmed by the presence of thyrotoxicosis, diffuse hyper functional goiter, and of autoantibodies to thyroid stimulating hormone (TSH) receptor and/or increased radioactive iodine uptake, at the moment of examination or in anamnesis.

Presence of overt thyrotoxicosis was based on levels of free T3 and free T4 above normal range and level of TSH lower than 0.1 mU/L.

Presence of subclinical thyrotoxicosis was based on level TSH lower than 0.1 mU/L with normal levels of free T3 and free T4.

For the start of Graves' disease was accepted the time of first registration of clinical signs of thyrotoxicosis with laboratory criteria (levels of free T3 and free T4 above normal range and level of TSH lower than 0.1 mU/L).

This study was approved by the local Ethical Committee of Almazov Federal Centre.

All patient were examined prior to the beginning of the thyreostatic therapy. All patients were treated by thioamides (mercasolil) in dose 30 mg and dose of thioamides was decreased to 10 mg (supporting dose) after restoration of euthyroidism (about 2-3 month treatment). In followup were 95 patients included; other patients were inaccessible to objective and laboratory inspection and were dropped from followup. These 95 patients were reexamined 1 and 2 years following the beginning of the therapy. In all followup visits, GD patients were checked for remission, according to the criteria associated with its high probability: normalization of thyroid volume (TV) and thyroid blood flow (TBF); absence of antibodies to TSH receptor; normalization of fT3, fT4, and TSH levels [17].

Remission was defined as the the time of registration of remission' criteria, but the patient was considered to reach remission only if remission fact was confirmed by the preservation of proof euthyreoidism in a year after cancellation of thyreostatic therapy [17]. In case of repeated thyreotoxicosis after the cancellation of thyrostatics the relapse was diagnosed.

A group of 135 age- and gender-matched euthyroid blood donors living in the same region constituted the control group.

2.2. Methods. Free thyroid hormones and antibody serum levels were measured by immune-enzyme assay using ACCESS 2 analyzer (Beckman Coulter, USA) and immunochemical test systems (UNICEL DXI 800 ACCESS, Beckman Coulter): free triiodothyronine (fT3, the normal range is 4.0–8.0 pmol/L), free thyroxin (fT4, the normal range is 10–25 pmol/L), TSH (the normal range is 0.25–3.5 mU/L), thyroperoxidase antibodies (TPOab, the normal range = 0–30 IU/mL), and antibodies to TSH receptor (rcTSHab, the normal range is <1.0 IU/L).

Ultrasound examination of thyroid gland was performed using ADR-2002 device with high-resolution 7.5 MHz sensors. Thyroid volume (TV) was calculated using the following method: (i) $TV_{lobe} = 0.5 \times l \times h \times m$ (l, lengths; h, height; m, width of thyroid lobe); (ii) volume of 2 thyroid lobes (left and right) is summarized as $TV = TV_{right\ lobe} + TV_{left\ lobe}$. Normal TV range for women and men is $\leq 18\,cm^3$ and $\leq 25\,cm^3$, respectively.

DNA was isolated from 200 μL volume of the peripheral blood by phenol-chloroform extraction. Genotyping was performed by polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) method using the

Table 1: Characteristics of the study groups.

	Graves' disease ($n = 180$)	Healthy controls ($n = 135$)
Age (years)	42.7 ± 0.72	46.2 ± 8.8
Sex (male/female)	36/144	24/110
Heart rate (bpm)	98.7 ± 1.44	72.6 ± 0.87
Blood pressure (mmHg)	130.5 ± 1.17/77.1 ± 0.67	125.3 ± 0.97/72.4 ± 0.71

following primers: RV: 5′-CTCAGGGCTGGCAAAGTCAAG; FW: 5′-CCACACTCTATTAGAGCCAATTG. Cycling conditions were as follows: the initial cycle of 30 seconds at 95°C, 30 seconds at 55°C, and 1 min at 72°C; 30 cycles and a final extension at 72°C for 5 minutes.

D2 genotype was identified by the endonuclease *Bsgl*-induced restriction (NEB, UK) of the PCR products at 37°C over night at the recommended conditions. Restriction fragments were resolved in a standard 1.5% agarose gel. To verify the genotyping method and the results the identification of genotypes from 30 random DNA samples was performed by direct sequencing.

2.3. Statistical Analysis. The results were expressed as frequencies, mean ± S.D., or median and percentiles 25–75 (P25–75). Allelic frequencies were determined by gene counting, and deviations from the Hardy-Weinberg equilibrium were verified using an χ^2 test. Clinical and laboratory data were addressed using χ^2 test, unpaired Student's t-test, Mann-Whitney U-test, ANOVA, Kruskal-Wallis H-test, Fisher exact test, or multiple logistic regression analysis, as appropriate. A two-tailed $P < 0.05$ was considered statistically significant, and all analyses were performed by STATISTICA 6.0 software package (StatSoft Inc., USA).

3. Results and Discussion

The baseline characteristics of age- and gender-matched groups of patients with Graves' disease (180 subjects) and controls (135 subjects) are presented in Table 1. A-allele (92Ala) frequency in patients with Graves' disease was 0.240, while it was 0.320 in the control group ($P < 0.0001$). In the Graves' disease group, 106 subjects (62.3%) were homozygous for 92Thr allele (TT genotype), 60 (33.3%) were heterozygous (TA), and 14 (7.8%) were homozygous for 92Ala-allele (AA). In the control group, 53 individuals (39.3%) had TT genotype, 79 (58.5%) were heterozygous (TA), and 3 (2.2%) had AA genotype. For the group of patients with Graves' disease, the genotypes were in Hardy-Weinberg equilibrium ($P = 0.52$; expected frequencies: TT = 57.2%, 36.9%, AA = 6.0%). However, in the control group, allele frequencies were in disequilibrium ($P = 0.0002$) (expected frequencies: TT = 46.9%, AT = 43.2%, AA = 9.9%), which can be partly explained by the low number of included subjects as well as the low frequency of A-allele.

The frequency of homozygotes for the T-allele (92Thr) was significantly higher in the group of patients with Graves' disease comparing to the control group (58.9% versus 39.3% resp.; $P < 0.0001$). Therefore, odds ratio (OR) was 2.20 (95% CI 1.40–3.47, $P = 0.0007$) for the TT genotype in Graves' disease patients. On the contrary, AT genotype produces inverse relationship, with a chance to develop disease being essentially lower in this group; OR = 0.36 (95% CI 0.24–0.57, $P < 0.0001$). The frequency of the minor A-allele (92Ala) was significantly more low in the group of patients with Graves' disease comparing to the control group (0.240 versus 0.320, resp.; $P < 0.0001$).

Characteristics of patients with Graves' disease ($N = 180$) having different genotypes are presented in Table 2. There was no significant age difference between the patients with different genotypes. It is worth noting that no males with AA genotype were identified in the study.

Characteristics of patients with Graves' disease having different genotypes were compared. Thyroid volume (TV) and fT3 level were significantly higher in patients with TT genotype, comparing to the patients with other genotypes ($P < 0.01$ and $P < 0.001$, resp.). Heart rate in patients with TT and TA genotypes (99.8 ± 3.01 and 96.7 ± 2.80 beats/min, correspondingly) was significantly higher, comparing to that in A-allele homozygous patients (AA genotype) (87.2 ± 5.30 beats/min, $P < 0.01$). There was no correlation of blood pressure parameters to the genotype. Notably, the duration of the disease was significantly longer in T-allele homozygous patients (TT genotype) ($P = 0.007$) (Table 3).

Negative correlation between disease relapse frequency and AA genotype was identified ($P < 0.01$) (Table 4). We therefore suggest that absence of A-allele predicts high rate of Graves' disease recurrence. We have addressed this hypothesis by performing analysis of the efficiency of conservative therapy in thyrotoxicosis patients with various D2 Thr92Ala genotypes. Dynamic followup was performed for 95 patients during 2 years of conservative treatment (repeated visits every 2-3 months), including the monitoring of hormone level and evaluation of remission. Criteria associated with high probability of remission were as follows: normalization of a thyroid gland volume and thyroid blood flow (TBF), reduction of TSH receptor antibody levels to the normal range, and euthyreoidism on the minimal dose of thyrostatics (10 mg mercasolil). Subsequently, followup was continued for those patients who had reached remissions and did not continue therapy during a year to prove the remission. We have found that frequency of AA genotype in the group of patients with euthyreoidism on the supporting dose of thyreostatics through the treatment was twice as high as that among patients with recurrence of thyrotoxicosis on the supporting dose of mercasolil (10 mg/d), 8.3% and 3.0%, respectively ($P < 0.01$). Distribution of the genotype frequencies within groups of Graves' disease patients is presented in Table 4. Most notably, these values correspond

Table 2: Characteristics of patients with Graves' disease, type 2 deiodinase polymorphism genotypes.

	D2 gene genotypes		
	TT ($n = 106$)	AT ($n = 60$)	AA ($n = 14$)
Genotype frequency	0.589	0.333	0.078
Age (years)	42.9 ± 0.99	42.2 ± 1.16	40.9 ± 3.10
Sex (male/female)	13/93	11/49	0/12
TV (cm^3)	33.5 ± 2.27	27.2 ± 2.55*	24.6 ± 2.35*
fT3 (pmol/L)	15.99 ± 1.55	11.56 ± 1.93*	10.7 ± 1.85*
fT4 (pmol/L)	48.4 ± 3.51	41.6 ± 3.91	40.2 ± 1.87
TSH (mU/L)	0.04 ± 0.007	0.04 ± 0.01	0.02 ± 0.007*
Heart rate (bpm)	99.8 ± 3.01	96.73 ± 2.80	87.2 ± 5.3*,**
Blood pressure (mmHg)	133.7 ± 2.19/77.6 ± 1.14	128.9 ± 2.01/77.4 ± 1.35	130.0 ± 2.8/78.0 ± 5.6

* $P < 0.01$ compared to the patients with TT genotype.
** $P < 0.01$ compared to the patients with AT and TT genotypes.

Table 3: Duration of disease in patients with Graves' disease, type 2 deiodinase gene Thr92Ala polymorphism genotypes.

	D2 gene genotypes			P
	T/T ($n = 106$)	A/T ($n = 60$)	A/A ($n = 14$)	
Total duration of the disease (months)	32.2 (7.9; 131.8)	16.8 (4.2; 67.1)	14.0 (5.6; 35.2)	0.007
Duration of overt thyrotoxicosis (months)	15.5 (5.9; 40.6)	10.9 (4.8; 25.0)	10.0 (4.2; 24.1)	0.04

Table 4: Remission and recurrence frequencies in patients with Graves' disease, type 2 deiodinase gene Thr92Ala polymorphism genotypes.

	D2 gene genotypes		
	TT ($n = 52$)	TA ($n = 30$)	AA ($n = 13$)
Recurrence	65.4%	60.0%	15.4%
Remission	34.6%	40.0%	84.6%

to OR = 7.90 (95% CI 2.0–32.3, $P = 0.002$) for T-allele in Graves' disease patients undergoing remission.

4. Conclusion

As functional activity of type 2 deiodinase is associated with polymorphism of D2 gene at 92 position [2], carriers of Ala92Ala genotype have lower activity of the enzyme and, accordingly, less active education of T3 in tissues. The key result of our study is the identification of the association of Ala92Ala genotype with high frequency of remission in Graves' disease patients (7.9-fold more frequent achievement of steady remission as a result of conservative therapy). The carriers of A-allele have milder thyrotoxicosis (i.e., lower levels of circulating thyroid hormones, low T3/T4 ratio, high level of TPO antibodies, and lower heart rate) that possibly facilitates achievement of remission in the subgroup of patients carrying A-allele. Data generated in this study suggest that AA genotype Ala92Thr polymorphism of D2 gene is protective, in regard to (1) the frequency of Graves' disease development, (2) severity of disease, and (3) rate of remissions in the patients.

Results of the present study provide further implications of the genetic factors in the variations of response to conservative therapy at thyrotoxicosis in Graves' disease patients.

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Research Article

Family History of Autoimmune Disease in Patients with Aicardi-Goutières Syndrome

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Purpose. The purpose of this study was to explore anecdotal evidence for an increase in the prevalence of autoimmune diseases in family members of patients with Aicardi-Goutières syndrome (AGS). *Methods*. Pedigrees of patients and controls were analyzed using chi-square and logistic regression to assess differences in reports of autoimmune disease among family members of cases and controls. Data was collected at Children's National Medical Center in Washington, DC, USA and at the International Aicardi-Goutières Syndrome Association Scientific Headquarters, C. Mondino National Institute of Neurology in Pavia, Italy. *Results*. The number of individuals with reported autoimmune disease is significantly related to having a family member with AGS ($\chi^2 = 6.25$, $P = 0.01$); 10% (35/320) of relatives of patients with AGS had a reported autoimmune disease diagnosis compared to 5% (18/344) of relatives of controls. There was a greater percent of maternal relatives of patients with AGS reporting autoimmune disease (14.6%), compared to controls (6.8%), with the association being statistically significant. The association was not significant for paternal relatives. *Conclusion*. The prevalence of autoimmune disease in relatives of children with AGS is significantly increased compared to controls. More research is needed to better understand this association.

1. Introduction

Aicardi-Goutières syndrome (AGS) is a heritable neurologic disorder with an immune basis. Patients most typically present early in life with increased cerebrospinal fluid (CSF) interferon alpha and markers of inflammation, elevated liver enzymes, thrombocytopenia, intracranial calcifications and leukoencephalopathy. Patients with AGS usually demonstrate severe neurologic dysfunction and life-long disability.

The immune basis of AGS was originally suspected by Aicardi and Goutières, as a persistent CSF pleocytosis was seen in affected infants. Infants presented with what appeared to be a congenital infection [1], and reports of elevations of CSF IFNα [2] and neopterin [3, 4] further suggested that an immune process was at play. However, it was not until the identification of AGS related mutations in nucleases, including TREX1 and the three constituent subunits of RNase H2, as well as a nonnuclease, SAMHD1, that the relationship between innate cellular immunity and AGS began to be more fully understood.

Discovery of the genes associated with AGS allowed for further definition of the phenotype. Most patients with AGS were found to have homozygous or compound heterozygous changes in these genes. AGS was also found to be allelic with Cree encephalitis and an inherited infantile systemic lupus erythematosus [5–8]. Heterozygous mutations in AGS related genes have also been found in rare patients with systemic lupus erythematosus (SLE) [9, 10] and familial chilblain lupus (FCL) [11–13], which are both autoimmune disorders. Studies in large populations of SLE patients suggest that *TREX1* single nucleotide polymorphisms may also be related to neurologic manifestations and the presence

of autoantibodies [10]. Additionally, mutations in *TREX1* have been reported in patients with retinal vasculopathy with cerebral leukodystrophy (RVCL) [14].

Of the five genes currently known to be associated with AGS, four, *TREX1* and *RNASEH2A-C*, encode proteins that have nucleic acid metabolizing functions, or nucleases. The fifth gene *SAMHD1*, encodes a protein that while not specifically a nuclease is thought to degrade nucleic acid precursors [15] and is upregulated by immune-stimulatory DNA [16]. Mutations in these genes are thought to result in the accumulation of endogenous nucleic acids. Growing evidence suggests that this accumulation provokes a type 1 interferon response that results in the development of pathogenic cytokines and autoantibodies that target the brain and other organs [17].

Anecdotal observation suggests an increase in the prevalence of various autoimmune diseases in the family history of children diagnosed with AGS. However, despite the evidence of AGS related mutations in some individuals from SLE populations, this has never been explored in families of AGS affected persons. The purpose of this study was to investigate this anecdotal observation to determine if there is an increase in prevalence of autoimmune disease in families who have children with a diagnosis of AGS, as compared to control families.

2. Materials and Methods

To investigate the possible increase in prevalence of autoimmune disease in family members of children with AGS, pedigrees were analyzed from families with AGS and controls who either had a definitive diagnosis other than AGS or who had unsolved (i.e., undiagnosed) leukodystrophies without any features of AGS (including clinical and neuroradiological findings). AGS cases were defined by having consistent clinical features (elevated alpha interferon and white blood cells in CSF, central nervous system calcifications, and leukoencephalopathy and no other identifiable etiology) and by confirmed mutations in any of the known AGS related genes.

Autoimmune disease is a broad term and, for the purposes of this study, was considered to include autoimmune thyroiditis leading to hypothyroidism, and other autoimmune thyroid dysfunction, inflammatory bowel disease, type I diabetes, rheumatoid arthritis, multiple sclerosis, Raynaud syndrome, psoriasis, scleroderma, Addison disease, and Kawasaki disease, [18–20] as well as the above mentioned autoimmune diseases (i.e., systemic lupus erythematosus and familial chilblain lupus) already known to be associated with AGS related genes.

The presence of autoimmune disease was investigated in case and control families using semistructured interviews to inquire about autoimmune disease generally and about the presence of specific autoimmune diseases (lupus, thyroid dysfunction, inflammatory bowel disease, type I diabetes, rheumatoid arthritis, multiple sclerosis, and autoimmune skin disorders). Data was collected between September 2008 and October 2011. Pedigrees for AGS subjects were drawn during AGS family clinic/conferences in 2008 and 2011 at Children's National Medical Center, or during clinical encounters in Washington DC or in Pavia, Italy. Pedigrees for control subjects were drawn during clinical encounters in Washington, DC by the same staff as performed AGS pedigrees. Pedigrees were excluded if there was insufficient information from one side of the family or if the patient was adopted and family history information was limited. Consanguineous families were included in both cases and controls. Pedigrees were drawn by genetic counselors or a pediatric neurologist with special expertise in biochemical genetics and leukodystrophy.

Data collection was part of a larger data collection effort within the European NIMBL (Nuclease Immune Mediated Brain and Lupus-like conditions) project. Italian families who participated to this study were enrolled in NIMBL in collaboration with the International Aicardi Goutieres Syndrome Association (IAGSA). United States families were enrolled in NIMBL in collaboration with the Myelin Disorders Bioregistry Project. The project was approved by the Institutional Review Board at Children's National Medical Center and the ethical review boards at the Child Neurology and Psychiatry Unit at the C. Mondino National Institute of Neurology, Pavia, Italy.

The prevalence of autoimmune disease was investigated all relatives combined, in parents only, and in second degree relatives only. In this study, half-siblings of parents were also considered to be second-degree relatives and were included in the analysis. Siblings and half-siblings of patients were not included in the analysis, as most of them were too young to have exhibited any features of autoimmune disease and may or may not develop autoimmune disease in the future.

Chi-square and/or logistic regression were used to assess differences between overall relatives, between maternal relatives and paternal relatives, between mothers and fathers, and between second-degree relatives in cases and controls.

3. Results

Pedigrees of families of children with AGS ($N = 31$) were compared with control pedigrees of families of children without AGS ($N = 31$). The US site collected family history information on 17 of the AGS families, while the Italian group collected family history on 14 families. Pedigrees were collected in a prospective fashion.

Controls were all from the Myelin Disorders Bioregistry at Children's National Medical Center in Washington, DC. Controls were patients who either had a clinical or molecular diagnosis other than AGS, or had an undetermined diagnosis but without features of AGS. Control diagnoses included Alexander disease, metachromatic leukodystrophy (MLD), hypomyelination with hypogonadotrophic hypogonadism and hypodontia (4H) syndrome, mucopolysaccharidosis IIIc, multiple sulfatase deficiency, congenital cytomegalovirus (CMV), acute disseminated encephalomyelitis (ADEM), and peroxisomal disorders.

Comparisons of autoimmune disease diagnoses were assessed in relatives of AGS patients and control patients using chi-square and logistic regression (Tables 1 and 2).

Table 1: History of autoimmune disease in parents of cases and controls.

Characteristic	AGS cases	Control	OR	*P* value	95% CI
Maternal history of AI (yes/no)					
No	26 (83.9%)	28 (90.3%)	1.00		
Yes	5 (16.1%)	3 (9.7%)	1.75	0.453	0.39–8.27
Paternal history of AI (yes/no)					
No	30 (96.8%)	31 (100.0%)	1.00		
Yes	1 (3.2%)	0 (0.0%)	—	0.313*	—
Maternal and/or paternal history of AI (yes/no)					
No	25 (80.7%)	28 (90.3%)	1.00		
Yes	6 (19.4%)	3 (9.7%)	2.24	0.288	0.51–9.91

*P value from chi-squared test due to zero control subjects with a paternal history of AI.

Table 2: History of autoimmune disease in second degree relatives of cases and controls.

Characteristic	AGS cases	Control	OR	*P* value	95% CI
All second degree relatives both matrilineal and patrilineal with history of AI (yes/no)					
No	11 (35.5%)	19 (61.3%)	1.00		
Yes	20 (64.5%)	12 (38.7%)	2.88	0.044	1.03–8.07

First, a chi-square test was performed to assess differences in reports of autoimmune diseases in the two groups. The number of relatives with reported autoimmune disease is significantly related to having a family member with AGS (χ^2 = 6.25, P = 0.01): there were 320 total relatives of patients with AGS, and 35 (10.9%) had reported autoimmune disease diagnoses, compared to 5.2% (18/344) controls. Thus, the prevalence of autoimmune disease in AGS families was more than twice that of the control families.

Next, a chi-square calculation was performed on maternal versus paternal relatives. There was a greater percent of maternal relatives with reported autoimmune disease (24/164, or 14.6%) compared to controls (12/176, or 6.8%), and this association was statistically significant (P = 0.03). For paternal relatives, the association between having diagnosis of an autoimmune disease and having a relative with AGS was not significant.

Using logistic regression, when looking at all first degree relatives (mothers and fathers of cases and controls) alone, patients with AGS were not significantly more likely to have a parent with a reported autoimmune disease (OR = 2.24; P = 0.29; 95% CI = 0.51–9.91). Of note, however, all of the parents who were reported to have autoimmune disease were in the US-based cohort of families (Table 1). If the US cohort is considered alone (Table 3), patients with AGS were statistically significantly more likely to have a parent with reported autoimmune disease (OR = 5.09; P = 0.040; 95% CI= 1.07–24.02). Reports of autoimmune diseases in first degree relatives of children with AGS included diagnoses of: thyroid dysfunction, psoriasis, lupus-like symptoms, and ulcerative colitis.

Logistic regression was again used for second-degree relatives alone (grandparents, aunts, and uncles of patients (Table 2)). Patients with AGS were significantly more likely to have at least one second degree relative with autoimmune disease compared to controls (OR = 2.88, P = 0.044, 95% CI = 1.03–8.07) (Table 2). A linear trend test was performed to determine if the odds ratio increased with increasing numbers of second degree relatives with reported autoimmune disease. There was evidence suggestive of a trend in the number of maternal family members (P = 0.086), but not in the number of paternal family members. Reports of autoimmune disease diagnoses in this group of second-degree relatives of AGS included: chilblains, sarcoidosis, psoriasis, lupus, inflammatory bowel disease, Raynaud's, scleroderma, multiple sclerosis, thyroid dysfunction, and rheumatoid arthritis. Individual numbers were too small to make meaningful comments about subsets of autoimmune disorders, specifically SLE, seen in this population.

Because there was a difference in the report of autoimmune disease diagnoses between females (mothers) versus males (fathers), an assessment of differences in female versus male second-degree relatives' report of autoimmune disease diagnoses was performed. Among the 29 second-degree relatives in the AGS group who were reported to have autoimmune disease diagnoses, 21 (72.4%) were female and 8 (27.6%) were male. Among the 15 individuals in the control group who were reported to have an autoimmune disease diagnosis, 10 (66.7%) were female and 5 (33.3%) were male.

4. Discussion

Anecdotal evidence of an increased prevalence of autoimmune disease in family members of patients with AGS prompted this analysis of pedigrees of AGS patients in comparison to control families. AGS is part of a growing group of inherited disorders in which increased alpha interferon is thought to play a substantial role in the pathogenesis [21]. Familial increases in alpha interferon has been shown to aggregate with increased risk of established autoimmune

Table 3: Comparison of autoimmune disease reported in first degree relatives in US-based cohort only.

Characteristic	AGS cases	Control	OR	*P* value	95% CI
Maternal and/or paternal history of AI (yes/no)					
No	11 (64.7%)	28 (90.3%)	1.00		
Yes	6 (35.3%)	3 (9.7%)	5.09	0.040	1.07–24.02

disorders such as SLE [22] and juvenile dermatomyositis [23]. Thus, it is not surprising that there is a greater percentage of relatives of patients with AGS with reported autoimmune disease compared to controls, and that this association was statistically significant. It should be noted that this statistically significant association held for maternal relatives, but not so for paternal relatives. Patients with AGS in the US cohort alone were shown to be statistically significantly more likely to have a parent with autoimmune disease compared to controls.

To explain the difference between the US and Italian cohorts, genotype was considered, since a larger proportion of in the Italian group of AGS patients had mutations in *RNASEH2B*, but removing those from the analysis did not result in any substantial change in findings. Thus, this difference is not understood and requires additional investigation. It is likely that the sample size is simply too small to detect meaningful differences.

Statistical analysis did show that children with AGS were significantly more likely to have at least one second degree relative with autoimmune disease as compared to controls. The finding of an increase in prevalence in females as compared to males was unexpected, although it is consistent with data showing that, in general, women are 2-3 times more likely to have autoimmune disease than men [18]. Although difficult to capture due to the variability of autoimmune diseases, the prevalence of autoimmune disease in the US may be approximately 1/31 or 3.2% [18]. Our control population was found to have a slightly higher prevalence of autoimmune diseases, but our case population was found to have significantly more than this rate. The slight increase in prevalence in controls may be due to the fact that our controls were drawn from children with other neurogenetic diseases. Assessing differences in prevalence of autoimmune diseases in pedigrees of AGS families and pedigrees of families without any neurogenetic disease may provide a more accurate comparison.

It is unclear at this time whether the increased prevalence of autoimmune disease in relatives of AGS patients is directly related to mutation status in these relatives. Of note, although parents were generally presumed to be carriers of mutations in AGS-related genes, the genotype of second degree relatives with reported autoimmune disorders was not known. In addition, it is unknown whether other genetic modifiers exist that could change the phenotypic expression of mutations in AGS related genes, which may predispose related individuals to autoimmune diseases. Finally, other factors may exist, including the fact that families with a diagnosis of AGS may be more attentive to symptoms of autoimmune disorders than control families, or that other familial confounders may exist, such as diet, environment or infection. This may explain differences seen between the US and Italian cohorts, for example. Therefore, there is not enough evidence or understanding at this time to claim that individuals with diagnosed autoimmune diseases should be screened for mutations in AGS-related genes, or that family members of AGS patients known to carry a heterozygous mutation should be screened for autoimmune disorders.

However, it is of note that *TREX 1* mutations have been found in patients with SLE. The relative risk for the development of SLE among those who carry *TREX1* variants has been found to be 25.3 (95% CI = 5.6–232.0) in one cohort ($N = 317$) [9]. In another recently studied large cohort of SLE patient ($N = 8730$), mutations in *TREX1* one occurred at a frequency of 0.5% [10]. This data, in addition to our finding of increased prevalence of autoimmune disease in family members of AGS patients, suggests the need for further research into genotype-phenotype correlations of AGS related mutations and autoimmune disease.

5. Limitations

Because family history data was obtained by report of parents of cases and controls, and not by primary analysis of family members' medical records, data may be inaccurate in both cases and controls. It is possible that both families of patients with AGS and control patients experienced recall bias or that they were not aware of autoimmune diagnoses in the family, and, thus, prevalence of autoimmune disease in both groups may be underreported. Additionally, because the semi-structured interviews for family history information were done at different times and by different investigators, not all cases and controls were asked about the same conditions in the same way, although every attempt was made to make the data as consistent as possible, including the use of the same group of investigators to collect control and AGS pedigrees.

One consanguineous case family and one consanguineous control family were included in the analysis. This was unlikely to have had an effect on the data, since second degree relatives of affected children were likely still only at 50% risk of having inherited a heterozygous mutation from a parent. There were no reports of affected individuals in either the case or control family in previous generations.

AGS is a rare disorder, and therefore, sample sizes are small. However, the disease may be underdiagnosed. As awareness of AGS grows and additional patients are diagnosed, more family history data will be available. Also, as the understanding of the autoimmune nature of this disease improves, more robust conclusions can be drawn about the association and risk for developing autoimmune disease in obligate or presumed carriers.

6. Conclusion

In this evaluation of family pedigrees from patients with Aicardi-Goutières Syndrome and control subjects, relatives of patients with AGS reported autoimmune disease diagnoses more frequently than family members of control patients; patients with AGS in the US-based cohort alone were statistically more likely to have a first-degree relative with an autoimmune disease and the entire cohort was found to be statistically more likely compared to controls to have at least one second-degree relative with autoimmune disease. Female relatives reported autoimmune disease diagnoses more frequently than male relatives. This data, in addition to the presumed autoimmune nature of AGS and the fact that genes that cause AGS (i.e., *TREX1* and *SAMHD1*) are allelic to those that can cause SLE and FCL, warrants further study into the association between heterozygous mutations in AGS-related genes and autoimmune disease. There is not enough data to suggest that heterozygous carriers of mutations in AGS-related genes are at risk for developing autoimmune disease, nor that those individuals with autoimmune disease should be screened for mutations in these genes, but further work is needed to better understand the association.

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Review Article

Interferon Regulatory Factor 5 in the Pathogenesis of Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by multiple genetic risk factors, high levels of interferon alpha (IFN-α), and the production of autoantibodies against components of the cell nucleus. Interferon regulatory factor 5 (IRF5) is a transcription factor which induces the transcription of IFN-α and other cytokines, and genetic variants of IRF5 have been strongly linked to SLE pathogenesis. IRF5 functions downstream of Toll-like receptors and other microbial pattern-recognition receptors, and immune complexes made up of SLE-associated autoantibodies seem to function as a chronic endogenous stimulus to this pathway. In this paper, we discuss the physiologic role of IRF5 in immune defense and the ways in which *IRF5* variants may contribute to the pathogenesis of human SLE. Recent data regarding the role of *IRF5* in both serologic autoimmunity and the overproduction of IFN-α in human SLE are summarized. These data support a model in which SLE-risk variants of IRF5 participate in a "feed-forward" mechanism, predisposing to SLE-associated autoantibody formation, and subsequently facilitating IFN-α production downstream of Toll-like receptors stimulated by immune complexes composed of these autoantibodies.

1. Introduction

Systemic lupus erythematosus (SLE) is a complex and heterogeneous disease characterized by a strong genetic contribution and activation of a number of immune system pathways [1–3]. Recent advances in human genetics and gene expression studies have increased our understanding of the immunopathogenesis of the disorder [4]. Interferon (IFN)-α is a pleiotropic type I IFN with the potential to break self-tolerance by inducing dendritic cell differentiation, which can lead to the activation of autoreactive T and B cells [5, 6]. Serum IFN-α levels are often elevated in lupus patients [7–9] and the "IFN-α signature" of gene expression in peripheral blood mononuclear cells is present in more than 50% of SLE patients [10–14]. High IFN-α levels are associated with more severe disease and presence of particular autoantibodies [9, 14, 15]. Additionally, high levels of IFN-α are common in unaffected SLE family members, suggesting that IFN-α is a heritable risk factor [8, 16]. Moreover, some patients given recombinant human IFN-α for viral hepatitis C or malignancy have developed *de novo* SLE and recovered after the IFN-α was discontinued [17–19]. This body of evidence suggests that IFN-α plays a key role in etiology and pathogenesis of SLE.

Interferon regulatory factor (IRF) 5 is a transcription factor that can induce transcription of IFN-α mRNA [20]. Perhaps not surprisingly, numerous genetic studies have supported an association between SLE and various single-nucleotide polymorphisms (SNPs) and functional variants in the *IRF5* gene. These genetic associations have been demonstrated across multiple ancestral backgrounds, although the

exact molecular mechanisms by which these polymorphisms contribute to human disease pathogenesis are still unclear [21–33]. Other autoimmune diseases such as rheumatoid arthritis, Sjogren's syndrome, systemic sclerosis, multiple sclerosis, and inflammatory bowel disease have also been associated with *IRF5* polymorphisms, suggesting a role of IRF5 in common autoimmune disease pathways [34].

Like other IRF family members, IRF5 has a prototypical helix-loop-helix and a conserved tryptophan repeat in its aminoterminal DNA-binding domain. IRF5 induces gene expression by binding to promoters containing the IFN-stimulated response element (ISRE), whose consensus sequence is GAAANN [35] and AANNNGAA [36]. IRF5 has been called the "master regulator of proinflammatory cytokines" [37] because of its role in upregulating expression of IL-6, IL-12b, IL-17, IL-23, TNF-α, IFN-β-IP-10, MCP1, and RANTES [38, 39] in addition to type 1 IFN [40]. Because *IRF5* is an IFN-induced gene, its expression can potentially be enhanced via a positive feedback loop, where IFN-α production could lead to increased IRF5 expression and subsequently additional IFN-α transcription [41]. In addition, IRFs play an important role in the regulation of cell growth and apoptosis as evidenced by IRF5 playing a role in the induction of apoptosis in cancer cells [42]. While IRF5 functions in cell cycle processes and apoptosis, for the purpose of this paper we will focus on how *IRF5* relates to IFN-α, and how IRF5 variants may influence the pathogenesis of SLE.

2. IRF5 and Infection

Early and accurate detection of microbial pathogens is a critical part of the immune response against pathogens. This is accomplished through the recognition of common microbial molecules called pathogen-associated molecular patterns (PAMPs) [43]. Pattern recognition receptors (PRRs) are cell surface proteins on innate immune cells that detect these PAMPs, bind them, and subsequently set off signaling cascades to initiate the immune response. PRRs include Toll-like receptors (TLRs), C-type lectin receptors, retinoic acid-inducible gene (RIG)-I-like receptors, and nucleotide-binding oligomerization domain-(NOD-) like receptors. Many downstream targets of PRRs are members of the IRF family. Type I IFN and pro-inflammatory cytokines produced downstream of PRR ligation coordinate the recruitment of other innate and adaptive immune cells, which enable the attenuation and eventual eradication of the infection.

Studies by multiple investigators show that IRF5 in particular can be induced in response to specific viral infections such as Newcastle disease virus (NDV), vesicular stomatitis virus, and herpes simplex virus type 1 [20, 41, 44]. IRF5 expression is mainly restricted to dendritic cells, B cells, macrophages, and monocytes [39, 41], a pattern which is unique from other IRF family members. Although IRF5 expression may be constitutive, its activity must be induced via several posttranslational modifications at multiple amino acid residues (discussed below).

3. IRF5 Is a Downstream Target of TLR7 and TLR9

Rather than being on the cell surface, TLR7, 8, and 9 are localized in the endosomal compartment, along with TLR3. TLR7 and TLR8 recognize single-stranded RNA viruses, while TLR9 recognizes double-stranded DNA (dsDNA) viruses or CpG motifs on bacteria. As shown in Figure 1, IRF5 is activated following engagement of TLR7 or 9, and perhaps TLR8. Of note, early studies in the characterization of TLR7 and TLR9 were performed in mutant mice [45, 46], but there is no mouse ortholog of TLR8. Therefore, less is known about the regulation and downstream signaling of TLR8, which is expressed only in humans.

In human plasmacytoid dendritic cells (pDCs), recognition of cognate TLR7 and TLR9 ligands leads to the activation of IRF5 [51], via the signaling intermediate MyD88. As an adaptor protein that has a Toll/interleukin (IL)-1 domain, MyD88 recruits interleukin-1 receptor associated kinase (IRAK)-4. IRAK-4 binds and phosphorylates IRAK-1, which in turn recruits tumor necrosis factor (TNF) receptor associated factor (TRAF) 6 [46–48]. TRAF6 is an E3 ubiquitin (Ub) ligase that adds K63-Ub chains to IRF5 [49]. Together, these events set the stage for the translocation of IRF5 into the nucleus.

4. Activation and Regulation of IRF5

Regulation of IRF5 activation is still not well understood. The C-terminal end of IRF5 has been shown to be autoinhibitory in an IFN-α reporter assay [41, 52, 53]. Upon stimulation, IRF5 is modified posttranslationally by phosphorylation and ubiquitination. Multiple phosphorylated residues have recently been identified (alignment positions based on IRF5 variant (v)5: T10, S158, S309, S317, S451, and S462) [54]. An additional putative phosphorylation site has been proposed at S430 on IRF5v4 (equivalent to S456 on IRF5v5) [55]. However, the importance of each phosphorylation event on IRF5 function is not clear. Chen et al. hypothesized that phosphorylation at these positions facilitated the unfolding of the auto-inhibitory structure of IRF5 monomers, promoting self-dimerization, and exposing a surface for CREB-binding protein (CBP)/p300 binding (see Figure 1 below) [55]. While there is no doubt that IRF5 is phosphorylated following stimulation through TLR7 or 9 [41], which downstream kinases and at what sites remains an area of active investigation. It is possible that pathway-specific IRF5 activation is achieved through the use of different kinases, each of which would presumably phosphorylate distinct amino acid residues. As described below, progress has been made addressing this issue in the context of RIG-I and NOD pathway regulation.

Evidence from viral stimulation and overexpression systems has shown that RIG-I pathway kinases IκB kinase (IKK)-ε and TANK-binding kinase (TBK) 1 can phosphorylate IRF5 [41, 44, 45, 56], but this phosphorylation is not sufficient for IRF5 nuclear translocation [56]. A recent study used mass spectrometry to identify residues S158 and S462

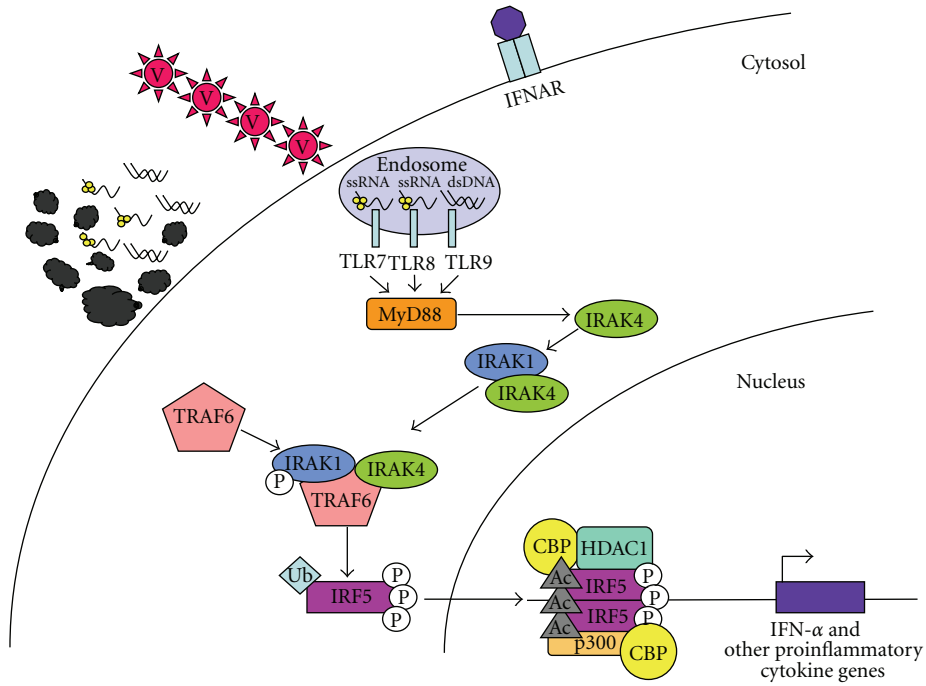

Figure 1: Schematic model forIRF5 activation. Cells use TLRs as sensors to detect the presence of viruses (V) via TLR7, -8, and -9. Alternatively, apoptotic debris (shown here as membrane blebs, ssRNA, and dsDNA) can also be a source of nuclear proteins and nucleic acids. Nuclear material is brought to the endosome, triggering TLR7, -8, and -9 signaling. Binding of cognate ligands to these TLRs recruits MyD88, a main signaling intermediate involved in TLR7, -8, and -9 signaling. MyD88 recruits interleukin-1 receptor associated kinase (IRAK)-4. IRAK-4 binds and phosphorylates IRAK-1, which in turn recruits Tumor necrosis factor (TNF) receptor associated factor (TRAF) 6 [46–48]. TRAF6 is an E3 ubiquitin (Ub) ligase that adds K63-Ub chains to IRF5 [49]. IRF5 is then shuttled to the nucleus and is acetylated by CBP and p300 [50]. Together, these events set the stage for the transcription of IFN-α and other pro-inflammatory cytokine genes.

on IRF5v5 as targets of TBK1, a kinase involved in the RIG-I pathway [54]. These events induced IL-6 transcription, but did not transactivate IFN-α promoter activity [44, 56]. Studies using viral stimulation have been less clear. Barnes et al. showed that NDV induced phosphorylation of IRF5 in 2fTGH cells transfected with IRF5 [41] as well as translocation into the nucleus and transactivation of an IFN-α reporter construct [20]. Cheng et al. demonstrated that NDV infection did not lead to phosphorylation of IRF5 in a HEC-1B/GFP-IRF5 system [44]. This discrepancy can perhaps be explained by the differences in cell type and/or cell tropism of the viruses. Interestingly, contrary to NDV infection, Sendai virus infection in 2fTGH cells led to activation of IRF3 and IRF7, but not IRF5 [20]. Moreover, IRF5 and 7 seem to have overlapping binding partners and functions, making it difficult to distinguish the dependence of either IRF on IFN-α transactivation [57]. To better understand the requirement of IRF5 on IFN-α regulation, biochemical studies need to be done in the context of *IRF7*$^{-/-}$ cells.

As with other IRF family members, IRF5 can form homodimers upon phosphorylation. This was demonstrated in a study in which GFP- and T7-tagged IRF5 were cotransfected with IKKε into HEC-1B cells. Pull-down assays with anti-T7 antibodies showed the presence of GFP-tagged IRF5 [44]. In support of this concept, crystallographic analysis of the C-terminal fragment of IRF5(v4) S430D showed the formation of stable homodimers [55]. In addition, like IRF3, IRF5 interacts with CBP/p300 [44, 55]. Size exclusion chromatography studies have shown two molecules of IRF5 S430D binding to two molecules of CBP, forming an $IRF5_2CBP_2$ complex [55]. IRF5 can also form dimers with IRF1, IRF3, and IRF7 [41, 57]. This interaction was enhanced upon stimulation with virus. Whereas binding of IRF3 with IRF5 synergistically augmented IFN-α reporter activity [41], IRF5/IRF7 heterodimers blocked each other's DNA-binding domains and prevented the ability of either to bind cognate DNA sequences, resulting in the repression of IFN-α promoter activity [57].

In addition to phosphorylation, ubiquitylation represents another important means of regulating protein expression and activity. Two types of poly-ubiquitin (Ub) chains dictate the fate of proteins: K48-Ub and K63-Ub, where the number refers to the position of the lysine (K) residue upon which the chains of Ub are built. E3 Ub ligases are responsible for adding Ub chains to either proteins destined for degradation (K48-Ub) or for activating signal transducing proteins (K63-Ub) [58]. The E3 Ub ligase TRAF6 is activated by TLR7 and 9 signaling via MyD88 and IRAK-1. The addition of K63-Ub on IRF5 by TRAF6 is necessary for nuclear translocation and IFN-α transactivation. Lysines 410 and 411 are putative targets of K63-Ub since mutagenesis of these lysines to arginines abolished nuclear translocation and IFN-α promoter activity [48].

K63-Ub-IRF5 could potentially be subjected to negative regulation by deubiquitinating enzymes such as TNF-α-induced protein 3 (TNFAIP3, also known as A20) [59]. With regard to type 1 IFN-induced gene activity, it is unknown whether TNFAIP3 can influence TLR7 and TLR9-mediated signaling via IRF5. IRF5 activity in an IL-12p40 luciferase reporter assay system was reduced with increased expression of TNFAIP3 [54]. This system utilized receptor interactive protein kinase 2 (RIP2), a kinase involved in the NOD signaling pathway.

Trafficking of molecules in and out of the nucleus is a tightly controlled process coordinated by importins and exportins on the nuclear membrane. These proteins recognize and bind to nuclear localization sequences (NLS) and nuclear export sequences (NES) encoded in the amino acid sequence. IRF5 has one NES (IRF5 v5 aa150-LQRMLPSLSLT-160 [44, 56] and two NLS's (IRF5 v4 aa12-PRRVRLK-18 and aa398-PREKKLI-404) [41, 55, 56, 60]. A specific inhibitor of the nuclear export protein CRM1, leptomycin B (LMB), has been used to monitor IRF5 nuclear trafficking. Treatment with LMB results in nuclear retention of IRF5 [56], indicating that IRF5 is continuously exported out of the nucleus.

Recently, investigators have presented evidence demonstrating the regulation of transcription factor activity by acetylation/deacetylation [61]. IRF1, 2, and 7 have been shown to be acetylated by histone acetylases [62, 63]. In a study by Feng et al., IRF5 appears to be one transcription factor subject to this form of regulation as well [50]. When 2fTGH cells expressing human IRF5 and either an ISRE- or an *IFNA1*-dependent luciferase reporter construct were stimulated with virus in the presence of trichostatin A (histone deacetylase (HDAC) inhibitor), luciferase activity was ablated. Furthermore, they showed that under uninfected conditions, IRF5 forms a multicomponent complex with the corepressors HDAC1, silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), and Sin3a to inhibit the luciferase reporter activity. Upon infection with NDV, IRF5 binds to histone acetylase (HAT) proteins p300, CBP, and PCAF while SMRT is exported out of the nucleus. It appears that IRF5 may be acetylated at several lysine residues since an antibody against acetylated lysine, which was used to immunoprecipitate overexpressed *IRF5* fragments, pulled out both N- and C-terminal IRF5 fragments. Taken together, IRF5 activity is highly regulated post-translationally. Multiple phosphorylation, ubiquitylation, and acetylation events must all be coordinated to induce IRF5 transactivation.

Not only is IRF5 activation regulated by different enzymes, but also IRF5 gene expression is complex. There are up to eleven distinct isoforms of IRF5 resulting from alternative splicing [22, 60]. Four different *IRF5* transcripts result from alternative usage of the first, noncoding exon (as shown in Figure 2(b)). In the study by Mancl et al., IRF5 isoforms were differentially expressed in various purified immune cell subpopulations, though more than one isoform could be expressed in the same subpopulation [60]. For example, pDCs constitutively expressed IRF5 variants 1–4 [60]. Moreover, different IRF5 isoforms activated the IFN-α and IFN-β promoters to varying degrees, where isoform 3/4 induced the highest levels of activity [60]. In summary, many points of IRF5 regulation are possible, and greater IRF5 activity could generate an IFN-α-rich environment which could lead to SLE disease susceptibility.

5. Genetic Variants in *IRF5* Are Associated with Systemic Lupus Erythematosus

The *IRF5* locus was first implicated in SLE through a candidate gene analysis involving patients of Nordic ancestry. The SNP rs2004640 which was associated in this study introduced a new donor splice site, suggesting alternate exon 1 splicing may occur in the context of this variant [21]. A subsequent study by Graham et al. strongly replicated the association of rs2004640 with SLE in multiple independent case-control cohorts, including cases and controls from Europe, North Americans of European ancestry, and a cohort from Argentina [22]. This study also confirmed that the risk allele allowed for alternate splicing of the first exon [22]. This study described three different alternate first exons (1A, 1B, and 1C) and showed that mRNAs containing 1B could only be made when the rs2004640 risk allele was present (Figure 2(b)). The first exon is not translated, so despite this clear impact upon splicing, the functional significance of exon 1B transcripts is not clear. Even when exon 1B transcripts are produced in the setting of the splice variant, they are present at levels which are 100 times lower than those derived from other exon 1 transcripts, such as exon 1A [22, 65].

A second SNP in the 3′ region of the *IRF5* locus was associated with increased IRF5 expression [22], and an SLE-risk haplotype was described that was composed of the high expression variant of this SNP along with the alternate splice variant of rs2004640. The high expression allele was not dependent upon the splice variant in this study, suggesting that there were multiple functional elements in *IRF5*. The high expression allele was correlated with a SNP in the 3′UTR region which introduces an alternate polyadenylation (poly-A) site and provides a potential explanation for higher *IRF5* mRNA abundance in the presence of this allele [23, 65, 66]. The SLE-risk allele of this SNP results in the production of a shorter poly-A tail, which is more stable and resistant to degradation, leading to a longer *IRF5* mRNA half-life and greater mRNA abundance (Figure 2) [23, 65].

6. Insertion/Deletion Polymorphisms in *IRF5*

In addition to the SNP variants detailed above, common insertion/deletion (indel) polymorphisms in *IRF5* have been reported, including a 30-base pair (bp) in-frame indel in exon 6, and a promoter indel [23, 28, 67]. The exon 6 insertion is present on both risk and nonrisk haplotypes. While this would suggest that it does not independently contribute to SLE-risk related to *IRF5*, the insertion is present on the risk haplotype and a cooperative role in pathogenesis cannot be ruled out. The exon 6 insertion is located in a proline-, glutamic acid-, serine-, and threonine-rich domain which can affect protein stability and function

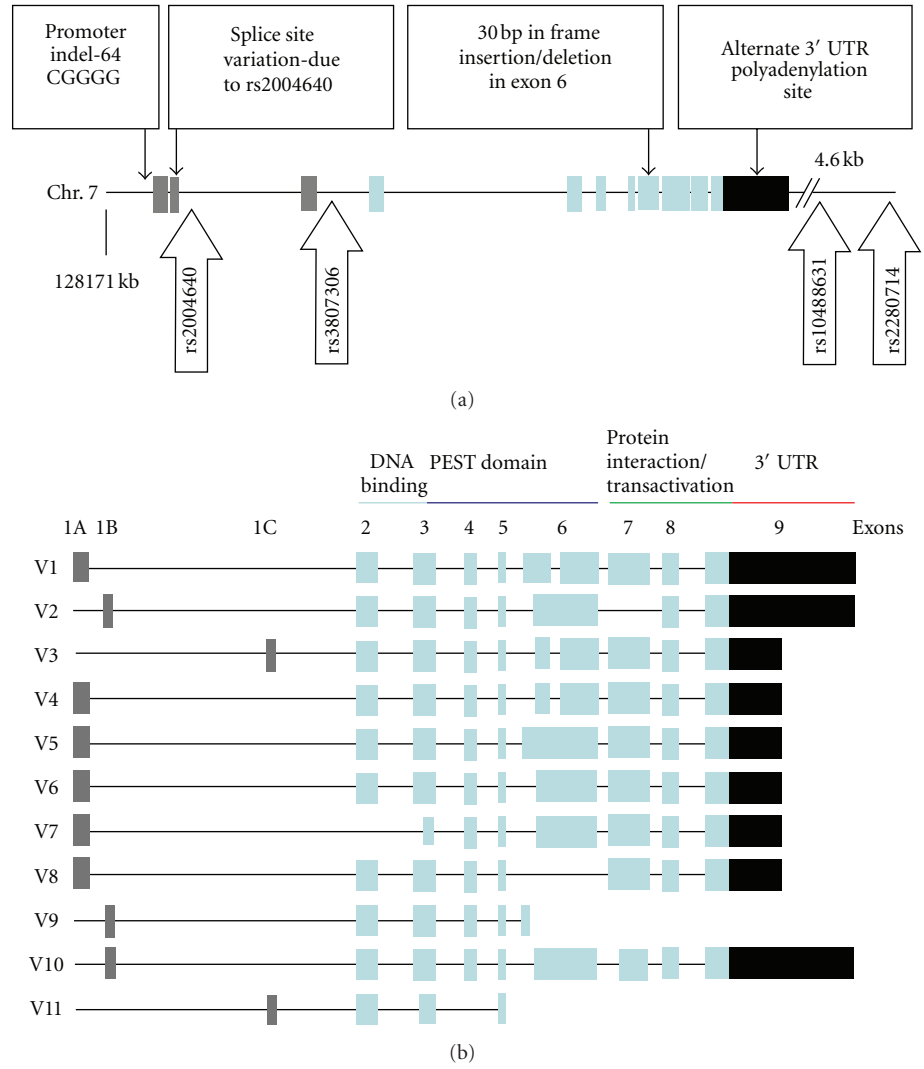

Figure 2: (a) *IRF5* gene marked with previously reported functional variants along with studied SNPs [64]. The first three grey boxes represent differentially spliced first exons (1A, 1B, and 1C), the next light blue boxes represent the exons 2–9, and the last black box indicates the 3′ UTR. SNPs rs2280714 and rs10488631 were used as proxies for rs10954213 in the 3′ UTR due to high LD. (b) *IRF5* mRNA isoforms [22]. There are eleven different variants. PEST, proline-, glutamic acid-, serine-, and threonine-rich.

of IRF5 (Figure 2(b)) [8, 23, 67]. Moreover, a promoter indel has been described, which is 5-base long (CGGGG/−), and this insertion polymorphism in the promoter is also present on the SLE-risk haplotype. This promoter variant confers risk of SLE independently from the risk haplotype presented by Graham et al. [23, 28], as shown in Table 1. The promoter indel is in high linkage disequilibrium (LD) with the exon 1 splice site variation, and it is possible that this variant could explain the risk signal from the 5′ region of the gene (Figure 2(a)). The SLE-associated insertion creates an additional SP1 transcription factor binding site and leads to increased *IRF5* expression [28]. Whether the promoter indel or the 3′ UTR variant is more important for *IRF5* mRNA abundance is not currently understood, and SLE-associated haplotypes carry both of these polymorphisms, suggesting that both may be required to result in risk of SLE. *IRF5* polymorphisms found to be associated with SLE in seminal candidate gene case-control studies are summarized in Table 1. Subsequent candidate gene and genome-wide association studies have strongly replicated these findings [24–27, 29–33].

7. Genetic Similarities and Differences by Ancestry

The risk alleles described above were initially found in European ancestry subjects, and while an association between *IRF5* and SLE has been subsequently confirmed in other ancestral backgrounds, the particular associated polymorphisms differ somewhat [24–27, 29]. For example, intron 1 SNPs (rs6953165 and rs41298401) but not exon 6 indel or 3′ UTR poly-A polymorphisms were found to be associated with SLE in Japanese population, and they were related to differential expression of several IFN pathway genes although

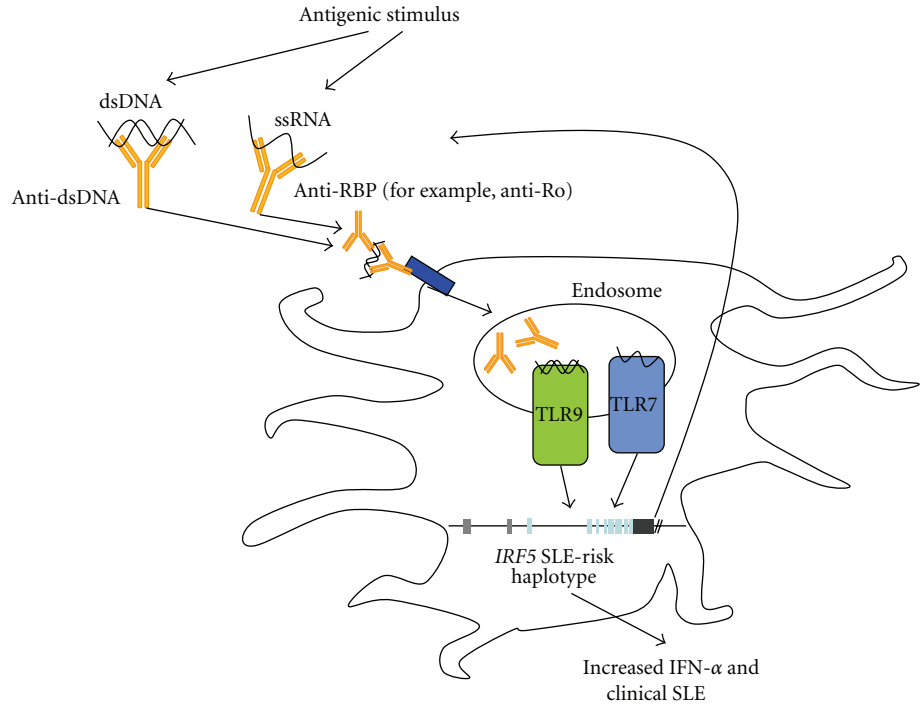

Figure 3: Diagram showing relationships between SLE-associated autoantibodies, *IRF5* genotype and IFN-α involved in the pathogenesis of SLE [64]. This suggests a "feed-forward" model in which specific auto-antibodies interact with particular *IRF5* risk variants which also predispose to the same antibody formation.

Table 1: Summary of genetic variants found in early seminal studies.

	Ancestry	Samples	Study type	Genetic variants	OR, *P* values	Functions
Sigurdsson et al., 2005 [21]	Swedish, Finnish	589 cases 377 controls	FB and CC association	rs2004640	OR = 1.59 $P = 7.1 \times 10^{-7}$	Altered exon 1 spicing
Graham et al., 2006 [22]*	Argentina, Spain, Sweden, USA	1661 cases 2508 controls	CC association	rs2004640	OR = 1.45 $P = 4.4 \times 10^{-16}$	Altered exon 1 splicing
Graham et al., 2007 [23]**	USA, UK, Sweden	555 trio pedigrees, 2188 cases 3596 controls	FB and CC association	Risk haplotype	OR = 1.78 $P = 1.4 \times 10^{-19}$	Altered exon 1 splicing, exon 6 in, short poly-A
				Protective haplotype 1	OR = 0.76 $P = 5.0 \times 10^{-8}$	Nonaltered exon 1 splicing, exon 6 in, long poly-A
				Protective haplotype 2	OR = 0.76 $P = 2.8 \times 10^{-5}$	Nonaltered exon 1 splicing, exon 6 del, short poly-A
Sigurdsson et al., 2008 [28]***	Sweden	485 cases 563 controls	CC association	CGGGG/–	OR = 1.69 $P = 4.6 \times 10^{-9}$	Promoter indel
				rs10488631	OR = 2.07 $P = 9.4 \times 10^{-10}$	Altered exon 1 splicing, exon 6 in, short poly-A

*The populations were mostly of European ancestry.
**Only the haplotype analysis is shown here. SNP rs2070197 was found to be a proxy for the risk haplotype.
***SNP rs10488631 is in high LD with rs2070197 and was used as a proxy for the risk haplotype. OR and *P* values are obtained from nonconditional analysis.
FB: family based, CC: case-control, OR: odds ratio, *P*: *P* value, poly-A: poly-adenylation, in: insertion, del: deletion, indel: insertion/deletion, LD: linkage disequilibrium.

Table 2: European ancestry case-case analysis showing *IRF5* haplotypes with associated functional elements and serological associations [64].

Tag SNP haplotype	Promoter indel	Splice variant	Exon 6 indel	Poly-A variant	Serologic association
(1) TACA	In	Present	In	Present	Anti-Ro: OR = 1.50 , $P = 2.0 \times 10^{-3}$ Anti-dsDNA: OR = 1.51, $P = 7.4 \times 10^{-3}$
(2) TATA	In	Present	Del	Present	Anti-dsDNA: OR = 1.68, $P = 4.9 \times 10^{-5}$
(3) TCTA	Del	Present	In	Absent	Anti-La: OR = 3.51, $P = 7.5 \times 10^{-3}$
(4) GCTA	Del	Absent	Del	Present	—
(5) GCTG	Del	Absent	In	Absent	—

The haplotypes are shown as each of the four alleles in order from 5′ to 3′ (rs2004640, rs3807306, rs10488631, rs2280714).
SNP: single nucleotide polymorphism, indel: insertion/deletion, Poly-A: poly-adenylation, In: insertion, Del: deletion, OR: odds ratio, *P*: *P* value.

not *IRF5* itself [26]. On the other hand, the European risk haplotype and its homozygosity appear more frequently in Mexican SLE patients compared to European patients [25], and in this ancestral background the European haplotype is a strong risk factor. In African Americans, a novel SNP rs3807306 was associated with SLE, although a functional role has not been defined [27]. We have performed follow-up work in African American and African populations which suggests that the European SLE-risk haplotype is present in African Americans due to European admixture and is associated with risk of SLE, but this haplotype was not present in African populations, and an African-derived SLE-risk haplotype was not observed in this study [23].

8. Autoantibodies, IFN-α and *IRF5* Variants

Further studies are needed to clarify how different combinations of the genetic elements of *IRF5* lead to SLE susceptibility, and what roles they play in the molecular pathogenesis of the disease. We have shown that the European risk haplotype is associated with increased serum IFN-α in SLE patients [68], and subsequent studies have supported this concept by showing that SLE-associated *IRF5* variants are associated with increased activation of the IFN-α pathway [69, 70]. However, the association between the risk haplotype and increased serum IFN-α in SLE patients was only observed in those patients who had anti-dsDNA or anti-RNA-binding protein (RBP) autoantibodies [68]. We expanded these findings in a study involving 1034 and 555 SLE patients with European and African ancestries, respectively [64]. The functional variants and SNPs studied are depicted in Figure 2(a). As shown in Table 2, the previously reported SLE-risk haplotype TACA [23] was associated with anti-dsDNA and anti-Ro antibodies, whereas the TATA haplotype which has previously been reported as a neutral haplotype [23] was associated with anti-dsDNA antibodies in case-case analysis. Similar patterns were detected in case-control analysis where the TACA and TATA haplotypes were associated with anti-dsDNA positive patients versus controls (Odds Ratio (OR) = 2.79, $P = 2.9 \times 10^{-20}$) and the TACA haplotype with anti-Ro positive patients versus controls (OR = 2.57, $P = 1.8 \times 10^{-14}$). The TACA haplotype is characterized by the presence of all four functional variants, the insertions in the *IRF5* promoter and exon 6, the spice variant, and the poly-A variant, whereas the TATA haplotype has all but the exon 6 insertion [64]. The fact that these two haplotypes which differ only at the exon 6 insertion are associated with different autoantibody profiles suggests a functional relevance of the exon 6 insertion. Functional studies of the exon 6 insertion to date support a role for exon 6 variants in altering its nuclear translocation, impacting apoptosis and cytokine production [67]. Moreover, our study showed that the haplotypes associated with particular autoantibodies resulted in increased levels of serum IFN-α only in the presence of that particular associated autoantibody. The above data support a pathogenic model in which these autoantibodies chronically stimulate the endosomal TLR system, and specific *IRF5* variants in conjunction with particular autoantibodies dysregulate IFN-α production, resulting in increased risk of SLE (Figure 3) [64].

The data presented above support a "gene + autoantibody = high IFN-α and risk of SLE" model, and presumably the associations between *IRF5* genotype and autoantibodies may be due to this interaction. Based upon these data, we cannot rule out the possibility that *IRF5* risk genotype could directly predispose to the formation of SLE-associated autoantibodies. In fact, *IRF5* knockouts of murine SLE models have decreased levels of SLE-associated auto-antibodies [71, 72]. This may be due to the role of IRF5 in regulating transcription of *Prdm1* which encodes Blimp-1, an essential regulator of plasma cell differentiation [73]. To answer this question in humans, we studied *IRF5* genotype in a unique cohort of anti-Ro autoantibody positive European subjects who carried a variety of diagnoses, including many who were asymptomatic and generally did not have high levels of circulating IFN-α [74]. We found that the *IRF5* SLE-risk haplotype was enriched even in these asymptomatic subjects with positive anti-Ro antibody, and that this enrichment was even greater (OR $\sim$ 5) in those initially asymptomatic Ro-positive individuals who later developed SLE [75]. Taken together, these data support a "feed-forward" hypothesis in which the risk haplotype predisposes to the formation of autoantibodies, and these autoantibodies subsequently lead to increased production of IFN-α in conjunction with the same *IRF5* variant (Figure 3) [75].

9. Conclusions

In this paper, we examined how IRF5 is regulated and activated, and how its genetic variants can influence the risk of

SLE by differentially activating the IFN-α pathway along with affecting the production of SLE-associated autoantibodies. The above data support an interesting novel model of SLE pathogenesis, in which genetic variations lead to serologic autoimmunity, subsequently creating a microenvironment which stimulates PRRs and results in high IFN-α [76].

A number of other SLE-associated genetic variants in the IFN-α and PRR pathways result in increased IFN-pathway activation [77–82], further supporting the concept that gain-of-function polymorphisms in the IFN-α and PRR pathways contribute to SLE susceptibility. While the exact initial trigger of autoimmunity in SLE remains unclear, possible antigenic sources include ultraviolet light, viruses, and demethylating drugs [83]. Recently, several studies point toward neutrophils as a factor in lupus pathogenesis [84, 85]. It has been hypothesized that chronic activation of neutrophils by immune complexes via Fc receptors induces them to release neutrophil extracellular traps (NETs) in a suicidal process called NETosis. NETs contain genomic DNA, providing a source of antigenic self-DNA. These would in turn stimulate TLRs on pDCs, putting in motion a vicious cycle of increased IFN-α and eventual autoimmune disease.

It is clear that IRF5 is a major pathogenic factor in human lupus, which will impact upon aspects of SLE diagnosis, prognosis, and management. Predictive models which include autoantibodies, IFN-α and other molecular measurements, and genetic variants may prove useful in diagnosis or prognosis. It seems unlikely that a purely genetic model will be sufficiently predictive, but the work summarized here demonstrates how other molecular phenotypes can greatly enhance the predictive capacity of genetic data. Additionally, the pathway in which IRF5 functions is currently being targeted by therapeutics directed at the endosomal TLRs and IFN-α [86, 87], and it is possible that *IRF5* genotype may help to define responder/nonresponder groups with respect to these therapies. The complexity demonstrated by this one disease-associated locus is staggering and suggests that we still have much work to do in understanding the genetic basis of human autoimmune disease.

Disclosure

The authors report no financial conflict of interests.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

C. M. Cham and K. Ko contributed equally.

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Review Article

Expression of the Autoimmune Regulator Gene and Its Relevance to the Mechanisms of Central and Peripheral Tolerance

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The autoimmune polyendocrine syndrome type 1 (APS-1) is a monogenic disease due to pathogenic variants occurring in the autoimmune regulator (*AIRE*) gene. Its related protein, AIRE, activates the transcription of genes encoding for tissue-specific antigens (TsAgs) in a subset of medullary thymic epithelial cells: the presentation of TsAgs to the maturating thymocytes induces the apoptosis of the autoreactive clones and constitutes the main form of central tolerance. Dysregulation of thymic *AIRE* expression in genetically transmitted and acquired diseases other than APS-1 may contribute to further forms of autoimmunity. As *AIRE* and its murine homolog are also expressed in the secondary lymphoid organs, the extent and relevance of AIRE participation in the mechanisms of peripheral tolerance need to be thoroughly defined.

1. Introduction

Pathogenic variants in the autoimmune regulator (*AIRE*) gene cause the autoimmune polyendocrine syndrome type 1 (APS-1), also called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), an autosomal recessive disease characterized by immunological disturbances such as difficulty to eradicate surface candidiasis and autoimmunity to various organs, mainly endocrine glands [1, 2]. *AIRE* is located in the region q22.3 of chromosome 21 [3], and its cloning dates back to the second half of the nineties [4, 5]. APS-1 is a rare model of monogenic autoimmune disease and in this quality provides unequivocal insights into the pathogenesis of more complex diseases of analogous nature [6, 7].

Due to its biochemical properties, AIRE protein activates the transcription of genes encoding for tissue-specific antigens (TsAgs) in a subset of medullary thymic epithelial cells (mTECs): this phenomenon, called promiscuous gene expression (PGE), is finalized to the promotion of central (thymic) tolerance [8]. The last step of the process is represented by the deletion (negative selection) of T-cell clones bearing T-cell receptors (TCRs) with critical degree of specificity for the corresponding TsAgs [9].

Nonetheless, the nature and extent of AIRE action remain unclearly defined. A rigorous mapping of *AIRE* gene expression is fundamental to the dissection of the protein role, but the current data show several incongruities, presumably due to differences in tissue substrate and sensitivity of the methods utilized.

Here an extensive review of the studies pertaining to the argument is reported, with an additional look at the relation between disturbances in *AIRE* expression and diseases other than APS-1 in human field and animal models.

2. The Initial Studies

2.1. Initial Mapping of AIRE Expression. The first two studies, which dealt with *AIRE* cloning and searched for *AIRE* mRNA in bulk tissue samples by Northern blotting (NB), gave disagreeing responses [4, 5]. Although the thymus showed the highest level of positivity in both cases, one research group found a further weak positivity only in lymph-nodal, fetal liver, and appendix samples [4], while the other one signaled also a moderate to strong positivity in the samples from bone marrow, spleen, peripheral blood lymphocytes (PBLs), and organs such as thyroid, pancreas,

adrenal gland, and testis, in other words the endocrine glands targeted by autoimmunity in APS-1 [5].

2.2. Identification of AIRE-Expressing Cell Lineages. Later, the same research groups stated that the highest amount of *AIRE* mRNA and AIRE protein could be identified, by in situ hybridization (isH) and immunohistochemistry (IHC), in rare cells scattered in the medulla and subcapsular area of the thymus, or buried in the Hassall's corpuscles [10, 11].

In immunofluorescence (IF), these cells were seen to express surface markers such as cytokeratins, molecules of the class-II major histocompatibility complex (MHC-II), and the clusters of differentiation CD80, CD86, and CD40, and for this reason were recognized as mTECs, the main components of the thymic stroma. A minority of thymic $AIRE^+$ cells expressed CD11c and CD83, which identify mature dendritic cells (DCs) of myeloid lineage [10].

In the secondary lymphoid organs, one research group detected AIRE only in the medulla and paracortical area of the lymph nodes, in the spleen and fetal liver: similarly to thymic DCs, lymph-nodal $AIRE^+$ cells expressed CD83, suggesting a common identity [10].

In contrast, the other group observed significant staining of medullary thymocytes, lymph-nodal and splenic red-pulp lymphocytes, and PBLs; other leukocyte populations, such as neutrophilic granulocytes and monocytes, were also $AIRE^+$. The results, obtained by IHC and immunocytochemistry (ICC), were confirmed by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) [11].

By IF, freshly isolated PBLs were positively stained for AIRE in the study of Rinderle et al. too [12].

3. *AIRE* Expression in the Cells of the Immune Response and in Nonlymphoid Organs

The initial studies set in motion the debate, still outstanding, on the existence of cell lineages, complementary to mTECs, in which *AIRE* would be expressed, and the related meaning. As expected, such debate primarily deals with *AIRE* expression in the cells of the immune response.

3.1. AIRE Expression in the Monocyte/DC Lineage. Measurable amounts of *AIRE* mRNA and AIRE protein were evidenced in $CD14^+$ cells sorted from peripheral blood, and in monocyte-derived DCs through in vitro differentiation [13–15], with isolated exceptions [16]. One of these research groups found *AIRE* mRNA also in plasmacytoid DCs isolated directly from the peripheral blood [15]. Members of the mitogen-activated protein kinase (MAPK) family would be involved in the signal-transduction pathway allowing *AIRE* expression in the monocyte/DC lineage [14, 17]. Interestingly, it was observed that DC maturation is paralleled by increasing AIRE levels and ordinary up-regulation of several genes [14].

Later, Poliani et al. detected *AIRE* expression in frozen samples of lymph nodes and gut-associated lymphoid tissue (GALT) from adult subjects, while the fetal samples were negative: the cells responsible for such positivity expressed surface markers typical of mature DCs [18].

3.2. AIRE Expression in the Lymphocyte Lineage. *AIRE* expression in the lymphocyte lineage remains quite uncertain: reappraising and partly correcting their previous findings [13], Nagafuchi et al. found, by RT-PCR, *AIRE* mRNA in PBLs belonging to the $CD4^+$ T-cell subset; the transcription level increased under antigen- or cytokine-mediated activation [19]. Another Japanese research group detected *AIRE* mRNA in thymic B lymphocytes and double-positive (DP) $CD4^+CD8^+$ thymocytes, while in the peripheral blood it was restricted to B lymphocytes only [20].

3.3. AIRE Expression in Nonlymphoid Organs. In two of the above studies, a large set of human organs was assayed by RT-PCR and IHC, and the authors agreed that organs of the endocrine, cardiovascular, respiratory, gastrointestinal, genitourinary, and nervous systems are either consistently negative or negligibly positive for *AIRE* expression [16, 18]. Based on the observation that mTECs and cancer cells share PGE, Klamp et al. included RT-PCR of samples from human cancers, but no *AIRE* expression was found [16].

So, to recapitulate, only Finnish researchers detected *AIRE* mRNA in human tissues such as endocrine glands and other nonlymphoid organs, albeit the cell lineages detaining such property were not defined [5].

3.4. Unexpected Localizations. An unexpected localization of *AIRE* mRNA was found by Harris et al., who studied two unrelated APS-1 adolescents with chronic abnormalities of endochondral ossification, characterized by irregular and radioopaque metaphyses, subjacent to the growth plates of long bones; *AIRE* expression, although not searched for in the bone samples of the patients, was assayed in the thymus, liver, and growth plates of healthy fetuses aborted at 13–18 weeks of gestational age, in chondrocytes and in two chondrosarcoma lines: in all examined tissues, *AIRE* expression resulted consistently active. In particular, in the growth plate of the knee, *AIRE* mRNA appeared at 15 weeks of gestational age and was still present at 18 weeks [21].

Similar considerations may be done for *AIRE* expression in epidermal keratinocytes, in keratinocytes of the outer and inner epithelial sheaths of the hair follicle, and in matrix melanocytes [22, 23]. At this level, AIRE is identifiable at 16 weeks of gestational age and colocalize with cytokeratin 17, a protein constitutive of the intermediate filaments [23].

The meaning of *AIRE* expression in chondrocytes and keratinocytes remains quite obscure. Intriguingly, Clark et al. had already proven that human skin cells (keratinocytes and fibroblasts), once cultured in a three-dimensional arrangement resembling the thymic architecture, have *AIRE* expression, synthesize a large set of TsAgs, and are able to perform a thymus-like function in de novo maturation and negative selection of T lymphocytes [24]. Currently, the demonstration that this observation may have some in vivo equivalence is lacking.

Findings pertaining to *AIRE* expression in human tissues are resumed in Table 1.

4. *Aire* Expression in the Mouse

Researches on the murine homolog (*Aire*, printed in lower case to avoid confusion) recapitulate most findings and incongruities encountered in dealing with human tissues, as resumed in Table 2.

4.1. Searching for Aire mRNA. Not surprisingly, the low level of *Aire* expression made some methods, such as NB, unsuitable to detect *Aire* mRNA even in the thymus [25–27].

On the other hand, RT-PCR gave consistently positive results on the thymus [26–33], lymph nodes [28–30], spleen [26, 28–32], and liver [28, 29, 32].

Performing RT-PCR on the cells obtained by enzymatic digestion of whole thymus and spleen, and sorted by flow cytometry, Heino et al. found *Aire* mRNA in mTECs and, to a lesser degree, in DCs: further analysis of the latter revealed *Aire* transcription in two thymic ($CD8\alpha^+$ and $CD8\alpha^-$) and three splenic ($CD4^+CD8\alpha^-$, $CD4^-CD8\alpha^-$ and $CD4^-CD8\alpha^+$) subsets of myeloid and lymphoid lineage [28].

RT-PCR demonstrated higher sensitivity than isH that, when employed to map *Aire* expression on tissue sections, detected *Aire* mRNA only in rare foci of mTECs of murine embryos (from 14.5 days after conception), and young and adult mice [25, 26, 32].

On the other hand, a Finnish research group observed, by isH, an additional staining of a small number of medullary thymocytes, and of the lymph-nodal paracortical zone, the splenic red pulp, and immature bone marrow elements belonging to various cell lineages [29]. In the same study, RT-PCR detected *AIRE* mRNA in a remarkable number of organ samples [29].

4.2. Searching for Aire Protein. Aire protein was found by Western blotting (WB) in the only thymus [28, 32]; by IHC, Heino et al. observed that $Aire^+$ cells belonged to a subset of mTECs, distributed among resting (60%) and activated (30%) elements, as revealed by CD95 and CD29, respectively. In embryonic thymus, $Aire^+$ cells appeared at 14 days after conception [28]. In the same study, IHC was unable to stain $Aire^+$ cells in any other tissue examined, albeit RT-PCR had detected *Aire* mRNA in the lymph nodes and spleen after the first round of amplification, and in the liver and various other organs after two rounds of the procedure [28].

Conversely, reproducing the results of RT-PCR, Halonen et al. found $Aire^+$ cells in several organs [29]. In a following study, the authors strengthened these results by comparing tissue reactivity in wild-type and *Aire*-deficient ($Aire^{-/-}$) mice [34]. The findings were later supported by a UK research group [32].

It should be underlined that almost all cited studies utilized polyclonal antibodies (Abs) from mouse or rabbit to stain $AIRE^+$/$Aire^+$ cells in human and murine tissues, respectively, while the use of monoclonal Abs was rare and gave a restricted positivity [10, 18].

In this sense, Hubert et al., using rabbit monoclonal anti-Aire Abs, found $Aire^+$ cells among murine mTECs only, albeit in the same study *Aire* mRNA had been detected also in thymic and splenic DCs [35]. Then the authors formulated an unifying theory, hypothesizing that the amount of *Aire* mRNA detected could be below the critical level useful to induce the translation into its protein, and that only monoclonal anti-Aire Abs would allow fine and unequivocal mapping of true *Aire* expression. It was not excluded that some extrathymic cells may express *Aire* in particular, not yet fully known, conditions [35].

4.3. Other Relevant Localizations. Two research groups identified, in the stroma of lymph nodes (and, in one instance, spleen), $Aire^+$ cells that exhibited a $CD45^-CD80^-CD86^-MHC\text{-}II^+$ phenotype with further, albeit not coincident, epithelial-cell markers. Such cells were able to induce the deletion of $CD8^+$ T-cell clones bearing TCRs specific for antigens encoded by Aire-dependent genes: the clones had been transferred into irradiated mice reconstituted with β_2-microglobulin-deficient ($\beta_2\text{-}m^{-/-}$) bone marrow to ensure that only radioresistant stromal cells of the secondary lymphoid organs could interact with them [36, 37].

Searching for *Aire* expression in lymph-nodal and splenic stroma by other research groups did not produce unequivocal results [31, 38–40]. The localization of Aire in secondary lymphoid organs may be connected to not yet defined mechanisms of peripheral tolerance integrating the thymic function by enlarging the set of controlled genes, deleting autoreactive T-cell clones that escape thymic deletion, or both [41, 42].

In another study, RT-PCR and IHC were used to demonstrate *Aire* transcription and translation in spermatogonia and early spermatocytes, where Aire would play a role in the program of early, scheduled apoptosis indispensable to the maintenance of germline stability [43].

Findings and controversies over the extrathymic expression of human *AIRE* and its murine homolog have been reviewed by Eldershaw et al. [44].

5. Regulation of *AIRE* Expression

5.1. Signal-Transduction Pathways. Besides to the integrity of the gene in itself, thymic *AIRE* expression requires that of two signal-transduction pathways enabling heterodimeric nuclear transcription factors known as NFs-κB: NF-κB1 includes a protein, p50 (from p105 precursor), and the transcription factor reticuloendotheliosis viral oncogene homolog A (RelA), while NF-κB2 includes protein p52 (from p100 precursor) and another member of Rel family, RelB. In epithelial-cell lineages, NFs-κB control cell proliferation, differentiation, and survival [45].

Thymic intercellular signaling promotes the pathways: at least three members of tumor necrosis factor (TNF)-receptor family represented on mTEC surface, namely CD40, receptor activator of NFs-κB (RANK) and LT-β receptor (where LT stays for lymphotoxin), are able, by interaction with their respective partners on $CD4^+$ thymocytes, namely

Table 1: *AIRE* expression (*AIRE* mRNA and AIRE protein) in human extra-thymic systems, organs, and tissues.

Systems, organs, tissues	*AIRE* expression negative or negligibly positive		*AIRE* expression moderately or strongly positive	
	Techniques	Cell types	Techniques	Cell types
Bone marrow	NB [4] RT-PCR [18] IHC [18]		NB [5]	
Lymph nodes	IHC [18] FC [20]	$CD14^-$ cells [20] B lymphocytes [18] T lymphocytes [18] Monocytes [18] Macrophages [18] DCs (plasmacytoid) [18] Epithelial cells [18] Endothelial cells [18]	NB [4, 5] isH [10] RT-PCR [16, 18] IHC [10, 11, 18] IF [10] FC [20]	Neutrophilic granulocytes [11] Lymphocytes [11] $CD14^+$ cells [20] DCs (myeloid-lineage) [10, 18]
Spleen	NB [4] RT-PCR [16, 18] IHC [18]		NB [5] isH [10] IHC [10, 11]	Neutrophilic granulocytes [11] Lymphocytes (red-pulp) [11]
GALT			RT-PCR [18] IHC [18]	
Fetal liver			NB [4, 5] isH [10] IHC [10]	
Adult liver	NB [4] isH [10] RT-PCR [16, 18] WB [12] IHC [10, 18]			
Peripheral blood leukocytes	NB [4] RT-PCR [13, 16, 19] ICC [10, 13] FC [20]	Neutrophilic granulocytes [13] PBMCs [16] $CD14^-$ cells [16] B lymphocytes [19] T lymphocytes [20] $CD4^+$ T lymphocytes [13, 20] $CD8^+$ T lymphocytes [19, 20] Monocytes [16, 19] DCs (myeloid-lineage) [16]	NB [5] RT-PCR [11, 13–15, 19, 20] ICC [11, 13] IF [11, 12] FC [20]	Neutrophilic granulocytes [11] PBMCs [11, 13, 19] PBLs [11, 12] B lymphocytes [20] T lymphocytes [19, 20] $CD4^+$ T lymphocytes [19] $CD14^+$ cells [20] Monocytes [11, 13, 14] DCs (myeloid-lineage) [13–15, 19] DCs (plasmacytoid) [15]
Skeletal muscle	RT-PCR [16, 18] IHC [18]			
Cartilage and bone	RT-PCR [16, 18] IHC [18]		RT-PCR [21]	Chondrocytes [21]
Heart and blood vessels	isH [10] RT-PCR [18] IHC [10, 18]			
Respiratory system (upper and lower tract)	isH [10] RT-PCR [16, 18] IHC [10, 18]			
Systems, organs, tissues	*AIRE* expression negative or negligibly positive		*AIRE* expression moderately or strongly positive	
	Techniques	Cell types	Techniques	Cell types
Gastrointestinal system (upper tract, small and large bowel, salivary glands)	RT-PCR [16, 18] IHC [18]		NB [4]	

Table 1: Continued.

Endocrine glands (parathyroid glands, thyroid, pancreas, adrenal gland)	NB [4] isH [10] RT-PCR [16, 18] WB [12] IHC [10, 18]	NB [5]	
Genito-urinary system, placenta, mammary gland	isH [10] RT-PCR [16, 18] IHC [10, 18]	NB [5]	
Skin and annexes	isH [10] RT-PCR [16, 18] IHC [10, 18]	RT-PCR [22] IF [22, 23]	Keratinocytes (epidermal and HF) [23]
Central and peripheral nervous system	RT-PCR [16, 18] IHC [18]		
Eye and annexes	RT-PCR [18] IHC [18]		

AIRE: autoimmune regulator, GALT: gut-associated lymphoid tissue, PBMCs: peripheral blood mononuclear cells, PBLs: peripheral blood lymphocytes, DCs: dendritic cells, HF: hair-follicle, CD: cluster of differentiation, NB: Northern blotting, isH: in situ hybridization, RT-PCR: reverse transcriptase-polymerase chain reaction, WB: Western blotting, IHC: immunohistochemistry, ICC: immunocytochemistry, IF: immunofluorescence, and FC: flow cytometry.

CD40 ligand, RANK ligand and LT-$\alpha_1\beta_2$, and by means of TNF-receptor-associated factors (TRAFs), to initiate the cascade of reactions ending in NF-κB activation [46–50].

5.2. Timing of AIRE Expression. *AIRE* expression is confined to a final stage of cell maturation, as shown in vitro and in vivo by the postmitotic status of murine Aire$^+$ mTECs [51, 52]; in addition, Aire$^+$ mTECs show a very limited life span [53, 54]. It is also indicative that Aire$^+$ mTECs, because of their degree of differentiation, are highly sensitive to the drug-mediated ablation of the thymic medulla, and that their regeneration follows an invariant pattern [55].

It has been suggested that *AIRE* expression, and consequently that of AIRE-dependent genes, are strategically delayed just to allow a full T-cell responsiveness [56].

5.3. Modulation of AIRE Expression. Currently, we do not know whether the level of *AIRE* expression is genetically set, and whether metabolic, or environmental, or other agents are able to modulate it. This phenomenon, if determining the amount of TsAgs encoded by AIRE-dependent genes, could influence the chances of the autoreactive T-cell clones to encounter their targets and impact the efficiency of negative selection.

Studies on *Aire*$^{-/-}$ mice showed that the thymic expression of the genes dependent on Aire is quantitatively related to the amount of it, and that, in heterozygous (*Aire*$^{+/-}$) mice, intermediate level of mRNAs condition the number of autoreactive T-cell clones escaping thymic deletion [57]. This led the researchers to suggest that, in human field, the condition of heterozygosity for pathogenic *AIRE* variants could confer a risk for the onset of sporadic autoimmune diseases, when acting in synergy with other susceptibility factors. Actually, no data support this hypothesis.

Chen et al. found that, in the murine thymus, baseline *Aire* expression is related to the genetic background, as mTECs of nonobese diabetic (NOD) mice displayed lower levels of mRNAs from *Aire* and three Aire-dependent TsAg-encoding genes, when compared to mTECs of Balb/c mice [58]; Heino et al. had already found that Aire$^+$ mTECs of NOD mice show an abnormal morphology [28].

Later, Venanzi et al. demonstrated that, in non-autoimmune-prone C57BL/6 mice, Aire activates more strongly the transcription of TsAg-encoding genes, and that the same genes are more severely downregulated in *Aire*$^{-/-}$ animals of the same strain. Unexpectedly, the percentage of Aire$^+$ mTECs was higher in the thymus of NOD mice [59].

According to these findings, autoimmune-prone mice would show a less strict regulation of dependence on Aire, more than a deficient amount of it.

6. Thymic Diseases and *AIRE* Expression in Human Field

6.1. Severe Combined Immunodeficiency (SCID). Omenn syndrome is characterized by peripheral expansion of oligoclonal T lymphocytes with autoreactive propensity. Impairment in various steps of T-cell maturation may cause the disease: the most frequent defect is caused by pathogenic variants in the recombinase-activating genes 1 and 2 (*RAG-1* and *RAG-2*, resp.) [60]. In all jawed vertebrates, RAG proteins induce a DNA rearrangement, called V(D)J recombination, that reassembles the exons encoding for the antigen-binding domains of TCRs from the native variable, diversity, and joining gene segments [61–63].

Some patients with Omenn syndrome and RAG deficiency have a marked decrease of circulating T and B lymphocytes, a condition referred to as T$^-$B$^-$SCID [64]. In either

Table 2: *Aire* expression (*Aire* mRNA and Aire protein) in murine extra-thymic systems, organs, and tissues.

	Aire expression negative or negligibly positive		*Aire* expression moderately or strongly positive	
Systems, organs, tissues	Techniques	Cell types	Techniques	Cell types
Bone marrow			isH [29] IHC [29]	Myeloblasts [29] Lymphoblasts [29] Megacaryocytes [29]
Lymph nodes	WB [28] IHC [28, 29]	Lymphocytes (germinal-center) [29]	isH [29] RT-PCR [28–30] IHC [29, 32]	Lymphocytes (germinal-center) [32] Lymphocytes (paracortical) [32] Lymphocytes (medullary) [29] DCs [29]
Spleen	NB [26, 27] isH [32] RT-PCR [27] WB [28] IHC [28, 29, 32]	Lymphocytes (red-pulp) [32] Lymphocytes (white-pulp) [29]	isH [29] RT-PCR [26, 28–32] IHC [29, 32] IF [32]	Neutrophilic granulocytes [29] Lymphocytes (red-pulp) [29] Lymphocytes (white-pulp) [32] B lymphocytes [32] T lymphocytes [32] DCs [29] DCs (myeloid-lineage) [28, 31] DCs (lymphoid-lineage) [28] Macrophages [29] Smooth-muscle cells [29]
Fetal liver			RT-PCR [29]	
Adult liver	NB [26, 27] isH [32] RT-PCR [27, 30] WB [28, 32] IHC [28, 32]		isH [29] RT-PCR [28, 29, 32] IHC [29, 34]	Hepatocytes [29, 34] Küpffer cells [29]
Peripheral blood leukocytes	RT-PCR [28]		ICC [29]	Neutrophilic granulocytes [29] PBLs [29] Monocytes [29]
Skeletal muscle	NB [26, 27] WB [28] IHC [28]		RT-PCR [26, 28]	
Heart	NB [26] isH [32] RT-PCR [27, 30] IHC [32]		RT-PCR [26, 32]	
Respiratory system (upper and lower tract)	NB [26] isH [32] RT-PCR [30] WB [28] IHC [28, 32]	Small-airway epithelial cells [32] Alveolar cells [32]	RT-PCR [26, 27, 32] IHC [29, 32]	Airway epithelial cells [29] Large-airway epithelial cells [32] Type-1 and type-2 pneumocytes [29] Alveolar macrophages [29]
Salivary glands	RT-PCR [30]		IHC [29]	Tubulo-acinar and duct cells [29]
Gastrointestinal system (upper tract)	NB [27] isH [32] RT-PCR [30]		RT-PCR [32] IHC [29]	Mucosal and glandular epithelial cells [29]
Gastrointestinal system (small intestine and large bowel)	NB [27] isH [32] IHC [32]	Enterocytes (small intestine) [32] Neuroendocrine cells [32]	RT-PCR [32] IHC [29, 32]	Mucosal and glandular epithelial cells [29] Goblet cells [32]
Hypophysis			IHC [29]	Anterior- and intermediate-lobe cells [29]
Thyroid	RT-PCR [27, 30]		IHC [29]	Follicular and parafollicular cells [29]
Pancreas	RT-PCR [27, 28, 30]		IHC [29]	Langerhans-islet cells [29] Acinar cells [29]

Table 2: Continued.

	Aire expression negative or negligibly positive		*Aire* expression moderately or strongly positive	
Systems, organs, tissues	Techniques	Cell types	Techniques	Cell types
Adrenal gland	isH [32] RT-PCR [30, 32] WB [28, 32] IHC [28, 32]		RT-PCR [27, 28] IHC [29]	Cortical-layer cells [29] Medullary chromaffin cells [29]
Urinary system	NB [26] isH [32] RT-PCR [26, 30] WB [28] IHC [28]		isH [29] RT-PCR [27, 29, 32] IHC [29, 32]	Glomerular and tubular epithelial cells [29, 32] Urinary-tract epithelial cells [29] Bladder smooth-muscle cells [29]
Male genital system	NB [26] isH [32] WB [28] IHC [28, 32]	Mature germinal cells [32]	isH [29] RT-PCR [26–29, 32] IHC [29, 32]	Germinal cells [29] Immature germinal cells [32] Sertoli cells [29] Leydig cells [29] Spermatic-tract epithelial cells [29]
Female genital system	isH [32] WB [28] IHC [28]		isH [29] RT-PCR [27, 30, 32] IHC [29, 32]	Oocytes [29] Follicular cells [29, 32] Luteal cells [29] Interstitial cells [29] Fallopian-tube epithelial cells [32] Endometrial cells [29] Myometrial cells [29]
Central and peripheral nervous system	NB [26] isH [32] RT-PCR [26]		isH [29] RT-PCR [29, 32] IHC [29, 32, 34]	Neurons of cerebral cortex, basal nuclei, brainstem nuclei, spinal cord [29, 32] Granular neurons, Purkinje cells [29, 34] Glial cells [29]
Eye and annexes	RT-PCR [30]		IHC [29]	Retinal-layer elements [29]

Aire: autoimmune regulator, PBLs: peripheral blood lymphocytes, DCs: dendritic cells, NB: Northern blotting, isH: in situ hybridization, RT-PCR: reverse transcriptase-polymerase chain reaction, WB: Western blotting, IHC: immunohistochemistry, ICC: immunocytochemistry, and IF: immunofluorescence.

thymus or peripheral blood mononuclear cells (PBMCs) of patients suffering from these diseases, a substantial AIRE reduction was found [65–67]. In this sense, such forms of immunodeficiency, the classical SCID included, confirm the crucial role of AIRE in the mechanisms of central tolerance [68, 69].

A reasonable interpretation of what happens in these conditions leads to suppose that the thymi of the patients bearing genetically transmitted defects of the molecules involved in the developmental steps of T lymphocytes (with privileged reference to the construction of TCR diversity), show abnormalities of TEC differentiation, and consequently of *AIRE* expression, that are proportional to the timing of intervention of the same factors; hypomorphic variants of the related genes would result in more subtle disturbances [70–72].

6.2. Thymomas. Thymomas are rare tumors derived from TECs that are often associated with autoimmune diseases, mainly myasthenia gravis, caused by Abs to the acetylcholine receptor (AChR). It has been hypothesized that thymoma-associated AIRE deficiency may impair the tolerance to AChR and other antigens [73]. In facts although *AIRE* expression in thymomas is clearly decreased in terms of *AIRE* mRNA and AIRE$^+$ cells, this datum does not correlate with the prevalence of myasthenia gravis [74, 75]. Thymoma patients do not exhibit the typical picture of APS-1 [76], albeit with isolated exceptions [77], but some resemblances between thymoma-associated myasthenia gravis and APS-1 exist [78, 79].

An interesting point of contact between APS-1 and thymomas is the presence of circulating Abs to various cytokines, such as interferons (IFNs) and interleukins (ILs): in the original article of Meager et al., sera from APS-1 patients showed high-titer neutralizing Abs to type-1 IFNs such as IFN-α, all subtypes included, and IFN-ω; IFN-β, another member of type-1 IFNs, as well as IFN-λ_1, a subtype of type-3 IFNs, were less frequently targeted [80]. The same Abs were found in a large number of thymoma patients with myasthenia gravis, albeit the titer was significantly lower [80].

These findings led the authors to hypothesize that in abnormal thymic microenvironments made vulnerable by AIRE deficiency, the process of autoreactivity focuses early on molecules, such as type-1 IFNs, that result to be abundantly in loco available [81].

In a second time, Abs to the cytokines produced by Th17 subset of T-helper lymphocytes, namely IL-17A, IL-17F, and

IL-22, were found in a high number of APS-1 patients; remarkably, the occurrence of such Abs in thymoma patients regarded mostly the restricted number of them that suffered from chronic mucocutaneous candidiasis, strengthening the similarities between the two diseases [82, 83].

In a further study, the same research group, utilizing a radioligand-binding assay, confirmed that Abs to IFN-ω occur in the totality of APS-1 patients and beat the prevalence of Abs to two subtypes of IFN-α, namely IFN-α_2 and IFN-α_8, that in turn are found in a high percentage of patients with thymoma-associated myasthenia gravis [84].

Leaving aside the autoimmune phenomena, the hypothesis that AIRE deficiency may contribute in thymomas to the tumor-promoting antiapoptotic features of TECs should not be discharged [85].

6.3. Down Syndrome. Down syndrome is characterized by thymic atrophy, and a decrease in $AIRE^+$ cells was found in the thymus of subjects with Down syndrome who had undergone surgical thymectomy because of congenital heart malformations [86]. These data seem to deny that susceptibility to autoimmunity in Down syndrome would be a consequence of the precocious ageing.

7. Thymic Diseases and *Aire* Expression in Animal Models

7.1. Experimental Blocks in Thymocyte Maturation. Engineering animal models in which thymic organogenesis is disturbed provide a relevant contribution to the comprehension of the phenomena observed in the corresponding human diseases. A strategic choice is the block, at various stages of the process, of the thymocyte maturation, with the study of the related consequences on the architecture of the thymus in its entirety, and on the developmental steps of TECs: Tgε26 and *Rag*-deficient ($Rag^{-/-}$) mice are examples of animal models utilized in such studies.

Tgε26 mouse expresses a high number of the invariant CD3-ε chain belonging to TCR complex, and its thymocytes are blocked at DN1-DN2 stages, where DN stays for double-negative and indicates a $CD4^-CD8^-$ condition, with subdivision based on the progressive expression of CD44 and CD25. $Rag^{-/-}$ mouse recalls the most frequent defect causing Omenn syndrome: as reported, RAG/Rag proteins are indispensable to create TCR diversity and an adequate T-cell repertoire; the consequence of their deficiency is an impaired thymocyte maturation, with a block at DN3 stage [87].

A prototypical study in this field was that of Zuklys et al.: as seen above, *Aire* mRNA and Aire protein are recovered in murine embryos at 14–14.5 days after conception, slightly anticipating DN3 stage of thymocyte maturation. Consistently with these data, the authors found that thymi from Tgε26 mice lacked an orthodox three-dimensional TEC network, and *Aire* mRNA could not be detected; conversely, the block of thymopoiesis in *Rag*-$2^{-/-}$ mice altered only partially the thymic compartmentalization, and the related mTEC differentiation and *Aire* expression [88].

As well as in human field, following studies suggest that the degree of thymic abnormalities, *Aire* expression included, depends on how precociously the factors damaged by pathogenic variants of the encoding genes act in the construction of TCR diversity [89–92].

7.2. Experimental Defects in NF-κB Signal-Transduction Pathways. Several studies have taken in account various murine constitutional and experimentally induced defects involving the molecules that participate to the signal-transduction pathways enabling NFs-κB, to elucidate the impact of each step impairment on mTEC properties, with particular regard to *Aire* expression [28, 88, 93–117]. A detailed report of such articles goes beyond the scope of the present work, but, as indicated, excellent reviews are available [46–50].

7.3. Other Experimental Diseases Targeting the Thymus. Also protozoan infections, such as that from *Trypanosoma cruzi*, target the thymus and are able to cause its atrophy: Morrot et al. studied a murine model of Chagas disease and found that thymic expression of *Aire* and TsAg-encoding genes was preserved, albeit this condition was accompanied by early release of activated T lymphocytes into the periphery [118].

8. Conclusions and Future Remarks

It is definitively proven that the highest level of *AIRE* expression, as well as that of its murine homolog, is seen in a subset of mTECs. The low-level transcription in thymic DCs is presumably finalized to increase the availability of TsAgs to be presented to the autoreactive T-cell clones.

The detection of *AIRE* mRNA in nonlymphoid organs remains questionable and could be due to the presence, in bulk tissue samples, of few *AIRE*-expressing cells (for example, elements of the monocyte/DC lineage) ordinarily inhabiting the organs, or contaminating the preparations. However, a barely detectable *AIRE* mRNA does not imply appreciable levels of translation.

In searching for AIRE protein, greater accuracy comes from the use of monoclonal anti-AIRE Abs, especially if joined to methods, such as flow cytometry, able to improve the purity of the cell samples.

In any case, thymic localization of AIRE remains the most relevant to its function: a confirmation of the hypothesis that, by modulating thymic *AIRE* expression, we would be able to condition the susceptibility to autoimmune diseases, could delineate promising opportunities in the fight against autoimmunity. There is growing evidence that, as suggested by animal models, secondary lymphoid organs (lymph nodes and spleen) repropose in the periphery the mechanisms of central tolerance. This phenomenon needs better characterization, starting from the most accurate definition of *AIRE* and TsAg-encoding gene expression in the stromal cell lineages of the involved organs.

Conflict of Interests

The author declares that he has no conflict of interests.

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Research Article

Genetics of Myasthenia Gravis: A Case-Control Association Study in the Hellenic Population

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Myasthenia gravis (MG) is an heterogeneous autoimmune disease characterized by the production of autoantibodies against proteins of the postsynaptic membrane, in the neuromuscular junction. The contribution of genetic factors to MG susceptibility has been evaluated through family and twin studies however, the precise genetic background of the disease remains elusive. We conducted a case-control association study in 101 unrelated MG patients of Hellenic origin and 101 healthy volunteers in order to assess the involvement of common genetic variants in susceptibility to MG. We focused on three candidate genes which have been clearly associated with several autoimmune diseases, aiming to investigate their potential implication in MG pathogenesis. These are interferon regulatory factor 5 (IRF-5), TNFα-induced protein 3 (TNFAIP3), also known as A20, and interleukin-10 (IL-10), key molecules in the regulation of immune function. A statistical trend of association ($P = 0.068$) between *IL-10* promoter single nucleotide polymorphisms (SNPs) and the subgroups of early and late-onset MG patients was revealed. No statistically significant differences were observed in the rest of the variants examined. As far as we are aware, this is the first worldwide attempt to address the possible association between *IRF-5* and *TNFAIP3* common genetic variants and the genetic basis of MG.

1. Introduction

Myasthenia gravis (MG) is an organ-specific autoimmune disease caused by autoantibodies directed against proteins of the postsynaptic membrane leading to impaired neuromuscular transmission. Clinically, MG is characterized by muscle weakness and rapid fatigue aggravated by exercise and relieved by rest. The main autoantigen, in 80–90% of MG patients, is the muscle acetylcholine receptor (AChR), a pentameric channel which mediates synaptic transduction at the neuromuscular junction [1]. In several of the remaining MG patients, autoantibodies to muscle-specific tyrosine kinase (MuSK) [2] or to lipoprotein-related protein 4 (LRP4) [3] are detected. Both MuSK and LRP4 form a receptor complex which binds the extracellular matrix proteoglycan agrin, resulting in AChR clustering, critical for neuromuscular junction function.

Although MG is a disease that affects both sexes, at all ages and in all races [4], evidence from several epidemiological studies have showed a sex- and age-dependent bimodal distribution of incidence rate, with one peak in the second and third decades of life, observed mostly in women, and the second one in the sixth and seventh decades of life occurring mainly in men [5]. The above observation led to the classification of MG into early onset which appears before 50 years of age and is usually related to thymus hyperplasia and late-onset MG (>50 years) with normal or atrophic thymus.

The extent of genetic contribution to MG susceptibility has been evaluated through family [6–8] and twin studies [9], reflective of the disease's familial clustering and subsequently of genetic inheritance. High concordance rates of MG observed among monozygotic twins compared with dizygotic twins strongly suggest the involvement of genetic factors in the pathogenesis of MG [9]. Moreover, several studies have reported that MG patients may be affected with another autoimmune disease, most frequently, thyroid disorders and rheumatoid arthritis [10]. This finding leads to the hypothesis that a more generalized disturbance of the immunological function occurs.

The human leukocyte antigen (HLA) complex is the prominent genomic region implicated in MG onset. HLA-A1 and B8 alleles for class I and DR3 for class II constitute an ancestral haplotype termed "8.1" which has been reproducibly associated with early onset MG and thymic hyperplasia [11]. Further research geared towards the dissection of this extended A1-B8-DR3 haplotype, led to the identification of the MYAS1 locus, a region of 1.2 Mb encompassing 36 genes, at the boundary of class III and proximal class I region, thus, excluding the class II loci and confirming the predominant association of B8 allele over that of DR3 [12].

Apart from the HLA, a number of HLA-unlinked genetic loci have also been investigated regarding their involvement in MG susceptibility. These findings have mainly been derived by candidate gene studies, while the genes that were reported to be associated or not associated with MG are discussed in detail in [13].

Interferon (IFN) regulatory factor 5 (IRF-5) is a member of the IRF family of transcription factors. IRF-5 is activated by IFN-α/B and upregulates a set of proinflammatory cytokines, such as IL-6, TNF-α, and IL-12, while it further induces IFN gene expression. Results from several studies, reviewed in [14], have implicated *IRF-5* as a susceptibility gene in SLE. Search for common variants that influence IRF-5 levels led to the identification of SNP rs10954213 (c.*555G > A), located within the polyA+ signal sequence AATAAA in the 3′ UTR. The G allele disrupts the polyadenylation site and transcription is terminated far downstream, thus producing longer and less stable IRF-5 mRNA transcripts [15]. Further, a 30-bp in-frame insertion-deletion variant (rs60344245) in the sixth exon of *IRF-5* determines the formation of two families of protein isoforms which have differential ability to initiate transcription of IRF-5 target genes [16].

The TNFα-induced protein 3 (TNFAIP3), also known as A20, is a key molecule in the negative feedback regulation of NF-κB-dependent responses. The inhibitory effect of TNFAIP3 on NF-κB signaling is generated from the cooperative activity of its two ubiquitin-editing domains: the N-terminal ovarian tumor domain (OTU), responsible for deubiquitinating receptor interacting protein 1 (RIP1), an essential adaptor protein of the TNF-induced signaling pathway, and the C-terminal zinc finger-containing domain, which functions as an E3 ubiquitin ligase promoting the proteasomal degradation of RIP1 [17].

The SNP rs13207033 (g.137965418G > A), located at 6q23 intergenic region, approximately 185 kb upstream of *TNFAIP3*, probably affects gene expression by the presence of potential regulatory DNA elements [18]. Another study indicated that a nonsynonymous coding SNP (c.380T > G, rs2230926) resulting in a phenylalanine-to-cysteine change at residue 127 (p.F127C), in the OTU domain of the TNFAIP3 protein, is associated with SLE among individuals of European ancestry [19].

Interleukin-10 (IL-10) is a pleiotropic cytokine secreted by different cell types, such as T cells and myeloid lineage cells. IL-10 has been characterized as an anti-inflammatory cytokine due to its stimulatory effects on T_H2 cells [20] and to the simultaneous suppression of T_H1 cells [21]. Moreover, IL-10 induces proliferation and differentiation of activated B lymphocytes [21] leading to further activation of humoral response. In experimental autoimmune MG (EAMG), IL-10 administration caused the increase of anti-AChR antibody levels, suggesting a disease-enhancing role of IL-10 [22].

Several variants have been noticed in the 5′ flanking sequence of the human *IL-10* gene. Three SNPs, namely, rs45552637 (A/C), rs1800872 (T/C), and rs1800896 (A/G), located at positions −592, −819, and −1082, respectively, determine the formation of three haplotypes (GCC, ACC, and ATA). The position of these SNPs is based on the previously published sequence U16720, deposited in the EMBL-EBI database. A study by Turner and coworkers reported correlation of these haplotypes with IL-10 protein production *in vitro* [23]. Specifically, GCC/GCC genotype was associated with high concanavalin A-induced IL-10 production, GCC/ACC and GCC/ATA genotypes with medium and ACC/ACC, ATA/ATA, and ACC/ATA genotypes with low IL-10 production.

In the current study, a hypothesis-driven approach was adopted in order to assess the involvement of certain common variants in MG susceptibility. Thus, we conducted a candidate gene case-control study, focusing on genes with a critical role in immune system function, aiming to identify whether previously reported associations between the above genes and other autoimmune diseases could hold true for MG.

2. Materials and Methods

2.1. Study Population. A total of 101 unrelated MG patients, all of Hellenic descent, were enrolled in this study. Blood samples from MG patients were collected at the Hellenic Pasteur Institute during routine diagnostic survey. Only AChR-positive MG patients were included in our study. The diagnosis of MG was based on the presence of anti-AChR antibodies in the patient's serum, using a radioimmunoprecipitation assay (RIPA). We intentionally excluded from the genetic analysis those patients who were identified as positive for autoantibodies against MuSK, in order to reduce the heterogeneity of the study group. Although sera of MG patients were not analyzed for anti-LRP4 autoantibodies, the absence of this test raises no issue of heterogeneity in the study population, because of the rare coexistence of anti-LRP4 and anti-AChR antibodies. The main characteristics of the anti-AChR MG group (age at onset and gender) are summarized in Table 1. Written informed consent was obtained by patients. The control group consisted of 101 ethnically and sex-matched healthy individuals.

2.2. Genotyping. Genomic DNA from each individual was extracted from peripheral venous blood sample using the QIAamp Blood Midi kit (Qiagen GmbH, Hilden, Germany). Polymerase chain reactions (PCRs) were performed with the KAPA2G Fast HotStart ReadyMix kit (KAPABIOSYSTEMS, Woburn, MA, USA). Primer sequences are presented in the supplementary materials as Supplementary Table 1 (see Table 1 in Supplementary Material available online at

Table 1: Characteristics of the MG study group.

	Anti-AChR MG patients ($N = 101$)		
	Early onset ($n = 45$)	Late onset ($n = 44$)	Unknown age at onset ($n = 12$)
Gender (male/female)	8/37	27/17	6/6
Age at onset (mean ± SD)	32.8 ± 8.9	65.8 ± 8.6	—

SD: standard deviation.

doi:10.1155/2012/484919), whereas reaction conditions are available upon request.

Amplification by PCR and agarose gel electrophoresis analysis were used to genotype the 30 bp insertion/deletion variant of *IRF5*.

PCR-based restriction fragment length polymorphism (RFLP) assay was used for the detection of SNP rs2230926 (T > G) in *TNFAIP3*. The amplified fragments of 549 bp were digested with the restriction enzyme *Fnu4HI* (New England Biolabs, Ipswich, MA, USA) and were then analyzed by electrophoretic separation on 2% w/v agarose gel. The G allele creates an *Fnu4HI* restriction site, resulting in the digestion of amplicons to 319 and 230 bp fragments.

The identification of *IL-10* promoter SNPs genotypes was performed by direct DNA Sanger sequencing. A fragment of 585 bp, including all three variants, was amplified by PCR. The PCR products were purified by the column-based PureLink PCR Purification kit (Invitrogen, Carlsbad, CA, USA). The sequence of the 585 bp fragment was determined using the BigDye Terminator chemistry v3.0 kit on an Applied Biosystems 3730 × l DNA sequencer (Applied Biosystems, Carlsbad, CA, USA). The primers used were the same as those for the amplification of this region.

Both SNPs, rs10954213 (A/G) in the 3′ UTR of *IRF5* and rs13207033 (G/A) located at a 6q23 intergenic region, were genotyped by real-time PCR and high resolution melting curve (HRM) analysis on a RotorGene Q real-time cycler (Qiagen GmbH, Hilden, Germany). The amplification of the fragment containing the SNP of interest was carried out using the Type-it HRM PCR kit (Qiagen GmbH, Hilden, Germany), according to manufacturer instructions. During HRM, the temperature increases from 65 to 95°C, with a heating rate of 0.1°C/2 sec, leading to the denaturation of PCR products and the generation of melting curves, characteristic for each genotype. Since a single base-pair change causes a significant shift in the melting temperature (T_m), genotyping is based on the analysis of the melting profiles: homozygotes for the A allele exhibit similar melting profiles and with a lower T_m, compared with the G/G homozygotes, whereas heterozygotes are differentiated by a change in the shape of the melting curve.

No template controls were meticulously included in all genotyping processes. Negative and positive control samples were initially identified by DNA sequencing and were, subsequently, used in all genotyping methods. Each sample was tested in duplicate, except for those analyzed by PCR-RFLP and DNA sequencing.

2.3. Statistical Analysis. The differences in genotype distribution and allele frequencies between cases and controls were calculated by χ^2 analysis or Fisher's Exact test. P values less than 0.05 were regarded as statistically significant.

3. Results

Genotype distributions of all variants were consistent with Hardy-Weinberg equilibrium in both MG patient and control groups (data not shown).

The allele and genotype frequencies of the *IRF-5* rs60344245 variant showed an akin distribution in the screened groups of 101 MG patients and 100 controls ($P = 0.76$). Regarding the *IRF-5* rs10954213 SNP, A/G genotype was found to be somewhat more frequent in MG patients than in controls (54.8% versus 45.5%), but χ^2 analysis revealed no significant difference ($P = 0.3$). Allele and genotype frequencies of both *IRF-5* variants are shown in Table 2.

The *TNFAIP3* rs13207033 G/G genotype frequency showed an increase in healthy controls (44.6%) compared to MG patients (33.3%). However, statistical analysis did not indicate any significant difference between the two groups ($P = 0.17$). In the case of rs2230926 coding SNP, genotypes are distributed similarly in the 73 MG patients and 81 controls examined, as it is inferred by the P value = 0.74. Genotype frequencies of the rs2230926 variant, in both MG patients and controls, are in accordance with those derived from samples of European ancestry (CEU) that are part of the international HapMap project. In addition, our study group exhibited rs13207033-genotype frequencies which are comparable to the frequencies reported in European populations, in the dbSNP database. Genotyping results of the two *TNFAIP3* variants are summarized in Table 3.

Age of disease onset was also evaluated by dividing MG patients into early- and late-onset patients. No significant difference in genotype distribution was detected between the two subgroups and the control group (data not shown).

DNA sequence analysis of *IL-10* promoter region showed that the ACC/GCC genotype was the most frequently observed genotype in both MG patients and controls (23.72% and 28%, resp.), followed by the low secretion genotype ATA/ACC, which was detected in 21.62% of total MG and 18% of controls (Table 4). However, the current study did not reveal any statistically significant difference in *IL-10* genotype distribution between the complete cohort of MG patients (i.e., total MG) and the control group ($P = 0.7$). Comparison between the subsets according to age at onset demonstrated that the high IL-10 secretion GCC/GCC genotype is found in a low frequency in early-onset MG (4%), while it is overrepresented in late onset MG cases (20%) (Table 4). A statistical trend of association ($P = 0.068$)

Table 2: Genotype and allele distribution of IRF-5 variants in MG patients and controls. Statistical values calculated by χ^2 test are also shown.

IRF-5 variants	Control	MG	P_{value}
rs60344245	$N = 100$ (%)*	$N = 101$ (%)	
Genotypes			
insertion/insertion	28 (28.0)	24 (23.8)	
insertion/deletion	48 (48.0)	53 (52.4)	0.76
deletion/deletion	24 (24.0)	24 (23.8)	
Alleles			
insertion	104 (52.0)	101 (50.0)	0.76
deletion	96 (48.0)	101 (50.0)	
rs10954213	$N = 101$ (%)	$N = 84$ (%)*	
Genotypes			
A/A	37 (36.7)	29 (34.5)	
A/G	46 (45.5)	46 (54.8)	0.3
G/G	18 (17.8)	9 (10.7)	
Alleles			
A	120 (59.0)	104 (62.0)	0.7
G	82 (41.0)	64 (38.0)	

* Analysis was not successful for a subset of samples.

Table 3: TNFAIP3 genotype and allele frequencies in MG patients and controls. Statistical values calculated by χ^2 test are also shown.

TNFAIP3 variants	Control	MG	P_{value}
rs13207033	$N = 101$ (%)	$N = 93$ (%)*	
Genotypes			
G/G	45 (44.6)	31 (33.3)	
A/G	44 (43.5)	53 (57.0)	0.17
A/A	12 (11.9)	9 (9.7)	
Alleles			
G	134 (66.0)	115 (62.0)	0.41
A	68 (34.0)	71 (38.0)	
rs2230926	$N = 81$ (%)*	$N = 73$ (%)*	
Genotypes			
T/T	77 (95.1)	68 (93.2)	
T/G	4 (4.9)	5 (6.8)	0.74
G/G	0 (0.0)	0 (0.0)	
Alleles			
T	158 (98.0)	141 (97.0)	0.74
G	4 (2.0)	5 (3.0)	

* Analysis was not successful for a subset of samples.

between the IL-10 phenotype distribution and the two subgroups of early and late onset was revealed (Figure 1).

4. Discussion

MG is a heterogeneous autoimmune disease with a clear genetic predisposition. In addition to the HLA loci, several common variants in HLA-unlinked genes have been associated with MG susceptibility [13]. Many of these risk-associated genes are widely distributed among various autoimmune diseases, supporting the notion that autoimmune diseases are characterized by shared pathogenetic pathways. In this study, we performed a case-control association study in order to investigate the contribution of common variants located in *IRF-5*, *TNFAIP3*, and *IL-10* genes to MG susceptibility. These genes were considered as good candidates because of their critical role in the regulation of immune response and their previously known implication in the autoimmune process [24]. Only patients with anti-AChR antibodies in their serum were included in the genetic analysis, as it has been expected that they represent a more homogenous subset than the broader MG group. This subgroup was further divided into two distinct disease entities: early-onset MG patients, comprising mostly women,

Table 4: IL-10 genotype frequencies in the complete cohort of MG patients (i.e., total MG), the subgroups of early and late onset MG and controls.

Phenotype	Genotypes	Total MG $N = 97$ (%)[a]	Early onset $N = 45$ (%)[b]	Late onset $N = 44$ (%)[b]	Control $N = 100$ (%)[a]
High IL-10 expression	GCC/GCC	11 (11.32)	2 (4.0)	9 (20.0)	13 (13.0)
Medium IL-10 expression	ACC/GCC	23 (23.72)	12 (27.0)	10 (23.0)	28 (28.0)
	ATA/GCC	24 (24.72)	13 (29.0)	9 (20.0)	17 (17.0)
Low IL-10 expression	ACC/ACC	12 (12.42)	5 (11.0)	6 (14.0)	15 (15.0)
	ATA/ATA	6 (6.2)	4 (9.0)	2 (5.0)	9 (9.0)
	ATA/ACC	21 (21.62)	9 (20.0)	8 (18.0)	18 (18.0)

[a]Analysis was not successful for a subset of samples.
[b]Eight MG samples were of unknown age at onset.

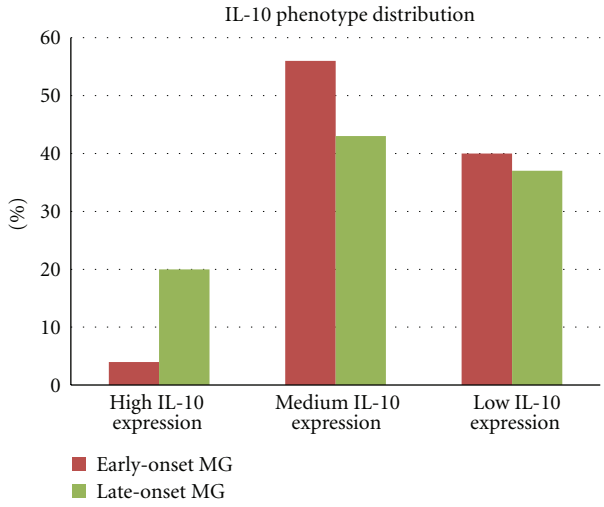

Figure 1: *IL-10* phenotype distribution in the subgroups of early- and late-onset MG patients.

and late-onset MG patients, showing a higher proportion of men.

As far as we are aware, this is the first study, in any population, to investigate the association between MG and common variants of *IRF-5* and *TNFAIP3* genes. According to previous studies, reviewed in [14], several variants in the *IRF-5* locus have been reproducibly associated with SLE implicating *IRF-5* as a susceptibility gene in lupus. The rs10954213 SNP has been found to influence mRNA polyadenylation, thus, impairing the levels of IRF-5 protein; A/A homozygotes express approximately 5-fold higher levels of immunoreactive IRF-5 compared to the G/G homozygotes [25]. As for the rs60344245 variant, the deletion of 30 bp (GGCCGCCTACTCTGCAGCCGCCCACTCTGC/−) removes 10 amino acids from the IRF-5 protein and alters a proline-, glutamic acid-, serine-, and threonine-rich (PEST) domain. In the IRF family of proteins, such domains participate in protein interactions [26] and they also cause rapid proteolytic degradation [27]. Despite their obvious functional role, the current study failed to demonstrate a significant association of the *IRF-5* rs10954213 and rs60344245 variants with MG ($P = 0.3$ and $P = 0.76$, resp.), suggesting that *IRF-5* may not be involved in MG pathogenesis.

Furthermore, recent findings from GWA studies have revealed significant associations between variants in the human *TNFAIP3* locus and a wide spectrum of autoimmune diseases. A GWA scan of rheumatoid arthritis patients, with anticitrullinated peptide antibodies, detected strong evidence of association of the rs13207033 SNP with the development of RA [28]. Similarly, a study by Musone and coworkers reported the association of the nonsynonymous coding SNP, rs2230926 with SLE [19]. Functional studies to determine the biological impact of rs2230926 demonstrated that the minor Cys127 protein shows a decreased inhibitory activity [19]. Yet, lack of association was observed between MG and *TNFAIP3* rs13207033 ($P = 0.17$) and rs2230926 ($P = 0.74$) SNPs.

Altogether, our data may indicate that the organ-specific MG might have a different genetic background leading to its separation from a wide cluster of systemic autoimmune diseases comprising SLE and RA [29]. An alternative explanation for the lack of association in our study could be related to the insufficient statistical power owing to relatively small sample sizes. As it is generally known, common variants account for a modest proportion of the genetic risk regarding the autoimmune diseases. In such cases, thousands of samples may be required in order to detect an association signal that can be distinguished from the background noise [30, 31]. However, in low prevalence diseases, such as MG, the recruitment of large sample sizes is very difficult. It is worth mentioning that the diagnostic unit of MG in the Hellenic Pasteur Institute is the only unit in Greece which has systematically received and analyzed blood samples since 1983. Therefore, our collection of MG DNA samples is currently the largest in Greece and it is constantly enriched by new cases.

Moreover, despite the specific selection of anti-AChR patients and their division into early and late onset, a further classification according to the thymus anomalies (thymoma or hyperplasia) could have been informative; however, histological data were not available.

Finally, an unexpected result was the lack of association of the *IL-10* promoter SNPs with MG. Since these variants are presumed to lie within the IL-10 promoter region, they may affect the binding of transcription factors that regulate IL-10 expression. A recent study by Alseth and coworkers, conducted on the Norwegian population, revealed

an association of ACC/ACC genotype with the subgroup of titin antibodies-positive MG patients, while a statistically significant increased ATA/ATA frequency was observed in early-onset MG patients [32]. In our group of Hellenic MG cases, no evidence of association was detected, when we compared the genotype distribution between the complete cohort of MG patients and the control group ($P = 0.7$). However, GCC/GCC genotype revealed a statistical trend of association with MG, in the distinct subgroups of early and late-onset MG patients ($P = 0.068$). Thus, further studies in larger sample sizes could uncover possible associations of MG with IL-10. It is also noteworthy the fact that allele frequencies for a given SNP may vary substantially across ethnic groups. The difference noticed in the frequency of the ACC/ACC genotype between the Norwegian and Hellenic control groups (3.4% versus 15%) is reflective of this condition.

Overall, this has been the first effort, to our knowledge, to address the possible association between common genetic variants of *IRF-5* and *TNFAIP3* and the genetic basis of MG, in any population, whereas further studies are needed to unravel the, yet largely unknown, genetic background of MG.

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Clinical Study

DNA Methyltransferase 3B Gene Promoter and Interleukin-1 Receptor Antagonist Polymorphisms in Childhood Immune Thrombocytopenia

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Primary immune thrombocytopenia (ITP) is one of the most common blood diseases as well as the commonest acquired bleeding disorder in childhood. Although the etiology of ITP is unclear, in the pathogenesis of the disease, both environmental and genetic factors including polymorphisms of TNF-a, IL-10, and IL-4 genes have been suggested to be involved. In this study, we investigated the rs2424913 single-nucleotide polymorphism (SNP) (C46359T) in DNA methyltransferase 3B (*DNMT3B*) gene promoter and the VNTR polymorphism of IL-1 receptor antagonist (*IL-1 Ra*) intron-2 in 32 children (17 boys) with the diagnosis of ITP and 64 healthy individuals. No significant differences were found in the genotype distribution of *DNMT3B* polymorphism between the children with ITP and the control group, whereas the frequency of allele T appeared significantly increased in children with ITP ($P = 0.03$, OR = 2, 95% CI: 1.06–3.94). In case of *IL-1 Ra* polymorphism, children with ITP had a significantly higher frequency of genotype I/II, compared to control group ($P = 0.043$, OR = 2.60, 95% CI: 1.02–6.50). Moreover, genotype I/I as well as allele I was overrepresented in the control group, suggesting that allele I may have a decreased risk for development of ITP. Our findings suggest that rs2424913 *DNMT3B* SNP as well as *IL-1 Ra* VNTR polymorphism may contribute to the susceptibility to ITP.

1. Introduction

Primary immune thrombocytopenia, commonly referred to as idiopathic thrombocytopenic purpura (ITP), is one of the most common blood diseases as well as the commonest acquired bleeding disorder in childhood. The affected children are young and previously healthy, and they typically present with a sudden onset of petechiae or purpura 2-3 weeks after a viral infection or immunization. Complete remission occurs in at least 2/3 of cases within 6 months of initial diagnosis [1, 2]. ITP is pathophysiologically characterized by a low circulating platelet count due to the production of autoantibodies against platelet glycoproteins, especially against $GPII_b/III_a$ and I_b/IX, followed by their destruction via the reticuloendothelial system [3, 4]. Although the development of autoantibodies by B cells remains central in the pathophysiology of ITP, a multidysfunction in cellular immunity and cytokine response may take place in the pathogenetic mechanisms of the disease [5–7].

Currently, it is generally accepted that both environmental and genetic factors are involved in the pathogenesis of ITP and, especially, interactions between genetic and epigenetic changes. Among the genetic factors, polymorphisms of inflammatory cytokine genes have been related with ITP [8–10]. In a study by Foster et al. [11], polymorphisms in Fc gamma receptors genes (*FCGR3A* and *FCGR3B*) and tumor necrosis factor-a (*TNF-a*) and lymphotoxin-a (*LTA*) genes were found to be associated with chronic childhood ITP. Satoh et al. [12] observed also an association between a polymorphism in *TNF-β* gene and chronic ITP in adults. In addition, Wu et al. [13, 14] found that IL-4 intron 3 and

IL-10 polymorphisms may contribute to childhood chronic ITP, while IL-1 Ra but not IL-1β exon 5 polymorphism is associated with childhood ITP.

Apart from the polymorphisms of inflammatory cytokine genes, environmentally induced epigenetic changes in gene expression have recently become a new field of research, and a lot of studies investigate the role of these changes in the loss of self-tolerance and the development of autoimmune diseases [15, 16]. Epigenetic mechanisms play an essential role in gene regulation by modifying chromatin structure, which in turn modulates gene expression. DNA methylation is a major epigenetic modification involving the addition of a methyl group to the 5′ position of a cytosine in a CpG dinucleotide and is catalyzed by DNA methyltransferases [17]. The family of DNA methyltransferases (DNMTs) consists of four independent methyltransferases, each of them playing a different functional role [18–20]. DNA methylation changes and DNMTs gene polymorphisms have been detected in several diseases, particularly cancer [21, 22]. DNMT3B promoter polymorphism has been reported to be associated with the risk of lung, colorectal, and head and neck cancers [23–26]. However, there is little information about the role of DNMTs polymorphisms in the development of autoimmune diseases [27, 28].

In the present study, we investigated the association of the rs2424913 single-nucleotide polymorphism (SNP) (C46359T) located into DNA methyltransferase 3B (*DNMT3B*) gene promoter and a VNTR polymorphism of IL-1 receptor antagonist (*IL-1 Ra*) intron-2 with an increased risk of ITP in children, in an attempt to elucidate the role of genetic and epigenetic mechanisms in the pathogenesis of such an autoimmune disease.

2. Patients and Methods

2.1. Patients and Control Subjects. The study group consisted of 32 children (17 boys and 15 girls) from unrelated families living in Crete, aged 7 months to 14 years, diagnosed with ITP, and hospitalized at the Department of Pediatric Hematology-Oncology of the University Hospital of Crete. The control group consisted of 64 individuals, sex and ethnically matched who had no history of autoimmune or other chronic diseases. The diagnosis of ITP had been made in all children based on history, physical examination, complete blood count, and examination of the peripheral smear, which should exclude other causes of thrombocytopenia. Bone marrow aspiration was performed, when necessary, to rule out other diseases. Ethnic bias within the population studied was minimized by excluding patients that were not of Cretan origin. Parents were informed that cells from the bone marrow would be used for *in vitro* research. The study had the University Hospital of Heraklion Ethics Committee approval.

2.2. DNA Extraction and Analysis of the DNMT3B and IL-1 Ra Gene Polymorphisms. Whole blood was collected in EDTA-containing tubes, and genomic DNA was extracted from the peripheral blood samples using DNA purification kit: Wizard Genomic (Promega, USA) according to the manufacturer's instruction. In the study group, the genomic DNA was extracted from bone marrow mononuclear cells (BMMNCs) using the same kit. The extracted DNA was stored at −20°C to be used for the genotyping.

The subjects enrolled in this study were genotyped using the polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) RFLPs method. In brief, the upstream primer 5′-TGCTGTGACAGGCAGAGCAG-3′ and the downstream primer 5′-GGTAGCCGGGAACTCCAC-GG-3′ were used to generate a region of 380 bp of the promoter of *DNMT3B* (Table 1). The amplification was carried out by using Taq polymerase provided by Invitrogen. An initial heating step at 95°C for 5 min was used, followed by 35 cycles of denaturing (at 95°C for 30 sec), annealing (65°C for 30 sec), and chain extension (72°C for 30 sec), followed by a final extension step at 72°C for 5 min. The PCR products were digested for 3 hrs at 37°C with 5 U *Avr*II (Fermentas), which digests the DNA amplified by the T allele into two bands of 207 bp and 173 bp. In contrast, fragments carrying the major C allele lacked the *Avr*II restriction site. Both undigested and digested PCR products were analyzed through electrophoresis on 2% agarose gel and visualized (with ethidium bromide staining) under ultraviolet (UV) light in reference to a molecular weight marker.

Similarly, the upstream 5′-CTCAGCAACACTCCTAT-3′ and the downstream 5′-TCCTGGTCTGCAGGTAA-3′ primers were used to generate the IL-1 Ra region harboring the 86-bp repeats (VNTR). The amplification was performed by using 2,5 U Taq polymerase (Invitrogen). An initial denaturation step at 95°C for 5 min was used, followed by 35 cycles of denaturation (at 95°C for 30 sec), annealing (58°C for 30 sec), and chain extension (72°C for 30 sec), and a final elongation step at 72°C for 5 min. PCR products were directly analyzed by electrophoresis on 2% agarose gel and visualized upon staining with ethidium bromide. Genotypes were scored blindly, and analysis of all ambiguous samples was repeated. Moreover, 10% of the samples were amplified twice for checking the accuracy of the results.

2.3. Statistical Analysis. Statistical analysis was performed using the GraphPad Prism statistical software method (GraphPad Software Inc., La Jolla, CA, USA). The distribution of the genotypes and alleles in the group of patients was compared to that of control group using the chi-squared test and Fischer's exact test where necessary, which was also used to determine whether the observed genotype frequencies conformed to Hardy-Weinberg expectations. The level of significance was set to 0.05. The association between polymorphisms and the risk of development of ITP was estimated by odds ratio (OR) and the 95% confidence intervals (CIs).

3. Results

3.1. Analysis of rs2424913 (DNMT3B) Polymorphism. The distribution of genotype and allele frequencies of rs2424913 *DNMT3B* SNP in 32 children with ITP and control group

Table 1: Allele types, PCR conditions, and PCR primers designed to amplify fragments harboring the polymorphic sites.

	DNMT3B promoter	IL-1 Ra
Type of polymorphism	Single-base C/T	86-bp VNTR
Site of polymorphism	Position 149	Intron 2
PCR primers		
Upstream	5′-TGCTGTGACAGGCAGAGCAG-3′	5′-CTCAGCAACACTCCTAT-3′
Downstream	5′-GGTAGCCGGGAACTCCACGG-3′	5′-TCCTGGTCTGCAGGTAA-3′
Digestion	*Avr*II	—
Allele size (bp)	C: 380	I: 410 II: 240
	T: 207 + 173	III: 325 IV: 500

Table 2: Distribution of rs2424913 *DNMT3B* allele and genotype frequencies in children with ITP and controls.

	ITP children $n = 32$ (%)	Controls $n = 64$ (%)	OR	95% CI	P value
Genotype frequency					0.07
C/C	12 (37.5)	37 (57.8)			
C/T	16 (50)	25 (39)	0.5	0.2–1.25	0.17
T/T	4 (12.5)	2 (3.2)	0.16	0.03–1.0	0.053
Allelic frequency					0.03*
Allele C	40 (62.5)	99 (77.34)			
Allele T	24 (37.5)	29 (22.66)	2	1.06–3.94	

*$P < 0.05$.

is presented in Table 2. Notably, no significant differences were found in the genotype distribution between the children with ITP and the control group. However, a significant difference between children with ITP and control group in allele frequencies has been observed. The frequency of allele T appeared significantly increased in children with ITP ($P = 0.03$, OR = 2, 95% CI: 1.06–3.94), thus indicating an apparent association between this allele and ITP in patients of Cretan origin.

3.2. Analysis of IL-1 Ra VNTR Polymorphism. In case of *IL-1 Ra* polymorphism, although we found four different alleles, we focused on alleles I and II and genotypes I/I, I/II, and II/II because of their higher prevalence. The genotype and allelic distribution of IL-1 Ra among children with ITP and the control group is presented in Table 3. A statistically significant difference was observed in the allele frequencies of *IL-1 Ra* between the two groups ($P = 0.042$). Children with ITP had a significantly higher frequency of genotype I/II, compared to control group (43.75% versus 23.44%, $P = 0.043$, OR = 2.60, 95% CI: 1.02–6.50). Moreover, genotype I/I as well as allele I was overrepresented in the control group (68.75 versus 50% and 84.43 versus 71.9%), suggesting that allele I may have a decreased risk for development of ITP, whereas the presence of allele II seems to increase 2.12 times the relative risk for disease development (OR = 2.12, 95% CI: 1.02–4.41).

4. Discussion

Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disease characterized mainly by the destruction of autoantibody-mediated platelets. Despite extensive research efforts during last years, the genetic basis of ITP remains largely unknown. In this study, by performing a case-control association study, we have investigated the possible association of the rs2424913 *DNMT3B* (C46359T) SNP and an *IL-1 Ra* VNTR polymorphism with susceptibility to ITP.

Epigenetic gene regulation has an essential role in determining individual gene function and activity. Epigenetic alterations lead to gene malfunction in a pathological context [15]. DNA methylation is a major epigenetic mechanism, which maintains chromosomal stability and regulates gene expression. It has been reported that DNA methylation plays a significant role in the development and progression of various cancers [21]. DNMT3B, analyzed in the present study, has been demonstrated to play important roles in tumorigenesis [26, 29] due to its ability to mediate de novo DNA methylation, which in turn might silence tumor suppressor gene expression through promoter hypermethylation [30]. In addition, there is an increasing interest in the role of epigenetic alterations in the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [31]. Of note, recent studies demonstrated that DNA hypomethylation has been

Table 3: Distribution of allele and genotype frequencies of IL-1 Ra VNTR polymorphism in children with ITP and controls.

	ITP children $n = 32$ (%)	Controls $n = 64$ (%)	OR	95% CI	P value
Genotype frequency					0.295
I/I	16 (50)	44 (68.75)	0.40[#]	0.15–0.98	
I/II	14 (43.75)	15 (23.44)	2.54[§] 2.60[#]	1.03–6.30 1.02–6.50	0.043*[#]
II/II	2 (6.25)	2 (3.13)			
III/III	0 (0)	0 (0)			
I/III	0 (0)	1 (1.56)			
I/IV	0 (0)	1 (1.56)			
II/IV	0 (0)	1 (1.56)			
Allelic frequency[†]					0.042*
Allele I	46 (71.9)	103 (84.43)	0.47	0.23–0.98	
Allele II	18 (28.1)	19 (15.57)	2.12	1.02–4.41	

*$P < 0.05$.
[†]Alleles from genotypes I/I, I/II, and II/II.
[#]Genotype I/I versus genotype I/II.
[§]Genotype I/II versus all genotypes.

implicated in the pathogenesis of SLE. Moreover, DNA methylation inhibitors are known to induce autoreactivity *in vitro* and the development of lupus-like syndrome *in vivo* [17, 32, 33]. There are only a few studies investigating the role of DNA methylation in the pathogenesis of ITP. Chen et al. [34] showed that there was no association between the *DNMT3B* promoter polymorphism and the susceptibility to ITP in Chinese population. However, another study reported that DNMT3A and DNMT3B mRNA expressions were significantly lower in ITP patients than in healthy controls, suggesting that aberrant DNA methylation patterns are possibly involved in the pathogenesis of ITP [35]. In the present study, we investigated the association between the rs2424913 *DNMT3B* SNP and the risk of ITP, but no significant differences were found in the genotype distribution between the children with ITP and the controls. However, we found a very low frequency of T/T genotype in our population, whereas in the Chinese population, there was found a distinct prevalence of the T/T genotype and absence of C/C genotype [34]. This finding pinpoints the importance of the racial origin in this type of studies, thus implying probably the different methylation status in different races. Moreover, we observed a significant difference in the allele distribution between children with ITP and controls. The presence of T allele seems to increase the relative risk for disease development. Altogether, our findings suggest that rs2424913 *DNMT3B* promoter SNP may be implicated to the pathogenesis of ITP. The *DNMT3B* C-to-T transition polymorphism (C46359T) examined, in *in vitro* assays, confers a 30% increase in promoter activity [23]. Although the mechanism of this association is unknown, it can be assumed that the "T" variant, by upregulating *DNMT3B* expression, may result in an aberrant *de novo* methylation of CpG islands in autoimmunity-mediating genes, thus leading to the development of ITP. However, the role of this gene in ITP seems to be a real "enigma" given that conflicting data have been presented so far. Thus, an aberrant DNA methylation status reflected by increased plasma SAH concentration and decreased expression levels of DNMT3A and 3B has been found in ITP [35], a situation that may play a crucial role in the pathophysiology of the disease. However, DNA hypomethylation (as demonstrated in the case of SLE) was found to induce autoreactivity *in vitro*. It is also possible that the "T" allele may be in linkage disequilibrium with other susceptibility loci. Altogether, the precise mechanism by which altered DNA methylation patterns induce ITP needs to be studied globally in the view of the concerted action of DNMT3A and DNMT3B, which results in a change of DNA methylation equilibrium in ITP patients.

As referred above, the pathogenesis of ITP is complicated with cellular immunity and cytokine response playing crucial roles in the pathogenesis [6, 7]. Abnormal serum cytokines levels have been reported in ITP patients [8]. The cytokine genes are polymorphic, which accounts for the different levels of cytokine production. A lot of studies have investigated so far the association between cytokine gene polymorphisms and different immunoinflammatory diseases [11, 36, 37]. IL-1 Ra, a major member of the IL-1 family (consisting of 11 members in total), is a natural anti-inflammatory molecule that neutralizes the effects of IL-1. The balance between IL-1 and IL-1 Ra is important in maintaining the homeostasis of immune system. As a result, *IL-1 Ra* polymorphisms may lead to changes in this IL-1 and IL-1 Ra balance and be associated with susceptibility of a variety of autoimmune diseases, such as rheumatoid arthritis, SLE, and ankylosing spondylitis [38–43]. There is only one study in the literature, which examined the *IL-1 Ra* polymorphism in Chinese children with ITP so far [14]. In the present study, we investigated the association between *IL-1 Ra* polymorphism and the susceptibility of ITP, and we found that *IL-1 Ra* polymorphism is associated with childhood ITP. The genotype I/II was more frequently detected in children with ITP than in controls. More specifically, we found that the presence of allele II seems to increase 2.12 times the risk for development

of ITP, thus assuming that *IL-1 Ra* polymorphism may be involved in the pathogenesis of ITP. The polymorphism under investigation is caused by the variable copy number of an 86-bp sequence, and the repeat region contains three potential protein-binding sites. Therefore, the variable copy number may have functional significance. Furthermore, allele II has been reported to be associated with more severe clinical outcome in several inflammatory diseases, including systemic lupus erythematosus [41], rheumatoid arthritis, and ulcerative colitis [44]. An increased frequency of allele II has also been described in diabetes patients with nephropathy [45]. The induction of IL-1Ra by IL-1beta is an important counterregulatory mechanism and may at least partially account for the increased IL-1Ra levels found in the carriers of allele II [46, 47]. Obviously, the IL-1Ra concentrations in ITP patients can be assessed in future experiments and, if they will be found decreased, then it may suggest a deficiency of this regulatory mechanism that may be particularly pronounced in allele II carriers, thus explaining the higher incidence of ITP.

A definite advantage of our study, particularly with respect to other association studies, was the attention paid on the selection of a genetically and ethnically homogeneous patient's cohort and control group. As a consequence, the results of this study are unlikely to be biased by sampling. Given that the incidence of pediatric ITP is on the order of 4–6 cases/100,000 population annually, it is extremely difficult to collect easily more patients of Cretan origin. Crete (situated 25°E and 35°N) is the largest island of Greece, with about 0.65 million inhabitants who share the same genetic and cultural background and a common environment. A possible weakness of our study deals with the limited sample size, a fact that is difficult to be overcome easily in a geographically isolated region.

In conclusion, our results provide evidence that rs2424913 *DNMT3B* SNP as well as the *IL-1 Ra* VNTR polymorphism may contribute to the susceptibility to ITP. However, it is in our short-term plans to collect and genotype samples from other geographical areas of Greece despite the substantial differences that may appear in the genetic background of subjects from the mainland Greece (due to increased migration or entrance of genetic material from the neighboring Balkan or west European countries in the gene pool of these Greek cohorts). In addition, further studies are needed in order to determine the functional role of the polymorphisms under study, aiming to gain insight regarding the mechanism(s) leading to ITP.

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Research Article

Mitochondrial Mutations are Associated with Atherosclerotic Lesions in the Human Aorta

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Somatic mutations of the human mitochondrial genome can be a possible determinant of atherosclerosis. To test this possibility, forty mitochondrial mutations were analyzed in the present study in order to see which of these mutations might be associated with atherosclerosis. Ten mitochondrial mutations belonging to mitochondrial genes MT-RNR1 (rRNA 12S); MT-TL1 (tRNA-Leu, recognizes UUR); MT-TL2 (tRNA-Leu, recognizes CUN); MT-ND1, MT-ND2, MT-ND5, and MT-ND6 (subunits 1, 2, 5, and 6, respectively, of NADH dehydrogenase); and MT-CYB (cytochrome b) were potentially associated with atherosclerosis. From 29% (2 of 7 aortic samples) upto 86% (6 of 7 aortic samples) of aortic samples had a significant difference between atherosclerotic plaques and unaffected tissue, with the respect to the level of heteroplasmy for each mutation. Further, the homogenates of affected and normal intimae of 22 aortas were compared to reveal the average level of heteroplasmy for the above-mentioned 10 mutations. For five mutations, the mean level of heteroplasmy was significantly different in atherosclerotic intimal homogenates in comparison with the unaffected tissue. These mutations were A1555G, C3256T, T3336C, G13513A, and G15059A. Thus, it was demonstrated that at least five mitochondrial mutations occurring in MT-RNR1, MT-TL1, MT-ND2, MT-ND5, and MT-CYB genes are associated with atherosclerosis.

1. Introduction

Atherosclerosis underlies the development of most cardiovascular diseases, which are the leading cause of death in the 21st century. The mechanisms involved in the development of atherosclerosis have been intensively studied and various mechanisms and factors responsible for atherosclerotic alteration of the arterial intima have been suggested. Accumulating evidence supports an autoimmune mechanism as one of the prime pathogenic processes involved in the development of atherosclerosis [1–4].

Recently we suggested that somatic mutations within the mitochondrial genome may be a probable cause of atherosclerosis development in humans [5]. In humans, the mitochondrial DNA (mtDNA) spans 16,569 DNA base pairs and is represented by a two-stranded circular molecule containing 37 genes. The two strands of mtDNA are differentiated by their nucleotide content, with the guanine-rich strand referred to as the heavy strand, and the cytosine-rich strand referred to as the light strand. The heavy strand encodes 28 genes, and the light strand encodes 9 genes. Of a total of 37 genes, 13 genes encode proteins (polypeptides), 22 genes encode transfer RNAs (tRNAs), and 2 genes encode the small and large subunits of ribosomal RNA (rRNA). Among the proteins, the subunits of complexes of a respiratory chain (cytochrome b, ATP synthase, cytochrome c oxidase, and NADH dehydrogenase) are encoded with mtDNA. Each mitochondrion contains several copies of its genome. Mitochondrial DNA is particularly susceptible to reactive oxygen species generated by the respiratory chain due to their

close proximity. Though mtDNA is packaged by proteins and harbors significant DNA repair capacity, these protective functions are less robust than those functions operating on nuclear DNA and therefore are thought to contribute to the enhanced susceptibility of mtDNA to oxidative damage. In some cases, mtDNA mutations can cause maternally inherited diseases, and some evidence suggests that they may be major contributors to the aging process and age-associated pathologies. Mutations of mitochondrial DNA can lead to a number of illnesses, including exercise intolerance and Kearns-Sayre syndrome, which causes the loss of full function of the heart, eye, and muscle.

The penetrance and expression of mitochondrial mutations vary greatly between relatives and depend mainly on a genotype and the level of heteroplasmy (a mixture of mutant and normal molecules of DNA). Therefore, both a qualitative and a quantitative evaluation of a mutant allele of mitochondrial genome are necessary for studying the association of mitochondrial mutations with human diseases.

Studying associations between somatic mitochondrial mutations and focal development of atherosclerotic lesions in the intimal layer of human arteries is of high theoretical and practical impact. Such mutations may result in defects in the protein chains of respiratory enzymes and tRNAs that are synthesized in mitochondria, therefore producing oxidative stress and increasing the probability of plaque formation. However, the physical association of mitochondrial mutations with atherosclerotic lesions remains obscure. The present study was undertaken to test the hypothesis that several mitochondrial mutations can be associated with atherosclerotic lesions and, therefore, help explain the focal and mosaic nature of atherosclerosis development.

2. Methods

Thoracic aorta samples were collected 1.5 to 3 h after sudden death at the autopsy from 22 males and females aged between 23 and 70 years. The study was carried out in accordance with the principles outlined in the Helsinki Declaration of 1975, as revised in 1983. The protocol was approved by the ethics committee of the Russian Cardiology Research and Production Complex and by the ethics committee of the Institute of Atherosclerosis Research, Moscow.

The vessels were opened longitudinally and washed with phosphate-buffered saline (PBS), pH 7.6. The grossly normal parts of the arteries and those regions with atherosclerotic lesions were identified macroscopically and classified according to the classification of the Atherosclerosis Council of the American Heart Association [6, 7] utilizing the corresponding histological evaluations. Unaffected areas were defined as tissue samples with smooth luminal surfaces. Zones with initial atherosclerotic changes (type I lesions) corresponded to the parts of arteries with a smooth yellowish surface with occasional small yellow spots. Small aggregates of extracellular lipid droplets were present in the connective tissue matrix. According to the histology, apart from resident cells, the initial lesion foci were characterized by an increased number of mononuclear cells, in contrast to the visually intact intima. Fatty streaks (type II lesions) were defined as yellow strips and spots that slightly protruded over the vessel surface, often merging into larger structures and forming lesion clusters. In tissue sections, the presence of lipids was identified inside both macrophage-like cells and smooth muscle cells. The connective tissue matrix also contained extracellular lipids. Lipofibrous plaques (type Va lesions) were defined as spherical or elliptic protrusions of yellowish or nacreous color. Microscopically, they included accumulated intracellular lipids and increased amounts of extracellular matrix. Lipofibrous plaques contained a bulky necrotic core covered by a connective tissue layer and also included zones that morphologically resembling fatty streaks. Fibrous plaques (type Vc lesions) were defined as considerably protruding, rounded, or oval, and pearl-colored formations. They were mostly composed of a crude connective tissue matrix with embedded cells. The lipid component was rare.

All analyzed 22 autopsy samples contained unaffected (nonatherosclerotic) zones which were estimated to constitute 10% to 45% of the luminal surface. All samples had zones with initial lesions and fatty streaks as well. Lipofibrous plaques were present in 12 aortic samples (55% cases) and occupied from 10% to 25% of luminal surface in these samples. Fibrous plaques were present only in 4 aortic samples (18% cases) and occupied from 3% to 12% of the luminal surface. Such a pattern of the distribution of atherosclerotic lesions throughout the luminal surface made it impossible, due to the low statistical power, to carry out an analysis of relation of heteroplasmy levels to the severity of atherosclerosis.

Homogenates of the affected (i.e., containing any above-mentioned lesion type or their combination) and normal intimae were compared to reveal an average level of heteroplasmy. To do this, all histologically verified segments of atherosclerotic intimae or unaffected regions were combined and homogenized, and after careful stirring, 10 μg of tissue was taken for DNA extraction.

DNA samples were obtained using commercially available kits for DNA extraction (BioRad, UK). For the amplification of fragments of mitochondrial DNA by polymerase chain reaction (PCR) method followed by pyrosequencing, the primers and conditions described elsewhere were used [7–25]. To quantitatively evaluate mutant alleles, a method of pyrosequencing [26–28] was adapted for conditions where both normal and mutant alleles were present in a biological specimen [25]. Briefly, the defective allele was quantified by analyzing the peak heights in the pyrogram of one-chained PCR fragments of a mitochondrial genome. The percent of heteroplasmy in DNA sample was calculated for each mutation, taking into account the expected sequence and the dimension of peaks for the homozygotes possessing either 100% of the normal or 100% of the mutant allele, as described elsewhere [25].

Statistical analysis was performed using SPSS v. 14 (SPSS Inc., USA). Wilcoxon statistics and frequency analysis were used for comparisons. The significance of differences was defined at a 0.05 confidence level.

3. Results

In this study we analyzed 40 mitochondrial mutations previously detected in such pathologies as coronary stenosis, some forms of diabetes, deafness, cardiac infarction, cardiomyopathy and stroke to reveal mutations associated with atherosclerosis [6–23]. At the first stage of this study we have analyzed DNA samples from segments of tissue from lipofibrous plaques and unaffected intimae of seven aortas. Thirty of analyzed mutations showed no difference in the level of heteroplasmy between atherosclerotic and normal tissues within the same aortic specimens.

Ten mitochondrial mutations belonging to the following genes: MT-RNR1 (rRNA 12S); MT-TL1 (tRNA-Leu, which recognizes UUR); MT-TL2 (tRNA-Leu, which recognizes CUN); MT-ND1, MT-ND2, MT-ND5, and MT-ND6 (resp., subunits 1, 2, 5, and 6 of NADH dehydrogenase); mt-CYB (cytochrome b) were identified, which were unevenly distributed in aortic tissue, as from 43% (3 of 7) to 100% (7 of 7) aortic samples differed in the level of heteroplasmy for these mutations between atherosclerotic and normal tissues (data not shown).

Moreover, these mutations also appeared to be associated with atherosclerotic lesions because from 29% (2 of 7) upto 86% (6 of 7), aortic samples had a significant difference in the level of heteroplasmy for the given mutations in lipofibrous plaques in comparison with normal (unaffected) intimae.

The demonstrated uneven distribution of mutations within aortic sample taken from single autopsy material could produce erroneous conclusion on the association of those mutations with atherosclerotic lesions due to random selection of tissue samples for mtDNA isolation. Therefore, further experiments compared PCR fragments of DNA extracted from the whole homogenates of the affected and normal intimae of all 22 aortas, focusing on the 10 mutations identified at the above stage of the study. Among these mutations, the level of heteroplasmy differed significantly in homogenates of affected and normal intimae for five of the mutations. These were single nucleotide substitutions A/G at position 1555, C/T at position 3256, T/C at position 3336, G/A at position 13513, and G/A at position 15059 (Table 1). The differences in the level of heteroplasmy did not reach statistical significance for nucleotide substitutions G/A at position 12315 and G/A at position 14459. Finally, there were no statistical difference in the level of heteroplasmy for mutations C/A at position 5178, G/A at position 14846, and InsG at position 652. The sample size ($n = 22$) was insufficient to provide valid examination of effects of confounding factors such as age, diabetes, and hypertension. However, regression and correlation analyses have been performed and showed that none of confounding factors possessed an explanatory value for heteroplasmy levels in the given data set.

Significant correlations were revealed between the levels of heteroplasmy for A1555G and C3256T ($r = 0.365$; $P = 0.015$), A1555G and T333C6 ($r = 0.417$; $P = 0.005$), A1555G and G15059A ($r = 0.400$; $P = 0.007$), between C3256T and T3336C ($r = 0.407$, $P = 0.006$), C3256T and G15059A ($r = 0.667$, $P < 0.001$), between T3336C and G13513A ($r = -0.461$, $P = 0.002$), between G5178A and G12315A ($r = 0.380$; $P = 0.011$), G5178A and G14459A ($r = 0.325$; $P = 0.032$), G5178A and G14846A ($r = 0.800$, $P < 0.001$), between G12315A and G14459A ($r = 0.362$, $P = 0.016$), G12315A and G14846A ($r = 0.478$, $P = 0.001$), and between G15059A and Ins652G ($r = -0.487$, $P = 0.001$).

Table 1: Comparison of the level of heteroplasmy for ten mitochondrial mutations in homogenates of unaffected intimal samples and atherosclerotic lesions.

Mutation	The level of heteroplasmy (%)*		P**
	Unaffected tissue	Lipofibrous plaque	
652insG	3 (4)	1 (3)	NS
A1555G	13 (8)	20 (11)	0.001
C3256T	8 (4)	18 (7)	<0.001
T3336C	2 (2)	6 (5)	0.006
C5178A	12 (11)	15 (17)	NS
G12315A	15 (12)	20 (14)	0.069
G13513A	26 (7)	19 (11)	0.019
G14459A	5 (3)	7 (4)	0.054
G14846A	8 (6)	10 (9)	NS
G15059A	11 (11)	28 (14)	<0.001

* The level of heteroplasmy is expressed as a mean, SD is shown in parentheses.
** The significance of differences was estimated by Wilcoxon signed-rank test; NS: nonsignificant differences.

4. Discussion

The association between mtDNA mutations and atherosclerotic lesions in the human aorta demonstrated by the present study is in agreement with a polygenic hypothesis of the origin and development of multifactorial diseases, which suggests that these pathologies may be the consequence of accumulated mutations. However, because some single mitochondrial mutations had higher prevalence in atherosclerotic tissue (i.e., the proportion of mtDNA copies bearing mutant allele was higher) and could possibly be the cause of the pathology, these results also support a monoclonal hypothesis of atherosclerosis. The last hypothesis considers the possibility of a somatic mutation appearing in a single smooth muscle cell that further proliferates and forms a monoclone; this monoclone could then expand into the vascular wall, followed by an intimal thickening and further development and growth of an atherosclerotic plaque [29]. It should be noted that the level of heteroplasmy for mutation G13513A was lower in atherosclerotic tissue as compared to unaffected aortic intima; this may allow offering a suggestion about atheroprotective role of this mutation which should be tested in further studies.

In contrast to comparisons of single lipofibrous plaques and unaffected intimal samples, in which the C5178A mutation seemed to be prevalent in normal tissue, the

controversial results were obtained in comparisons of whole homogenates. There exists an assumption that C5178 mutation protects the intima from atherosclerosis [13]. However, our data do not confirm this assumption. In our research, the level of heteroplasmy for C5178A mutation has appeared to be lower in the whole homogenate of unaffected intima as compared to homogenates of atherosclerotic lesions.

On the basis of the obtained data, we conclude that at least five mitochondrial mutations, A1555G in MT-RNR1, G12315A in MT-TL2, G14459A in MT-ND6, C5178A in MT-ND2, and G15059A in MT-CYB are associated with atherosclerotic lesions in human aortic intima. Obviously, one of the limitations of our study is a lack of the demonstration of functional relationship between the presence of mtDNA mutations and the respiratory chain function (e.g., alteration of expression or enzymatic activities of respiratory complexes). However, it is worth to noting here that the investigation of functional relationship between the presence of mtDNA mutations and the respiratory chain function would require an independent expansive study.

Heteroplasmy is defined as the presence of a mixture of more than one type of an organellar genome within a cell or individual. Pathogenic mtDNA mutations are usually heteroplasmic, with a mixture of mutant and wild-type mtDNA within the same organism. A woman harboring one of these mutations transmits a variable amount of mutant mtDNA to each offspring.

Heteroplasmy is common in humans and has been associated with aging and disease. Mitochondrial DNA is present in hundreds to thousands of copies per cell and also has a very high mutation rate. New mtDNA mutations arise in cells, coexist with wild-type mtDNA (heteroplasmy), and segregate randomly during cell division. The vast majority of deleterious mtDNA point mutations are heteroplasmic, and their mutant load can vary significantly among different tissues, even in the same subject. Heteroplasmic mtDNA defects are considered an important cause of human disease with clinical features that primarily involve nondividing (postmitotic) tissues. The amount of mutant mtDNA in a cell, called the heteroplasmy level, is an important factor in determining the amount of mitochondrial dysfunction and thus the disease severity. Both qualitative (presence or absence of a mutation) and quantitative (heteroplasmy level) estimations of mutant alleles in the mitochondrial genome are necessary for studying the association between mitochondrial mutations and human diseases, including atherosclerosis [5].

The cells that inhabit the subendothelial space in arteries participate in the processes of inflammation and atherosclerotic plaque formation. Increased levels of mtDNA heteroplasmy in arterial wall lead to a higher likelihood that cell function is inhibited due to the presence of mutations in the coding region of mtDNA. Impaired cell function, in turn, may lead to local oxidative stress and other pathologic events, which could promote atherosclerosis formation. Because free radicals and lipid peroxidation have been previously shown to be relevant in the etiology of atherosclerosis and coronary heart disease [30], among genetic factors, we hypothesize that mitochondrial mutations have a role in atherosclerosis [5].

5. Conclusion

Based on the data obtained in the present study, we now suggest that mtDNA heteroplasmy, which is a biomarker of defective mitochondrial function, can also be employed as a novel biomarker of atherosclerosis and consequent clinical manifestations of this disease.

Conflict of Interests

The authors declare that they have no potential conflict of interests.

Acknowledgment

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Review Article

Coinhibitory Molecules in Autoimmune Diseases

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Coinhibitory molecules such as CTLA-4, PD-1 and BTLA negatively regulate immune responses. Multiple studies indicate that the deficiency or mutation of coinhibitory molecules leads to the development of autoimmune diseases in mice and humans, indicating that the negative signals from coinhibitory molecules are crucial for the prevention of autoimmunity. In some conditions, the administration of decoy coinhibitory receptors (e.g., CTLA-4 Ig) or mAb against coinhibitory molecules suppresses the responses of self-reactive T cells in autoimmune diseases. Therefore, modulation of coinhibitory signals seems to be an attractive approach to induce tolerance in autoimmune diseases in humans where the disease-inducing self-antigens are not known. Particularly, administration of CTLA-4 Ig has shown great promise in animal models of autoimmune diseases and has been gaining increasing attention in clinical investigation in several autoimmune diseases in humans.

1. Introduction

The immune system has developed multiple mechanisms to prevent harmful activation of immune cells. One such mechanism is the balance between costimulatory and coinhibitory signals delivered to T cells. The B7-1 (CD80)/B7-2 (CD86)-CTLA-4 pathway is the best-characterized inhibitory pathway for T-cell activation [1–3]. Another inhibitory pathway involves programmed death-1 (PD-1), which interacts with PD-L1 (B7-H1) and PD-L2 (B7-DC) and negatively regulates T cell activation [1, 3, 4]. B and T lymphocyte attenuator (BTLA), the third coinhibitory molecule for T-cell activation, is a cell surface molecule with similarities to CTLA-4 and PD-1 [5]. The ligand for BTLA is herpesvirus-entry mediator (HVEM), a TNF receptor family protein, and the ligation of BTLA with HVEM attenuates T-cell activation [6–9]. Since these inhibitory coreceptors inhibit proliferation and cytokine production of T cells *in vitro* and *in vivo*, they are thought to play important roles in maintaining immunological homeostasis and tolerance [10–12].

Autoimmune diseases occur because of a failure of the immune system to maintain nonresponsiveness or tolerance to self-antigens. Accumulating evidence indicates that coinhibitory molecules are key in the prevention of autoimmune diseases, because a defect or a functional mutation in these molecules promotes autoimmunity and polymorphisms of these genes are associated with genetic susceptibility to autoimmune diseases in humans.

Once an autoimmune disease developed, whether it is organ specific or nonorgan specific, in most cases corticosteroids and/or immunosuppressants are used for treatment. Refractory autoimmune diseases are sometimes treated with biological agents such as TNFα blockers, anti-IL-6 receptor antibody, and anti-CD20 antibody. However, immunosuppressive therapy occasionally causes serious adverse effects such as infection and malignancy. Therefore, novel immunomodulating agents for autoimmune diseases that have fewer adverse effects are desired.

This review is intended to give an overview of the immunobiology of the coinhibitory molecules and their roles in autoimmune diseases. We also review the advantages and limitations that should be discussed to translate the targeting of coinhibitory pathways into successful therapeutic interventions.

2. CD28/CTLA-4-B7 Pathway in the Regulation of Immune Responses

Numerous studies have demonstrated the importance of CD28-B7 costimulation for TCR-MHC-mediated T cell activation [13]. The interaction between CD28 on T cells and the B7 family molecules [B7-1 (CD80) and B7-2 (CD86)] on antigen presenting cells (APCs) plays a central role not only in the activation of normal (protective) T cell responses but also for the activation of pathological (self-reactive) T cell responses [1, 14]. CD28 is constitutively expressed on naïve and activated T cells. B7-1 is expressed in low levels on resting APCs and is upregulated with prolonged interaction with T-cells, whereas B7-2 is constitutively expressed and rapidly upregulated on APCs. Thus, B7-2 is likely to be mainly involved in mediating initial T cell activation, while B7-1 may play an important role in propagating the immune responses. After activation, T cells express CTLA-4 (CD152), which has higher affinity for B7-1 and B7-2 than CD28 does [15, 16]. Engagement of CTLA-4 delivers negative signal into T cells, resulting in inhibition and/or termination of T cell responses. CD28-B7 interactions are also important for the expansion and maintenance of $CD4^+CD25^+$ Tregs [17].

3. Roles of CTLA-4 Pathway in the Maintenance of Self-Tolerance

A defect in the negative signals from coinhibitory molecules may lower the threshold of autoreactive lymphocyte activation and thus may lead to the development of autoimmune diseases. This notion has been first evidenced by the autoimmune phenotype or lymphocyte hyperreactivity in mice lacking CTLA-4. CTLA-4-deficient mice rapidly develop a lymphoproliferative disease with multiorgan lymphocytic infiltration and tissue destruction and die by 3-4 weeks of age [18, 19]. In humans, CTLA-4 has been suggested to be associated with various autoimmune diseases including Grave's disease, autoimmune hypothyroidism, type I diabetes, systemic lupus erythematosus (SLE), and celiac disease [20–24]. Interestingly, Cunninghame Graham et al. have shown that although the 3′ flanking region of CTLA4 is an important region for association to both SLE and Graves' disease, the pattern of association to SLE is distinct from that seen in Graves' disease and the variants contributing to the association in SLE are more distal to CTLA4 than those in Graves' disease [23]. These findings suggest that CTLA-4 plays critical roles in the prevention of autoimmunity in multiple organs through multiple mechanisms.

4. Blockade of CD28-B7 Pathway as a Therapy for Autoimmune Diseases

It is anticipated that therapies directed against the B7 molecules would selectively affect T cells that are in the process of antigen-induced activation but would not affect resting T cells. Thus, in patients with autoimmune diseases, blockade of B7-CD28 interactions might preferentially inhibit lymphocytes that are in the process of responding to self-antigens without affecting resting T cells that recognize other antigens.

To develop the agents that would block signaling through CD28, investigators have taken advantage of the fact that CTLA-4 binds B7-1 and B7-2 with much higher affinity than CD28 does [15, 16]. A fusion protein consisting of the extracellular domain of CTLA-4 and the constant region of IgG blocks the interaction between B7 molecules and CD28 and thereby inhibits T-cell activation [16]. This fusion protein, designated CTLA-4 Ig, has been used successfully in mice to block T cell responses, to inhibit B-cell differentiation into plasma cells, to facilitate organ transplantation, and to induce anergy to self-antigens [25–27]. As for treatment of autoimmune disease models, CTLA-4 Ig treatment prevents autoantibody production, reduces the severity of lupus nephritis, and prolongs survival in NZB/NZW F1 mice [28]. CTLA-4 Ig treatment also prevented experimental autoimmune encephalomyelitis (EAE) induced by either active immunization or adoptive transfer of activated antigen-specific T cells [29, 30].

In vivo studies using anti-CD80 mAbs and anti-CD86 mAbs have suggested that CD80 and CD86 differentially regulate the development of autoimmune disease. Actively induced EAE is ameliorated by treatment with anti-CD80 mAb and is exacerbated by treatment with anti-CD86 mAb [31]. On the other hand, the development of diabetes in NOD mice, a model for insulin-dependent diabetes mellitus (IDDM), is exacerbated by treatment with anti-CD80 mAb and is blocked by treatment with anti-CD86 mAb [32]. In rheumatic disease models, both anti-CD80 mAb and anti-CD-86 mAb are required to suppress disease manifestations in lupus mice [33] or collagen-induced arthritis (CIA) models [34]. These conflicting results can be explained by the influence of the timing of the treatment or the differences of the pathology of disease models. The fact that these treatments block the signaling not only from CD28 but also from CTLA-4 may explain the reason why the treatment with these agents exacerbates autoimmune responses in certain situations.

5. Clinical Application of CTLA-4 Ig for Human Autoimmune Diseases

Based on the encouraging results in murine models, the efficacy of CTLA-4 Ig has been examined in patients with autoimmune diseases. Abatacept is a fusion protein composed of the Fc fragment of a human IgG1 linked to the extracellular domain of CTLA-4 [35]. Abatacept has shown efficacy in a broad spectrum of rheumatoid arthritis (RA) patients from early stage to refractory diseases that are resistant to TNF blockers [36, 37]. Abatacept treatment results in significant improvement in the signs and symptoms of RA including the inhibition of the structural damage [38]. Abatacept has also demonstrated efficacy in patients with juvenile idiopathic arthritis (JIA) who have not responded to traditional DMARDs or TNF blockers [39].

In addition to RA and JIA, Abatacept has shown clinical efficacy in patients with psoriasis in a phase I trial [40].

Abatacept has also demonstrated efficacy in patients with psoriatic arthritis including those who exhibit an inadequate response to TNF blockers [41]. In a study conducted on SLE patients, the efficacy of Abatacept on musculoskeletal manifestations has also been demonstrated [42].

Belatacept (LEA29Y) is another human CTLA-4 Ig that differs from Abatacept by substitution of two amino acids, which confers a stronger binding avidity to B7 and a greater inhibition of T-cell activation. The treatment with Belatacept has been shown to be as effective as cyclosporine in preventing acute rejection after renal transplant and in helping preserve glomerular filtration rate [43, 44]. Phase I/II clinical trial of multiple-dose of Belatacept versus Abatacept versus placebo in RA has revealed preliminary efficacy of Belatacept in the treatment of RA.

6. PD-1-PD-L1/PD-L2 Pathway Inhibits T Cell Activation

PD-1 (CD279) is another coinhibitory receptor belonging to CD28 family [45, 46]. PD-1 is expressed on activated T cells, B cells, regulatory T cells, and monocytes and binds to two ligands of the B7 family, PD-L1 (B7-H1), and PD-L2 (B7-DC) [47, 48]. The ligation of PD-1 with these ligands inhibits proliferation of CD4 T cells and CD8 T cells by arresting the cell cycle [49]. Whereas PD-L2 expression is mostly restricted to innate immune cells such as dendritic cells (DCs) and macrophages, PD-L1 is expressed not only on hematopoietic cells including T cells, B cells, mast cells, DCs, monocytes, and macrophages but also on several parenchymal tissues including the vascular endothelium and epithelium of multiple organs [1, 4, 46, 50, 51]. The expression of PD-L1 in nonhematopoietic cells suggests that PD-L1 suppresses self-reactive T cells or B cells in peripheral tissues and may regulate inflammatory responses in the organs.

Unlike CTLA-4-deficient mice, PD-1 deficiency leads to autoimmune disorders later in life. PD-1-deficient mice on a C57BL/6 background spontaneously develop lupus-like glomerulonephritis and proliferative arthritis [52]. In addition, PD-1-deficient mice on a BALB/c background develop dilated cardiomyopathy [53], which is associated with the production of autoantibody against cardiac troponin I, and die of congestive heart failure [54].

7. Blockade of PD-1-PD-L1/PD-L2 Pathway in Autoimmune Diseases

Targeting PD-1 with an agonist could be an alternative approach for the treatment of autoimmune diseases. However, so far more effort has been directed at blocking this pathway to relieve PD-1-mediated immune suppression in the context of chronic viral infection and tumor immunotherapy [4]. The first study for blockade of PD-1 pathway in autoimmune diseases has reported that blockade of PD-1 and PD-L1, but not of PD-L2, accelerates the onset of the diabetes in NOD mice [55]. On the other hand, blockade of PD-L2 but not of PD-L1 augments EAE in C57BL/6 mice with minimal and delayed expression of PD-L2 in the central nervous system [56]. In contrast, blockade of PD-L1 but not of PD-L2 significantly increases the incidence of EAE in BALB/c mice upon immunization with MOG peptide [57]. These results suggest that PD-L1 and PD-L2 differentially regulate the susceptibility and chronic progression of EAE in a strain specific manner.

Thus far, more than 30 single nucleotide polymorphisms (SNPs) have been identified within *PD-1* gene. Many reports have highlighted that some regulatory SNPs in *PD-1* might affect the expression and transcription of the gene [58, 59]. The SNPs have been studied as a part of attempts to identify the pathogenesis of several autoimmune diseases including SLE [58–60] and RA [61]. For instance, Prokunina et al. have shown that one intronic SNP in *PD-1* gene, which alters a binding site for the runt-related transcription factor 1 (RUNX1) located in an intronic enhancer, is associated with development of SLE [58]. SNPs in the *PD-1* gene have also been studied in multiple sclerosis (MS) [62], ankylosing spondylitis (AS) [63], and Graves' disease [64]. In Japanese and Filipino populations, a higher frequency of a specific SNP in the *PD-1* gene has been demonstrated in patients with subacute sclerosing panencephalitis (SSPE) [65]. Currently, two anti-PD-1 mAbs are undergoing Phase II trials to determine the efficacy against tumors. One of them, MDX-1106, has shown clinical efficacy in renal cell carcinoma and melanoma without serious toxicity [66]. The efficacy of anti-PD-1 mAb or PD-1 Ig treatment in the patients with autoimmune diseases is needed to be elucidated in the further basic and clinical studies.

8. BTLA-HVEM Pathway Is the Third Inhibitory Pathway for Lymphocyte Activation

BTLA (CD272) is the third inhibitory coreceptor, which has been identified as an inhibitory receptor on $CD4^+$ T cells and B cells with similarities to CTLA-4 and PD-1 [5]. Later analyses have revealed that BTLA is expressed not only on $CD4^+$ T and B cells but also on a wide range of hematopoietic cells including $CD8^+$ T cells, NKT cells, NK cells, macrophages, and dendritic cells at various levels [9, 67–69]. Moreover, it has recently been demonstrated that BTLA is highly expressed on follicular B helper T cells (Tfh cells) [70]. The ligand for BTLA is the TNF receptor family member HVEM [7, 8, 71], which is broadly expressed on hematopoietic cells, including T cells, macrophages, and DCs [71]. Ligation of BTLA induces its tyrosine phosphorylation and SHP-1/SHP-2 association and then attenuates IL-2 production and proliferation of T cells [5, 72]. These findings suggest that BTLA functions as an inhibitory coreceptor through the interaction with HVEM.

9. Relevance of BTLA-HVEM Pathway in Autoimmune Diseases

Recent analyses suggest that BTLA is crucial for dampening immune responses. BTLA-deficient mice exhibit enhanced specific antibody responses and sensitivity to EAE [5], rapid rejection of partially MHC-mismatched cardiac allograft

[73], and acceleration of experimental colitis [74]. We have shown that the deficiency of BTLA also causes the breakdown of self-tolerance, resulting in the development of an autoimmune hepatitis- (AIH-) like disease and lymphocytic infiltration in multiple organs [75]. We have also shown that BTLA plays a protective role in autoimmune diseases in MRL-lpr mice and that AIH-like disease develops in BTLA-deficient mice even in the absence of Fas-dependent signaling [76].

It has been reported that combined treatment with anti-BTLA mAb and CTLA-4 Ig in a fully MHC-mismatched islet transplant model induces donor-specific tolerance [77]. The effect of combined treatment with anti-BTLA mAb and anti-PD-1 mAb on autoimmunity has also been examined in NOD mice [78]. This study has shown that the onset of diabetes is delayed when anti-BTLA mAb is given to 10-week-old NOD mice. In addition, anti-BTLA mAb inhibits anti-PD-1 mAb-induced acceleration of diabetes in NOD mice [78]. Moreover, Ishida et al. have demonstrated that anti-BTLA mAb treatment during the induction phase of ragweed-induced experimental conjunctivitis significantly increases eosinophil infiltration and Th2 cytokine production from T cells [79]. These data support the notion that BTLA pathway is involved in the regulation of immune responses not only to self-antigens but also to non-self-antigens and suggest the efficacy of the modulation of BTLA pathway in multiple immune diseases.

The role of HVEM-BTLA pathway in the pathogenesis of autoimmune diseases in humans is still largely unknown. However, Lin et al. have recently shown the significant association between the SNP (C+800T) in the BTLA gene with the RA susceptibility in a Chinese population [80]. We have also shown that a functional polymorphism of BTLA gene, which lacks the inhibitory activity, is significantly associated with RA susceptibility in a Japanese population [81]. As of today, no active clinical trial of agents targeting BTLA-HVEM pathway is reported. We assume that the enhancement of BTLA signaling is applicable to the treatment of autoimmune diseases and that the blockade of this pathway may be useful for the treatment of the reduced immune responses against tumors or infection.

10. Concluding Remarks

The manipulation of signals exchanged between APCs and T-cells has considerable clinical relevance. T cell responses are context-dependent and are influenced by signals from their environment through a variety of receptor-ligand interactions. These signals amplify and modify the original TCR signal received by antigenic stimulation in T cells, regulate expansion and differentiation of activated T cells, or control effector functions in a particular environment. The agents regulating the signals through coinhibitory molecules including CTLA-4, PD-1, and BTLA have the potential to regulate autoimmunity and responses to tumors and chronic infections. Coinhibition, by its very nature, is not antigen specific, and therefore will not be specific for self-reactive T cells. However, because immune responses in the various autoimmune diseases and in normal responses may differ in their requirement for costimulatory and coinhibitory signals, selective costimulation blockade, or coinhibition boost by the administration of the agents against coreceptors may have a therapeutic potential in the treatment of autoimmune diseases.

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Research Article

The Impact of Osteopontin Gene Variations on Multiple Sclerosis Development and Progression

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Osteopontin is a proinflammatory molecule, modulating TH1 and TH17 responses. Several reports suggest its involvement in multiple sclerosis (MS) pathogenesis. We previously reported that OPN gene variations at the 3′ end are a predisposing factor for MS development and evolution. In this paper, we extended our analysis to a gene variation at the 5′ end on the −156G > GG single nucleotide polymorphism (SNP) and replicated our previous findings at the 3′ end on the +1239A > C SNP. We found that only +1239A > C SNP displayed a statistically significant association with MS development, but both +1239A > C and −156G > GG had an influence on MS progression, since patients homozygous for both +1239A and −156GG alleles displayed slower progression of disability and slower switch to secondary progression than those carrying +1239C and/or −156G and those homozygous for +1239A only. Moreover, patients homozygous for +1239A also displayed a significantly lower relapse rate than those carrying +1239C, which is in line with the established role of OPN in MS relapses.

1. Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system characterized by an autoimmune response against the myelin sheaths and axons, resulting in progressive neurological dysfunction [1]. Patients with MS display variable clinical course; at onset, approximately 10% of patients display a primary progressive form (PP), whereas the remainder start out with a relapsing remitting form (RR), and most of them switch to a secondary progressive form (SP) within 10–30 years [2]. Both genetic and environmental factors are involved in the development/progression of MS, and several studies point to a complex inheritance involving interactions between combinations of loci that may influence the immune response [3, 4]. An increasing bulk of data suggest that osteopontin (OPN) may play a role in the pathogenesis of MS [5]. OPN is a 60 kDa-secreted phosphoprotein functioning as a free cytokine in body fluids or as an immobilized extracellular matrix molecule in mineralized tissue [6]. OPN serum levels are increased in several autoimmune diseases and may influence development of these diseases through the OPN immunoregulatory effects enhancing the proinflammatory T helper type 1 (TH1) and TH17 cell responses and inhibiting the TH2 responses [7].

OPN transcript is abundant in plaques dissected from brains of patients with MS, whereas it is absent in control

brain tissue; this finding has been confirmed in rat experimental autoimmune encephalomyelitis (EAE) by microarray cDNA analysis of spinal cord tissue [8]. OPN serum levels are higher in relapsing-remitting than in progressive patients, particularly during the relapse [9, 10]. Chowdhury et al. reported a correlation between cerebrospinal fluid (CSF) OPN levels and disease activity in patients with MS. These levels did not correlate with disability status but were higher in patients with active disease [11].

The human OPN gene (*OPN*) is located on chromosome 4q22.1, and single nucleotide polymorphisms (SNPs) are associated with development and/or disease activity of several autoimmune diseases [12–14]. A link between the gene and protein data was suggested by the correlation between some *OPN* genotypes and OPN serum levels [15]. Four SNPs of the *OPN* gene (+282T > C in exon VI: rs4754; +750C > T in exon VII: rs11226616; +1083A > G: rs1126772 and +1239A > C: rs9138) in 3′ UTR form three haplotype combinations: haplotype A (282T-750C-1083A-1239A), haplotype B (282C-750T-1083A-1239C), and haplotype C (282C-750T-1083G-1239C). Carriers of haplotype B and C displayed higher OPN serum levels and higher risk of developing autoimmune diseases than haplotype A homozygotes. Several data suggested that the high OPN levels were due to increased stability of the mRNA coded by haplotype B and C [15]. Regarding MS, we previously found that haplotype A homozygotes displayed about 1.5 lower risk of developing MS and lower OPN serum levels than haplotype B or C carriers. Moreover, clinical analysis showed that haplotype A homozygous patients displayed slower switching from a RR to a SP form and milder disease with slower evolution of disability than patients carrying haplotype B or C [16].

Interindividual differences of OPN expression may be also influenced by variations in the promoter region of *OPN* that may modulate its transcriptional activity. This role has been suggested for the −66T > G [17], −156G > GG (rs7687316), and −443>T > C [17] SNPs by Giacopelli et al. [18], and we detected a combined effect of −156G > GG and +1239A > C on risk of systemic lupus erythematosus (SLE) development [14].

According to these findings, the aims of this study were (1) to replicate our previous findings on the +1239A>C SNP, (2) to investigate the role of the −156G > GG SNP, (3), to assess the impact of these variations on disease evolution.

2. Materials and Methods

2.1. Patients. We analyzed 728 Italian patients (278 males, 450 females; M/F: 0.62) with MS diagnosed according to the revised McDonald criteria [19] and 1218 randomly selected ethnically and age-matched healthy controls. Patients were consecutive patients enrolled from the Multiple Sclerosis Centers of the "Amedeo Avogadro," University of Eastern Piedmont (Novara), the University of Milan, IRCCS Policlinico Hospital (Milan), the Don C Gnocchi Foundation, IRCCS, S Maria Nascente (Milan), and the "Santa Croce e Carle" Hospital (Cuneo), Italy. Their clinical and demographic features were similar to those of other series [20, 21]. Controls were consecutive Italian donors obtained from the transfusion services of the respective hospitals. Patients and controls were unrelated, Caucasian and Italian, matched for age and gender, with no family history of autoimmune diseases in first degree relatives. According to their clinical course, patients were defined as follows [22]:

RR: occurrence of exacerbations, each lasting at least 24 h and separated by at least one month of inactivity, with full recovery or sequelae ($n = 447$);

PP: steady worsening of symptoms and signs from onset for at least 6 months, whether superimposed with relapses or not, with occasional plateau and temporary minor improvements; ($n = 71$);

SP: initial RR course followed by steady worsening of symptoms and signs for at least 6 months, whether superimposed with relapses or not, with minor remissions, and plateau ($n = 210$).

We performed an analysis of the following outcome measures: time to reach Kurtzke expanded disability status scale [23] (EDSS) score > 3.0 and time to reach a progressive course, since it was previously shown that OPN SNPs at the 3′ UTR region may influence these measures in MS patients [16]. According to Hawkins and McDonnell [24], disease of patients who, after at least 10 years from onset, had a mild disability, that is, EDSS score ≤ 3.0, was defined benign MS. Patients who reached secondary progression within 10 years from onset were defined fast progressive. Patients who did not reach the endpoints were excluded.

In RR patients, EDSS score was assessed in remission phase.

The annual relapse rate before treatment was collected in 327 patients with bout onset (RR patients and SP patients) [21]. Only relapses that occurred in the first three years of disease were included in the analysis.

Samples from patients with RR were drawn during remission. All patients gave their informed consent according to the Declaration of Helsinki [25]. The research was approved by the local ethical committee.

2.2. DNA Analysis. Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using standard methods and primers used to evaluate OPN SNPs were the following: 5′-gccgtgaattccacagccatg-3′ (OPN F) 5′-ttgaatgtaataagaatttggtgg-3′ (OPN R)(for +1239 SNP) and 5′-agccctctcaagcagtcatc-3′ (promo 1F) 5′-cctgtgttggtggaggatgt-3′ (promo 1R) (for −156 SNP). PCR products were purified with the EXO/SAP kit (GE, Healthcare, Piscataway, NJ, USA). Sequencing was performed with the ABI PRISMR BigDyeTM Terminator kit (Applied Biosystems, Foster City, CA) on an automatic sequencer (Applied Biosystems 3100 Genetic Analyser) according to the manufacturer's instructions.

2.3. OPN ELISA Assay. Serum OPN concentrations were evaluated in a capture enzyme-linked immunoadsorbent assay (ELISA) according to the protocol provided by the manufacturer (Calbiochem, San Diego, CA). The optical density was measured at 450 nm with a microplate reader (Bio-Rad, Hercules, CA). The I-smart program was used to

create a regression curve. All assays were performed in duplicate, and the observer (E.O.) was blinded to the diagnosis.

2.4. Statistical Analysis. Allelic frequencies and outcome measures were compared with the chi-square test with the Yate's correction. Relapse rate was compared with the Mann-Whitney *U*-test. For the ELISA experiments, the approximation of population distribution to normality was tested by using statistics for kurtosis and symmetry. Results were asymmetrically distributed and consequently presented as median values and percentiles. ELISA data comparisons were performed with the nonparametric Mann-Whitney *U* test. All *P* values are 2-tailed and the significance cut-off was $P < 0.05$.

3. Results

We typed the +1239A > C SNP in 728 patients and 1218 controls and the −156G > GG SNP in 728 patients and 912 controls, not overlapping with the cohorts analyzed in our previous study [16]. The +1239A > C SNP was analysed because it allows to discriminate between the A and non-A haplotypes (not carrying versus carrying the +1239C allele, resp.).

Frequency of +1239A homozygotes was decreased in MS patients compared to controls (46% versus 52%; $P = 0.011$), and +1239A homozygotes displayed 1.27 lower risk of MS than +1239C carriers (Table 1). These findings confirmed our previous results on different groups of 425 patients and 688 healthy controls, showing that carriers of the +1239A display a slight protection against MS development. Conversely, no statistically significant difference between patients and controls was found for the −156G>GG SNP (Table 2).

Genotypic distribution did not deviate significantly from the Hardy-Weinberg equilibrium in any group (data not shown).

The next step was to assess the impact of these variations on MS evolution, since we previously reported that +1239A homozygotes displayed slower disease progression and milder disability over time compared to +1239C carriers [16]. According to our previous work, disease progression was evaluated by comparing patients switching from RR to SP within 10 years from onset (fast progressive, $n = 184$) and those remaining RR over 10 years (slow progressive, $n = 444$) and disease severity was evaluated by comparing patients with an EDSS score ≤ 3.0 ten years or more after onset (benign MS, $n = 194$) and those who reached a score > 3.0 within ten years (non-benign MS, $n = 446$).

Table 3 shows that the proportion of slow progressive patients was significantly higher in +1239A homozygotes than in +1239C carriers (80% versus 63%, $P < 0.0001$), whereas no difference was found between −156GG homozygotes and −156G carriers (73% versus 70%, $P = 0.3$). Patients homozygous for both +1239A and −156GG showed a significantly higher proportion of slow progressive patients than those carrying +1239C and/or −156G (95% versus 68%, $P < 0.0001$) and those homozygous for +1239A only (95% versus 80%, $P = 0.0094$).

Table 3 also shows that the proportion of benign MS patients was significantly higher in +1239A homozygotes than in +1239C carriers (38% versus 24%, $P = 0.0001$) and in −156GG homozygotes than in −156G carriers (46% versus 28%, $P = 0.0018$). Patients homozygous for both +1239A and −156GG showed a significantly higher proportion of benign MS patients than those carrying +1239C and/or −156G and those homozygous for +1239A only (52% versus 38%, $P = 0.038$).

To further evaluate the clinical impact of *OPN* variations, we analyzed the relapse rate in bout-onset patients carrying different genotypes. Data were available for 327 patients (157 AA, 170 non-AA). The relapse rate was significantly lower in +1239A homozygotes than in +1239C carriers (0.5/yr versus 1.3/yr, $P = 0.01$), whereas no difference was found between −156GG homozygotes and −156G carriers (0.8/yr versus 1.1/yr; $P = 0.09$) or between subjects carrying both protective genotypes and those carrying at least one predisposing genotype (0.6/yr versus 1.2/yr; $P = 0.06$) (Table 4).

Finally, we explored whether OPN serum levels varied in patients displaying different outcomes. Consistently, we found that benign patients, as well as slow progressive patients, showed significantly lower protein levels compared to nonbenign and fast progressive patients, respectively

Table 1: Frequency distribution of OPN +1239A > C genotypes in MS patients and healthy controls.

Genotype SNP + 1239	*MS ($n = 728$)	†Controls ($n = 1218$)
AA	335 (46)	634 (52)
AC	314 (43)	486 (40)
CC	79 (11)	98 (8)
AA	335 (46)	634 (52)
Non-AA	393 (54)	584 (48)
	‡OR = 1.27 $P = 0.011$ (95% CI: 1.05–1.54)	

*Multiple sclerosis patients.
†number of subjects and proportions are shown in the brackets. Genotypic distribution did not deviate significantly from the Hardy-Weinberg equilibrium in any group (data not shown).
‡Odds ratio (OR), 95% confidence limits (95% CI), χ^2 test calculated on allelic frequencies, and *P* values are 2-tailed.

Table 2: Frequency distribution of OPN-156G/GG genotypes in MS patients and healthy controls.

Genotype SNP −156	†MS ($n = 728$)	†Controls ($n = 912$)
GG/GG	78 (10.7)	112 (12.3)
G/GG	304 (41.8)	384 (42.1)
GG	346 (47.5)	416 (45.6)
	‡OR = 0.91 $P = 0.25$ (95% CI: 0.79–1.06)	

*Multiple sclerosis patients.
†Number of subjects, proportions are shown in the brackets. Genotypic distribution did not deviate significantly from the Hardy-Weinberg equilibrium in any group (data not shown).
‡Odds ratio (OR), 95% confidence limits (95% CI), χ^2 test calculated on allelic frequencies, and *P* values are 2-tailed.

Table 3: Frequency distribution of different outcomes in MS patients carrying different OPN genotypes.

Outcome	Genotypes					
	+1239A > C		−156GG > G		+1239A > C −156G > GG	
	AA	C	GG/GG	G	AA GG/GG	C G
Fast progressive[b]	57 (20)	127 (37)	18 (27)	166 (30)	3 (5)	181 (32)
Slow progressive[a]	228[c] (80)	216 (63)	58 (73)	386 (70)	57 (95)	387 (68)
	$P < 0.0001$[d]		$P = 0.311$		$P < 0.0001$	
Benign MS[a]	110 (38)	84 (24)	36 (46)	158 (28)	35 (52)	159 (28)
Non benign MS[b]	183 (62)	263 (76)	42 (54)	404 (72)	32 (48)	414 (72)
	$P = 0.0001$		$P = 0.0018$		$P = 0.0002$	

[a]Patients displaying RR form (slow progressive) or EDSS ≤ 3 (benign MS) after 10 years from onset.
[b]Number of patients displaying that disease status; proportions are shown in brackets.
Patients displaying either RR course and less than 10 years of followup (29/728) or PP course (71/728) were excluded from the analysis of progression. Patients displaying EDSS ≤ 3.0 and less than 10 years of followup (88/728) were excluded from the analysis of disability.
[c]Patients switching to SP form (fast progressive) or reaching EDSS > 3 (non-benign MS) within 10 years from onset.
[d]Statistical analysis was performed by comparing the different outcomes with the χ^2 test.
Total number in the analysis of progression: 628 patients: 285 AA; 343 non-AA; 76 GG; 552 non-GG; 60 AAGG; 568 non-AAGG.
Total number in the analysis of course 640 patients: 293 AA, 347 non-AA; 78 GG, 562 non-GG; 67 AAGG, 573 non-AAGG.

Table 4: Relapse rate in patients with bout onset displaying different OPN genotypes.

Outcome measure	Genotype					
	AA $N = 153$	C $N = 174$	GG/GG $N = 33$	Non-GG $N = 294$	AAGG $N = 26$	CG $N = 301$
Relapse rate	0.5[a] (0.2–1)	1.3 (0.6–1.7)	0.8 (0.4–1.2)	1.1 (0.5–1.3)	0.6 (0.2–1.3)	1.2 (0.5–1.5)
	$P = 0.01$[b]		$P = 0.09$		$P = 0.06$	

[a]Median values; interquartile ranges are shown in the brackets.
[b]Mann-Withney U test.

(median value 132 versus 237 ng/mL, interquartile range 94–164 versus 189–289 ng/mL, $P < 0.0001$; median value 154 versus 280 ng/mL, interquartile range 100–207 versus 228–341 ng/mL, $P =< 0.0001$).

4. Discussion

This work stems from our previous observation of a protective effect of +1239A homozygosity at the 3′UTR of *OPN* for MS development and evolution. In our previous paper, this genotype decreased the risk of MS development by 1.56-fold [16]. The parallel observation of a combined effect of +1239C and −156G on risk of (SLE) development [14] prompted this work extending the *OPN* analysis in MS to −156GG > G.

The current data, obtained on a much larger independent population, replicated our previous findings on +1239A > C, showing that the frequency of +1239A homozygotes was decreased in MS patients and that these subjects displayed 1.27 lower risk of MS development than +1239C carriers. The same SNPs in the 3′ UTR region of the OPN gene have been studied in 326 Spanish MS patients and 484 controls by other authors. They did not find statistically significant differences between patients and controls, and this apparent discrepancy might be explained by differences in both size and ethnic background of the population under study [26].

By contrast, analysis of −156G > GG SNP did not detect statistically significant differences between patients and controls (OR 0.91, $P = 0.25$), which indicated that this genetic variation was not associated to MS development. To our knowledge, this is the first paper on this SNP in the MS population.

The most intriguing results were those on the role of these SNPs on the MS course. On the one hand, this study not only confirmed the correlation between +1293A > C and disease progression, but also strengthened this finding showing that +1239A homozygotes displayed a lower relapse rate than the other patients. On the other hand, it detected an additional effect of −156G > GG on disease progression since patients homozygous for both +1239A and −156GG displayed a milder disease, with slower progression of disability and slower switch to secondary progression, than those carrying +1239C and/or −156G and those homozygous for +1239A only. Therefore, −156GG homozigosity in the 5′ end of the gene conferred a further protection especially in subjects also carrying the protective genotype at the 3′ end of the gene.

These protective effects might be related to functional outcomes of these *OPN* variations. In our previous work, in fact, we showed that +1293C was associated with a high

"baseline" production of serum OPN, possibly related to increased stability of the OPN mRNA [15]. Moreover, position −156 seems to fall in a putative binding site for a component of the RUNX family of transcription factors and might influence osteopontin expression [18].

A further point supporting a protective role of AA genotype is provided by the analysis of OPN serum levels in patients displaying different disease outcomes. As a matter of fact, patients showing increased frequency of AA genotype, that is, benign and slow progressive MS patients, displayed lower OPN levels. Moreover, our findings are in line with the work by Kariuki SN et al. who reported that *OPN* gene variants modulate cytokine levels in SLE [27].

In conclusion, this work confirms that osteopontin and the *OPN* gene may be involved in MS development and, especially, progression. These observations suggest that this cytokine may be a therapeutic target to counteract MS progression supporting the finding of Steinman et al. showing that anti-OPN antibodies ameliorate the disease course in experimental autoimmune encephalomyelitis [28].

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Research Article

Gene Expression Profiling in Dermatitis Herpetiformis Skin Lesions

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Dermatitis herpetiformis (DH) is an autoimmune blistering skin disease associated with gluten-sensitive enteropathy (CD). In order to investigate the pathogenesis of skin lesions at molecular level, we analysed the gene expression profiles in skin biopsies from 6 CD patients with DH and 6 healthy controls using Affymetrix HG-U133A 2.0 arrays. 486 genes were differentially expressed in DH skin compared to normal skin: 225 were upregulated and 261 were downregulated. Consistently with the autoimmune origin of DH, functional classification of the differentially expressed genes (DEGs) indicates a B- and T-cell immune response (LAG3, TRAF5, DPP4, and NT5E). In addition, gene modulation provides evidence for a local inflammatory response (IL8, PTGFR, FSTL1, IFI16, BDKRD2, and NAMPT) with concomitant leukocyte recruitment (CCL5, ENPP2), endothelial cell activation, and neutrophil extravasation (SELL, SELE). DEGs also indicate overproduction of matrix proteases (MMP9, ADAM9, and ADAM19) and proteolytic enzymes (CTSG, ELA2, CPA3, TPSB2, and CMA1) that may contribute to epidermal splitting and blister formation. Finally, we observed modulation of genes involved in cell growth inhibition (CGREF1, PA2G4, and PPP2R1B), increased apoptosis (FAS, TNFSF10, and BASP1), and reduced adhesion at the dermal epidermal junction (PLEC1, ITGB4, and LAMA5). In conclusion, our results identify genes that are involved in the pathogenesis of DH skin lesions.

1. Introduction

Dermatitis herpetiformis (DH) is an autoimmune subepidermal blistering skin disease characterized by intense pruritic papulovesicular eruptions mainly localized on extensor surfaces [1]. DH typically develops in patients with celiac disease (CD). The two conditions share the same genetic background (HLA genes DQ2–DQ8), improve following a gluten-free diet (GFD), and are mediat-ed by IgA autoantibodies [2]. IgA antibodies against tissue transglutaminase (tTG) are detectable both in CD and DH, while autoantibodies directed against epidermal transglutaminase (eTG) are a typical serological marker of patients with DH [3].

The key feature of DH is a granular deposition of IgA within the tips of dermal papillae and along the basement membrane of perilesional skin. eTG has been shown to colocalize with such IgA deposits [4]. Typical histopathologic features of DH consist of accumulation of neutrophils and a few eosinophils with formation of papillary microabscesses which then coalescence to form a subepidermal bulla.

Moreover, a perivascular cellular infiltrate composed mainly by CD4+ lymphocytes is also present [5].

In DH, blister formation is associated with epidermal splitting due to destruction of basement membrane components and proteolysis of adhesion molecules at the dermal epidermal junction. A comprehensive analysis of the molecular mechanisms that coordinate the initiation and progression of the pathological process is still lacking. Our approach consists in the use of a gene array strategy that allows the simultaneous detection of thousands of genes in a given sample. We have examined gene expression directly in the skin tissue of patients with DH to analyze the transcriptional events that culminate in the skin lesion formation. We report here patterns of transcripts in 6 DH patients using DNA

microarrays that characterize injured skin and identify signatures of gene expression that are involved in the pathogenesis of blister formation. The analysis of modulated genes provides evidence for the intervention of genes involved in immune activation, inflammation, impaired adhesion and cell death, considered key features in the pathogenesis of the disease.

2. Materials and Methods

2.1. Patients. Six adult patients (3 men and 3 females; mean age 51 years, median age 52 years, and age range 36–59 years) with DH and CD, showing all clinical and immunopathological features of the diseases, were included in this study. All patients had the typical clinical features of DH, with erythematous papules and vesicles symmetrically distributed on the extensor surfaces of the upper and/or lower extremities and buttocks. The duodenal histological damage of the 6 patients at diagnosis ranged from grade 2 to 3b, according to Marsh's classification [6]. In particular, three patients had a grade 2 damage, and three patients had grade 3b damage. Five out of six patients suffered from gastrointestinal symptoms (diarrhoea, abdominal distension, and pain); one patient had extraintestinal symptoms (iron deficiency anaemia and weight loss).

Skin biopsies presented classical histopathologic features of DH, including subepidermal cleft with neutrophils and/or eosinophils at the tips of the dermal papillae and granular deposits of IgA at the tips of derma papillae on direct immunofluorescence.

Serologically, five out of six patients had serum anti-tTG and antiendomysium (EMA) IgA antibodies without gluten-free diet. The seronegative patient had a duodenal biopsy with a grade 3b histological damage and was affected by IgA deficiency. Indeed anti-tTG IgG were detected in this patient.

All patients were on normal gluten-containing diet and were not taking Dapsone at the moment of skin biopsy. Two punch biopsies of 6 mm each were performed at the diagnosis on each one of the 6 patients from early lesional skin (grouped erythematous papules surmounted by vesicles) following local anaesthesia (1% lidocaine with 1/100,000 epinephrine). Skin specimens for biopsy were obtained from elbows (2 patients) and from buttocks (4 patients).

Normal skin biopsies were obtained from 6 sex- and age-matched healthy adult subjects (3 males and 3 females, mean age 50 years, median age 53 years, age range 34–60 years) with no evidence of gastrointestinal or skin disease. Specimens were snap-frozen in liquid nitrogen immediately after biopsy.

All the subjects (patients and controls) were of Caucasian origin from Northwestern Italy.

The patients included showed no evidence of other coexisting autoimmune diseases.

Written informed consent was obtained in each case. The study was conducted according to the Declaration of Helsinki Principles and was approved by the local ethical committee.

2.2. Samples Preparation. Tissue samples from every single patient were frozen in liquid nitrogen immediately after dissection and stored at −70°C until homogenization. Frozen samples were homogenized in TRI REAGENT (1 mL per 50–100 mg of tissue) in a Potter-type mechanical homogenizer with Teflon pestle. RNA extraction, preparation of cRNA hybridization, and scanning of probe arrays for each samples were performed according to the protocols of the manufacturer (Affymetrix, Santa Clara, CA, United States) by Cogentech Affymetrix microarray unit (Campus IFOM-IEO, Milan, Italy) using the human genome U133A 2.0 gene chip (Affymetrix). The human genome U133A gene chip is a single array representing 14,500 well-characterized human genes and including more than 22,000 probe sets and 500,000 distinct oligonucleotide features.

2.3. Gene-Array Analysis. The different gene expression patterns were analyzed by using Gene Spring software, version 11.0 (Agilent Technologies, Santa Clara, CA, United States).

The normalized background-corrected data were transformed to the $\log_2$ scale. A signal $\log_2$ ratio of 1.0 indicates an increase of the transcript level by twofold change (2 F.C.), and −1.0 indicates a decrease by twofold (−2 F.C.). A signal $\log_2$ ratio of zero would indicate no change.

The unpaired t-test was performed to determine which genes were modulated at a significance level ($P < 0.05$), and P values were corrected for multiple testing by using Bonferroni correction.

Finally, statistically significant genes were selected for final consideration when their expression was at least 1.5-fold different in the test sample versus control sample.

Genes that passed both the P value and the F.C. restriction were submitted to a functional classification according to the Gene Ontology (GO) annotations (http://www.geneontology.org/).

3. Results

In order to identify genes involved in the pathogenesis of the typical skin lesions of DH, the gene expression patterns of 6 skin biopsies from 6 patients affected by DH were compared with 6 skin biopsies from 6 healthy controls.

A P value criterion ($P < 0.05$) and a fold change criterion (FC > 1.5) were both applied to the signal variation of every single gene to select robust and statistically significant changes between baseline and experimental arrays.

For statistical comparison, an unpaired t-test was calculated, and after a Bonferroni correction, 1191 transcripts resulted statistically significantly modulated ($P < 0.05$).

Among these transcripts, 486 also fulfilled the fold change criterion, since they were differentially expressed 1.5 fold or more; in particular 225 and 261 transcripts resulted, respectively, to be up- and downregulated.

Such transcripts were classified in functional categories according to Gene Ontology annotations, including immune response, apoptosis, cell growth, proliferation and differentiation, inflammatory response, production and remodelling of the extracellular matrix, and metabolism.

Table 1 shows a detailed representation of genes within the above-mentioned clusters. The table also includes GeneBank accession numbers and fold changes.

Table 1: Annotated genes differentially expressed in DH versus healthy controls grouped according to their function.

Functional class	Probe set ID	F.C.	Regulation	Gene symbol	Gene title	Accession number
Immune response	206486_at	1.5	Up	LAG3	Lymphocyte-activation gene 3	NM_002286
	204352_at	1.6	Up	TRAF5	TNF receptor-associated factor 5	NM_004619
	205821_at	1.7	Up	KLRK1	Killer cell lectin-like receptor subfamily K, member 1	NM_007360
	203717_at	2.4	Up	DPP4	Dipeptidyl peptidase 4	NM_001935
	203939_at	3.8	Up	NT5E	5′-nucleotidase, ecto (CD73)	NM_002526
	204502_at	2.0	Up	SAMHD1	SAM domain and HD domain 1	NM_015474
Inflammation	206332_s_at	3.1	Up	IFI16	Interferon, gamma-inducible protein 16	NM_005531
	217738_at	2.0	Up	NAMPT	Nicotinamide phosphoribosyltransferase	NM_005746
	203176_s_at	2.2	Up	TFAM	Transcription factor A, mitochondrial	NM_003201
	205870_at	2.2	Up	BDKRB2	Bradykinin receptor B2	NM_000623
	204655_at	2.2	Up	CCL5	Chemokine (C-C motif) ligand 5	NM_002985
	209392_at	2.3	Up	ENPP2	Ectonucleotidepyrophosphatase	L35594
	202859_x_at	2.3	Up	IL8	Interleukin 8	NM_000584
	211272_s_at	2.4	Down	DGKA	Diacylglycerol kinase, alpha 80 kDa	AF064771
	207177_at	2.5	Up	PTGFR	Prostaglandin F receptor	NM_000959
	208782_at	2.9	Up	FSTL1	Follistatin-like 1	BC000055
	204563_at	7.3	Up	SELL	Selectin L	NM_000655
	206211_at	4.3	Up	SELE	Selectin E	NM_000450
	217800_s_at	1.9	Up	NDFIP1	Nedd4 family interacting protein 1	NM_030571
	214475_x_at	2.8	Down	CAPN3	Calpain 3, (p94)	AF127764
	201859_at	3.1	Up	SRGN	Serglycin	NM_002727
	201110_s_at	5.2	Up	THBS1	Thrombospondin 1	NM_003246
Apoptosis	202558_s_at	1.5	Up	STCH	Stress 70 protein chaperone	NM_006948
	217786_at	1.5	Down	PRMT5	Protein arginine methyltransferase 5	NM_006109
	204781_s_at	1.5	Up	FAS	TNF receptor superfamily, member 6	NM_000043
	202693_s_at	1.7	Up	STK17A	Serine/threonine kinase 17a	NM_004760
	201912_s_at	2.6	Up	GSPT1	G1 to S phase transition 1	NM_002094
	202887_s_at	2.6	Down	DDIT4	DNA-damage-inducible transcript 4	NM_019058
	202687_s_at	2.9	Up	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	NM_003810
	202411_at	3.1	Up	IFI27	Interferon, alpha-inducible protein 27	NM_005532
	202391_at	3.1	Up	BASP1	Brain abundant, membrane-attached signal protein 1	NM_006317
Cell proliferation	208676_s_at	1.5	Up	PA2G4	Proliferation-associated 2G4, 38 kDa	U87954
	205937_at	1.5	Up	CGREF1	Cell growth regulator with EF-hand domain 1	NM_006569
	1773_at	1.5	Down	FNTB	Farnesyltransferase, CAAX box, beta	L00635
	202886_s_at	2.2	Up	PPP2R1B	Protein phosphatase 2, regulatory subunit A, beta isoform	M65254
	202167_s_at	1.9	Down	MMS19	MMS19 nucleotide excision repair homolog	NM_022362
	203108_at	2.1	Up	GPRC5A	G protein-coupled receptor, family C, group 5, member A	NM_003979
	202454_s_at	2.7	Down	ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3	NM_001982
	204798_at	1.6	Up	MYB	v-myb myeloblastosis viral oncogene homolog	NM_005375
	218717_s_at	1.7	Up	LEPREL1	Leprecan-like 1	NM_018192
	209765_at	1.8	Up	ADAM19	ADAM metallopeptidase domain 19	AF311317
	202381_at	1.8	Up	ADAM9	ADAM metallopeptidase domain 9	NM_003816
	203044_at	2.1	Up	CHSY1	Chondroitin sulfate synthase 1	NM_014918

Table 1: Continued.

Functional class	Probe set ID	F.C.	Regulation	Gene symbol	Gene title	Accession number
Extracellular matrix	205479_s_at	2.1	Up	PLAU	Plasminogen activator, urokinase	NM_002658
	210845_s_at	2.1	Up	PLAUR	Plasminogen activator, urokinase receptor	U08839
	201995_at	2.2	Up	EXT1	Exostoses (multiple) 1	NM_000127
	205828_at	3.4	Up	MMP3	Matrix metallopeptidase 3 (stromelysin 1)	NM_002422
	203936_s_at	2.2	Up	MMP9	Matrix metallopeptidase 9	NM_004994
	202620_s_at	2.4	Up	PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	NM_000935
	207316_at	2.8	Up	HAS1	hyaluronan synthase 1	NM_001523
	203343_at	3.2	Up	UGDH	UDP-glucose dehydrogenase	NM_003359
	204620_s_at	4.0	Up	VCAN	Versican	NM_004385
	202766_s_at	5.6	Up	FBN1	Fibrillin 1	NM_000138
	202404_s_at	4.1	Up	COL1A2	Collagen, type I, alpha 2	NM_000089
	201852_x_at	2.9	Up	COL3A1	Collagen, type III, alpha 1	NM_000090
	211980_at	2.4	Up	COL4A1	Collagen, type IV, alpha 1	NM_001845
	221730_at	2.7	Up	COL5A2	Collagen, type V, alpha 2	NM_000393
	207134_x_at	2.2	Up	TPSB2	Tryptase beta 2	NM_024164
	210084_x_at	2.1	Up	TPSAB1	Tryptase alpha/beta 1	AF206665
	214533_at	3.5	Up	CMA1	Chymase 1, mast cell	NM_001836
	205624_at	2.1	Up	CPA3	Carboxypeptidase A3 (mast cell)	NM_001870
	206871_at	3.3	Up	ELA2	Elastase 2, neutrophil	NM_001972
	205653_at	5.0	Up	CTSG	Cathepsin G	NM_001911
	202376_at	1.7	Down	SERPINA3	Serpin peptidase inhibitor, clade A, member 3	NM_001085
	201147_s_at	1.8	Down	TIMP3	TIMP metallopeptidase inhibitor 3	NM_000362
	206243_at	2.8	Down	TIMP4	TIMP metallopeptidase inhibitor 4	NM_003256
Dermal-epidermal junction	216971_s_at	1.5	Down	PLEC1	Plectin 1, intermediate filament binding protein	Z54367
	214292_at	1.5	Down	ITGB4	Integrin, beta 4	AA808063
	210150_s_at	1.5	Down	LAMA5	Laminin, alpha 5	BC003355
Metabolism	207786_at	1.9	Down	CYP2R1	Cytochrome P450, family 2, subfamily R, polypeptide 1	NM_024514
	211019_s_at	2.1	Down	LSS	2,3-oxidosqualene-lanosterol cyclase	D63807
	205676_at	2.5	Up	CYP27B1	Cytochrome P450, family 27, subfamily B, polypeptide 1	NM_000785

Among genes involved in the immune response, upregulated genes play a role in T lymphocyte activation, for example, lymphocyte-activation gene 3 (LAG3) [7] and dipeptidyl-peptidase 4 (DPP4) [8], or in B and T lymphocyte migration, for example, 5′-nucleotidase and ecto-CD73 (NT5E) [9].

Other upregulated genes involved in the immune response belong to the CD40 signalling pathways, including the TNF receptor-associated factor 5 (TRAF5) or play a role in innate immunity such as the killer cell lectin-like receptor subfamily K, member 1 (KLRK1, better known as NKG2D), or SAM domain and HD domain 1 (SAMHD1) [10].

Moreover, a cluster of genes that have a role in the inflammatory process was upregulated. This cluster encompasses the interferon, gamma-inducible protein 16 (IFI16), bradykinin receptor B2 (BDKRB2), chemokine (C-C motif) ligand 5 (CCL5), ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2, also called autotaxin), interleukin 8 (IL8), prostaglandin F receptor (PTGFR), follistatin-like 1 (FSTL1), selectin L (SELL), selectin E (SELE), thrombospondin 1 (THBS1), and serglycin (SRGN).

Moreover, a downregulation of the diacylglycerol kinase, alpha 80 kDa (DGKA) [11], a negative regulator of the respiratory burst in normal polymorphonuclear cells, and of calpain 3 (CAPN3) that downregulates cell migration in resting monocytes, was observed.

Many genes coding for protein involved in apoptosis and/or in apoptosis regulation resulted to be modulated in pathological samples. Among these, several proapoptotic genes were upregulated such as TNF receptor superfamily, member 6 (FAS), tumour necrosis factor (ligand) superfamily, member 10 (TNFSF10) brain abundant, membrane-attached signal protein 1 (BASP1), stress 70 protein chaperone microsome associated (STCH) [12], serine/threonine kinase 17a (STK17A), G1 to S phase transition 1 (GSPT1) and interferon, and alpha-inducible protein 27 (IFI27) [13].

On the other hand, genes coding for the antiapoptotic protein arginine methyltransferase 5 (PRMT5) and DNA-damage-inducible transcript 4 (DDIT4) were downregulated.

Antiproliferative genes were upregulated in DH skin samples including the cell growth regulator with EF-hand domain 1 (CGREF1) and the tumor suppressor genes named proliferation-associated 2G4 (PA2G4/EBP1) [14].

Moreover positive regulators of cell growth, such as MMS19 nucleotide excision repair homolog (MMS19) and v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (ERBB3), resulted downregulated.

Several genes involved in extracellular matrix components synthesis as well as in wound healing and tissue repair were upregulated.

These genes are involved in the synthesis of collagen such as procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), or in the production of hyaluronan as hyaluronan synthase 1 (HAS1) [15].

Four genes coding for different collagen molecules were also upregulated, and these are collagen, type I, alpha 2 (COL1A2), collagen, type III, alpha 1 (COL3A1), collagen, type IV, alpha 1 (COL4A1), and collagen, type V, alpha 2 (COL5A2).

Moreover, we also observed an up-regulation of versican (VCAN) [16] and fibrillin 1 (FBN1) genes.

When we analyzed genes involved in extracellular matrix remodeling, we observed an upregulation of several proteases such as matrix metallopeptidase 3 (MMP3) [17], matrix metallopeptidase 9 (MMP9), ADAM metallopeptidase domain 9 (ADAM9), ADAM metallopeptidase domain 19 (ADAM19), plasminogen activator, urokinase (PLAU) [18], and its receptor PLAUR.

Moreover, among proteolytic enzymes, we found an increased expression of genes coding for proteins that belong to the neutrophil and mast cell secretory repertoire such as tryptase alpha/beta 1 (TPSAB1), tryptase beta 2 (TPSB2), chymase 1 (CMA1), carboxypeptidase A3 (CPA3), elastase 2 (ELA2), and cathepsin G (CTSG).

On the contrary, the alpha-1 antiproteinase (SERPINA3) and the metallopeptidase inhibitors 3 and 4 (TIMP3 and TIMP4) were downregulated.

Three genes coding for protein that are present at the dermal-epidermal junctions were downregulated. These transcripts are plectin 1, intermediate filament binding protein 500 kDa (PLEC1) [19], integrin, beta 4 (ITGB4), and laminin, alpha 5 (LAMA5).

4. Discussion

Despite the huge effort in elucidating the pathogenesis of DH, a detailed understanding of the molecular events involved in DH lesion formation is still lacking. In the present work we provide for the first time a comprehensive analysis of the transcriptome within DH lesional skin.

First of all, we observed the modulation of genes, that are involved in the regulation of both immune response and inflammation.

Consistently with the autoimmune origin of DH, we found an overexpression of genes involved in T and B immune response (LAG3, TRAF5, DPP4, and NT5E) [7–9].

Lymphocyte activation gene-3 (LAG-3; CD223) is a negative costimulatory receptor that modulates T-cell homeostasis, proliferation, and activation; it is a CD4 homolog that is required for maximal regulatory T-cell function and for the control of CD4(+) and CD8(+) T cell. Interestingly, it may be required for the control of autoimmunity [20, 21]. In this setting, the overexpression of LAG-3 can be considered a mechanism to control the autoimmune response.

Many proinflammatory genes were found to be upregulated in DH samples and some of them with high fold changes (Table 1). These transcripts included IFI16, a gene that is activated by oxidative stress and mediates ICAM-1 stimulation by TNF-alpha [22], FSTL1, a proinflammatory protein enhancing IFN-gamma pathway [23, 24], PTGFR, the receptor of prostaglandin F2 alpha that is thought to be increased in skin blisters of DH [25], and the chemokine CCL5 and the bradykinin receptor BDKRB2, both involved in inflammatory cell recruitment and proinflammatory cytokine production [26].

Particular attention deserves the upregulation of selectin-E (SELE) and IL8 (Table 1); indeed Hall et al. [27] demonstrated that patients with DH have an increased serum level of IL-8 that is associated with cutaneous endothelial cell activation and increased expression of SELE [28]. IL-8 triggers inflammatory leukocyte recruitment as well as angiogenesis and cell proliferation [29, 30]. Human neutrophils are the major components of DH inflammatory infiltrate and are able to produce high levels of IL-8 in response to various inflammatory stimuli. Their ability to firmly adhere to the endothelium prior to roll and extravasate into tissue requires the expression of adhesion proteins such as SELE that are expressed at low level on resting endothelial cell surfaces [31, 32]. Interestingly, SELE is upregulated in DH skin samples indicating local endothelial cell activation.

Hall et al. [28] hypothesized that the presence of mucosal inflammation in the gut of patients with DH may be critical in priming both neutrophils and cutaneous endothelial cells through the production of elevated levels of proinflammatory cytokines such as IL8. Our results indicate also a local production of IL8 most probably released by activated neutrophils.

Interestingly, we found overexpression of ENPP2/autotaxin, a molecule that exacerbates inflammation by increasing chemotaxis through the upregulation of neutrophil integrins [33]. We also observed an increased expression of SRGN/serglycin that is important for the retention of key inflammatory mediators inside neutrophil storage granules and secretory vesicles [34].

The downregulation of the two anti-inflammatory genes, DGKA and CAPN3, may be also linked to increased neutrophil migration [35].

Apoptosis is thought to play a role in the pathogenesis of cutaneous lesions, and increased apoptotic events in basal and suprabasal keratinocytes were observed within lesional and perilesional skin of DH [36]. Consistently with this observation, we found overexpression of proapoptotic genes

such as FAS and TNFSF10/TRAIL and downregulation of two antiapoptotic transcripts, namely, PRMT5 and DDIT4. These genes may be correlated also to the unique form of apoptotic cell death of neutrophils, called "NETosis," that has been recently associated with autoimmune phenomena in systemic lupus erythematosus and possibly in other autoimmune diseases [37]. Since neutrophils play a pivotal role in DH skin lesions, we can speculate that NETosis may play a role also in DH.

We noticed a remarkable modulation of genes coding for several components of the extracellular matrix such as collagen type III, IV, and V. An elevated level of collagen type III, IV, and V has been described in the DH blisters of the papillary derma [38]. The gene coding for fibrillin (FBN1) was also upregulated in our DH skin samples. This protein constitutes the major backbone of multifunctional microfibrils in elastic and nonelastic extracellular matrices and may be one of the structural components bound by IgA-reactive deposits in the skin of patients with DH [39].

Matrix degradation at the dermal-epidermal junction has been thought to contribute to DH blister formation [40]. During this assault to the extracellular components, proteases secreted by keratinocytes, macrophages, and neutrophils act in concert.

We found an increased expression of neutrophil and mast cell enzymes such as TPSB2, TPSAB1, CMA1, CPA3, ELA2, and CTSG that are thought to be involved in the splitting up of epidermis from dermis [41]. Noteworthy high levels of ELA2 have been described in vesicle fluid obtained from patients with DH [42].

Proteases secreted by granulocytes and mast cells could mediate the development of DH cutaneous lesions either directly or indirectly by the activation of metalloproteases [43].

Several genes coding for metalloproteases resulted upregulated in our DH skin samples including MMP3/stromelysin, MMP9/gelatinase B, ADAM9/meltrin gamma, and ADAM19/meltrin beta. It has been demonstrated that MMP3 participates to blister formation by degrading basement membrane components [17].

Airola et al. reported an increased secretion of this enzyme by basal keratinocytes surrounding neutrophil abscesses [17]. MMP9 is another molecule produced by eosinophils and neutrophils that are attracted to the basement membrane zone by integrins and selectins.

It has been suggested that the formation of blisters may be induced by an overexpression of local enzymes [43], and indeed the results of our gene array experiments indicate an increased production of proteolytic enzymes within the skin lesions. Macrophage metalloelastase (MMP12) was found abundantly expressed in subepithelial macrophages of DH skin lesions by *in situ* hybridisation [44]. The MMP12 transcript was also detected in all our samples; however, only in 4/6 DH samples, the level of expression of this enzyme was significantly upregulated when compared to the controls. For this reason, MMP12 has not been included in the list of upregulated genes. This discrepancy could be ascribed to the different detection methods used (*in situ* hybridisation versus gene array). In the paper by Salmela et al. [44] the increased mRNA expression of MMP12 was confined to subepithelial macrophages. This increase may be diluted in mRNA samples derived from the total biopsy specimen composed by a large number of different cell types. Moreover, the relative content of macrophages may vary in the different skin biopsies used for the gene array analysis.

Interestingly we found a strong downregulation of genes coding for tissue inhibitors of proteases such as SERPINA3, TIMP3 and TIMP4.

Therefore, our gene analysis confirms that an important role in the maintenance and amplification of the immunological processes underlying blister formation may be played by an imbalance between the activities of MMPs and their tissue inhibitors, as previously hypothesised by Zebrowska et al. [45].

Another molecule involved in the degradation of basement membrane is the plasminogen activator urokinase (PLAU) that has been found to be highly expressed in keratinocytes in experimentally induced DH lesions [18]. This molecule may also have an activating role in MMP9 in early phase of blister formation [43]. Interestingly, PLAU and its receptor PLAUR were upregulated in our DH skin samples; moreover, we found an increased expression of thrombo spondin 1 (THBS1). THBS1 can downregulate PAI, the inhibitor of plasminogen activator [46], thus eventually reinforcing the final physiological effect of the PLAU overexpression.

It is tempting to speculate that an overexpression of PLAU may lead to increased production of plasmin that in turn activates MMP9, as seen in experiments carried out in mice [47].

We also observed a downregulation of genes coding for proteins involved in the network that anchor the keratin filaments of cells cytoskeleton to the underlying dermis at the dermal-epidermal junctions. These molecules are: PLEC/plectin 1, ITGB4, and LAMA5/laminin alpha 5.

Plectin is a large 200 nm long protein found in hemidesmosomes and whose function is to bind keratin intermediate filaments to the hemidesmosome, and specifically to transmembrane collagen XVII and $\beta 4$ integrins [19]. It has been demonstrated that a defective expression of plectin/HD1 may predispose to blister formation in human skin [48].

Laminin 5 is essential for adhesion of keratinocytes to basement membrane [18], and integrins such as ITGB4 are the main laminin receptor [49].

In DH skin lesions, proteins within the dermal epidermal junction are target of proteolytic enzymes released by neutrophils. In addition, the decreased expression of the abovementioned molecules might worsen the damage induced by granulocytic enzymatic activity.

Overall, the results obtained support the hypothesis that during blister development, the inflammatory reaction evoked by the autoimmune response typical of the disease is associated to a local overexpression of proteolytic enzymes leading to the detachment of the dermal-epidermal junction. The consequent tissue damage may be amplified by a reduced production of protease inhibitors.

Moreover, our data suggest that an increased rate of apoptosis and a reduced expression of anchoring proteins

at dermal-epidermal junction are key features in DH skin lesions.

In conclusion, we believe that our study on gene expression gives a better understanding of the molecular mechanisms involved in the pathogenesis of skin lesions in DH.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Gene-Expression-Guided Selection of Candidate Loci and Molecular Phenotype Analyses Enhance Genetic Discovery in Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is a highly heterogeneous autoimmune disorder characterized by differences in autoantibody profiles, serum cytokines, and clinical manifestations. We have previously conducted a case-case genome-wide association study (GWAS) of SLE patients to detect associations with autoantibody profile and serum interferon alpha (IFN-α). In this study, we used public gene expression data sets to rationally select additional single nucleotide polymorphisms (SNPs) for validation. The top 200 GWAS SNPs were searched in a database which compares genome-wide expression data to genome-wide SNP genotype data in HapMap cell lines. SNPs were chosen for validation if they were associated with differential expression of 15 or more genes at a significance of $P < 9 \times 10^{-5}$. This resulted in 11 SNPs which were genotyped in 453 SLE patients and 418 matched controls. Three SNPs were associated with SLE-associated autoantibodies, and one of these SNPs was also associated with serum IFN-α ($P < 4.5 \times 10^{-3}$ for all). One additional SNP was associated exclusively with serum IFN-α. Case-control analysis was insensitive to these molecular subphenotype associations. This study illustrates the use of gene expression data to rationally select candidate loci in autoimmune disease, and the utility of stratification by molecular phenotypes in the discovery of additional genetic associations in SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a severe multisystem autoimmune disease of unknown etiology. Genetic factors clearly play a role in susceptibility, and a number of genetic loci have been implicated in the disease [1]. Despite the successes of recent genetic association studies, only a fraction of the genetic liability for SLE has been explained to date. SLE is a heterogeneous disease clinically, and there is strong evidence that the molecular pathogenesis of the condition varies considerably between individuals as well. For example, specific autoantibodies are formed in some patients and not others, and these autoantibody specificities have been associated with clinical features of the disease [2, 3]. In addition, approximately half of adult patients with SLE demonstrate overactivity of the interferon alpha (IFN-α) pathway in their peripheral blood [2, 4]. Interestingly, high IFN-α and SLE-associated autoantibodies are heritable as traits in SLE families and can be found in family members who are not affected by SLE [5, 6]. Autoantibodies can be found in sera for many years prior to the clinical diagnosis of SLE [7], and it is thought that some of the autoantibodies may be themselves directly pathogenic. IFN-α is a cytokine involved in viral defense, capable of bridging the innate and adaptive immune systems [8]. Interestingly, when recombinant human IFN-α has been given as a treatment for chronic

viral hepatitis, some treated individuals have developed de novo SLE, which frequently resolves upon discontinuation of the IFN-α [9, 10]. These data support the concept that both IFN-α and SLE-associated autoantibodies represent causal factors in human SLE. Additionally, both IFN-α and SLE-associated autoantibodies are heritable within SLE families supporting a genetic contribution, and thus the idea that these molecular measurements could be used as a phenotype in genetic studies.

In previous work, we have begun to map genetic variants which are associated with high IFN-α and with the presence of particular autoantibodies in SLE patients [11–13]. Some well-established genetic risk factors for SLE have been associated with one or both of these molecular phenotypes [14–18]. In addition, we have performed a genome-wide association study (GWAS) using these two molecular traits as phenotypes to enable discovery of novel genetic variants associated with IFN-α and SLE-associated autoantibodies [19]. A number of novel genes have been validated from this screen to date [19, 20], although much of the variance in both serum IFN-α and the presence or absence of particular autoantibodies remains to be explained.

In prioritizing genetic variants to be followed up in our GWAS scan, we used gene ontogeny and expert literature search to prioritize variants which were in or near genes related to immune responses. This was based upon the supposition that SLE is an autoimmune disease, and many of the well-validated loci which have emerged from unbiased studies to date encode genes with immune function. This approach has some limitations, as genetic variations which were not near known genes were not prioritized, nor were those which did not have known function within the immune system. It is clear that genetic variants can sometimes impact the expression of a gene which is not nearby, and these genetic variants may be assigned to irrelevant nearby genes in gene ontogeny analysis. Additionally, many genes which could be critical to human disease pathogenesis may still be unstudied and unknown, and thus unlikely to be prioritized in follow up candidate studies.

To address these possibilities in our GWAS validation, we searched our top 200 SNPs in a public database which links genome-wide SNP data from the HapMap project to genome-wide gene expression data from the HapMap lymphoblastoid B-cell lines (SCAN) database, [21]. Genes which are disease associated are more commonly associated with alternate gene expression than genes which are not disease associated [22], and thus genes from our top 200 which were strongly associated with differences in gene expression should be more likely to be true associations. In this study, we leverage gene expression data sets to prioritize additional candidates from our trait-stratified GWAS for validation in an independent cohort. We found eleven SNPs which were significantly associated with alternate gene expression of multiple transcripts in public databases, and had not been prioritized for followup in our initial GWAS screen. Four of these eleven SNPs were significantly associated with the important molecular subphenotypes IFN-α and SLE-associated autoantibodies in our independent validation cohort, validating this method of genetic discovery.

2. Methods

2.1. Initial GWAS Study Description. The initial cohort of SLE patients studied in the GWAS scan was obtained from the Hospital for Special Surgery Lupus Registries, and consisted of 104 SLE patients [19]. This study was designed as a case-case analysis to compare SNP frequencies in SLE patients with high versus low IFN-α and those with and without SLE-associated autoantibodies. Patients were selected in an extremes-of-phenotype design from the top 33% and bottom 33% of serum IFN-α activity and were additionally stratified for the GWAS study by ancestry and the presence or absence of anti-RBP or anti-dsDNA antibodies. A study design incorporating multiple ancestral backgrounds was chosen as both autoantibodies and serum IFN-α levels are heritable pathogenic factors which are shared between all ancestral backgrounds. The top 200 SNPs were examined in detail using expert review of public databases, and seven top SNPs chosen for replication using a gene-centric algorithm demonstrated strong associations with either serology or serum IFN-α in an independent cohort, as would have been expected based upon the initial GWAS study design [19].

2.2. Validation Cohort. The independent validation cohort of 453 SLE patients was obtained from the University of Chicago Translational Research in the Department of Medicine (TRIDOM) registry and Rush University Medical Center and consisted of 282 African-American and 171 European-American SLE patients. All patients met the revised 1982 ACR criteria for the diagnosis of SLE [23]. Samples from 418 controls were obtained from the TRIDOM registry, including 300 African-American and 118 European-American subjects who were individually screened for the absence of autoimmune disease by medical record review. The subjects in this study were not related to each other. Informed consent was obtained from all subjects at each site, and the study was approved by the IRB at each institution.

2.3. SCAN Database Query. We searched the top 200 SNPs from the GWAS described above as query terms in the SNP and CNV Annotation (SCAN) database (http://www.scandb.org/) [21]. This database is a searchable index of genome-wide gene expression data linked to genome-wide SNP genotype data from the HapMap project. Gene expression data is derived from studies in which gene expression arrays were run on Epstein-Barr virus-transformed lymphoblastoid cell lines from individuals genotyped in the HapMap project. The SCAN database contains expression data from both European (Centre d'Etude du Polymorphisme Humain or CEPH) and West African (Yoruba or YRI) HapMap reference populations. We used a threshold P value of $P < 9 \times 10^{-5}$ and searched both CEPH and YRI population datasets for each SNP. Because SNPs associated with alternate gene expression are more likely to be disease or trait associated [22], we selected SNPs which were associated with alternate expression of 15 or more transcripts in the SCAN database. This resulted in 11 SNPs,

and for each SNP at least one of the 15 or more associated transcripts was involved in immune function.

2.4. SNP Genotyping in the Validation Cohort. Individuals in the validation cohort were genotyped at the rs9521996, rs11199974, rs7785392, rs9568401, rs4892122, rs4778708, rs1340981, rs1408806, rs4894215, rs1569428, and rs1159916 SNPs. Genotyping was performed using ABI TaqMan Assays-by-Design primers and probes on an ABI 7900HT PCR machine with >98% genotyping success. Scatter plots were all reviewed individually for quality, and genotype frequencies did not deviate significantly from the expected Hardy-Weinberg proportions ($P > 0.01$ in controls across all ancestral backgrounds).

2.5. Reporter Cell Assay for IFN-α. The reporter cell assay for IFN-α has been described in detail elsewhere [5, 24]. Reporter cells were used to measure the ability of patient sera to cause IFN-induced gene expression. The reporter cells (WISH cells, ATCC #CCL-25) were cultured with 50% patient sera for 6 hours and then lysed. mRNA was purified from cell lysates, and cDNA was made from total cellular mRNA. cDNA was then quantified using real-time PCR using an Applied Biosystems 7900HT PCR machine with the SYBR Green fluorophore system. Forward and reverse primers for the genes *MX1*, *PKR*, and *IFIT1*, which are known to be highly and specifically induced by IFN-α, were used in the reaction [5]. *GAPDH* was amplified in the same samples to control for background gene expression. The amount of PCR product of the IFN-α-induced gene was normalized to the amount of product for the housekeeping gene *GAPDH* in the same sample. The relative expression of each of the three tested IFN-induced genes was calculated as a fold increase compared to its expression in WISH cells cultured with media alone. Results from the IFN-α assay were standardized to a healthy multiancestral reference population as previously described, and a serum IFN-α activity score was calculated based upon the mean and SD of the reference population [5]. This assay has been highly informative when applied to SLE as well as other autoimmune disease populations [5, 25–27].

2.6. Measurement of Autoantibodies. Antibodies to anti-Ro, anti-La, anti-Sm, and anti-RNP were measured in all samples by ELISA methods using kits from INOVA Diagnostics (San Diego, CA), and anti-dsDNA antibodies were measured using *Crithidia luciliae* immunofluorescence, with detectable fluorescence considered positive. All samples were assayed in University of Chicago clinical laboratory by the same personnel that test clinical samples. For the ELISA assays, the standard cutoff points for a positive test designated by the manufacturer were used to categorize samples as positive or negative.

2.7. Statistical Analysis. To control for population structure and effects related to admixture, we used a principal component analysis of SNPs which varied in frequency by ancestral background. All subjects in the study had genotype data

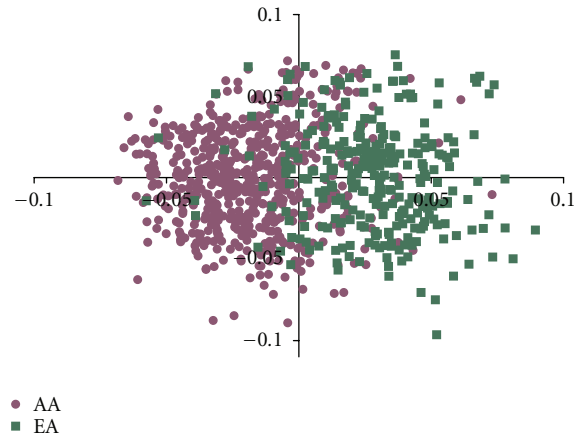

Figure 1: Principal component analysis of SNPs genotyped in all cases and controls. Component 1 is shown on the x-axis, and component 2 is shown on the y-axis. Each dot represents one subject, and the dots are color-coded by the self-reported ancestry of that subject.

available for 30 such SNPs, and principal component analysis was performed using the PCA option in the Cluster program by Eisen et al. [28]. The first two principal components are shown plotted on the x and y axes, respectively, in Figure 1, and the first component provides a strong separation of those subjects of self-reported African-American ancestry from those of self-reported European-American ancestry. We included the first and second principal components as covariates in all subsequent association analyses to provide control for differences in proportional ancestry in both cases and controls.

Logistic regression models were used to detect associations with SLE in case-control analysis or in case-case analyses examining the SLE-associated autoantibody traits and serum IFN-α activity. The SLE-associated autoantibodies anti-Ro, anti-La, anti-Sm, anti-RNP, and anti-dsDNA were all tested for association with each SNP in logistic regression models. Serum IFN-α was binned as high or low, using 2SD above the mean of healthy donor sera as the cutoff point, and then used as the outcome variable in logistic regression. Significant relationships observed in this regression were then explored by comparing quantitative IFN-α data between genotype categories. The IFN-α data was nonnormally distributed, and nonparametric Mann-Whitney U was used to compare quantitative IFN-α data between genotype subgroups. P values shown in the paper are uncorrected for multiple comparisons. To establish significance and account for multiple comparisons, we used a threshold P value of $P < 4.5 \times 10^{-3}$ to allow for a type I error rate of 0.05 following a Bonferroni correction for the number of SNPs tested in this study.

3. Results

3.1. Three of Eleven SNPs Demonstrate Association with Autoantibody Traits in SLE Patients. We used logistic regression to detect associations between autoantibody traits and

Table 1: Summary of SNPs associated with autoantibody traits.

SNP	Chr.	Nearby Gene	Ancestry	Autoantibody	Odds ratio	P value
rs9521996 C	13	ANKRD10	AA	Anti-RNP	2.01	8.0×10^{-4}
rs1408806 G	9	TYRP1	EA	Anti-Sm	3.48	1.5×10^{-3}
rs4894215 G	2	—	EA	Anti-Ro	2.16	2.5×10^{-3}

SNP: single nucleotide polymorphism, chr.: chromosome, autoantibody: the antibody specificity associated with the particular SNP, odds ratio and P-value are calculated from the logistic regression model.

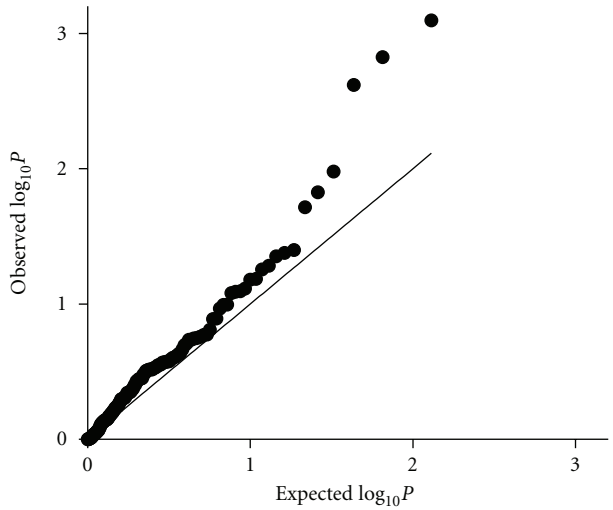

Figure 2: Q-Q plot showing the observed versus expected P values in the autoantibody analysis. P values that would be expected under the null hypothesis (no association between SNPs and autoantibody traits) are represented by the line, and the observed P values are represented by dots, one for each tested SNP-autoantibody association.

genotype at each of the 11 SNPs in each ancestral background separately. Three SNPs demonstrated associations which would withstand a Bonferroni correction for multiple comparisons correcting for the number of SNPs tested ($P < 4.5 \times 10^{-3}$, Table 1). Figure 2 shows a Q-Q plot of the distribution of probabilities observed in the antibody analyses versus the null distribution. In Figure 2, the top three SNP-antibody associations highlighted in Table 1 are represented by the three dots with the highest values on the y-axis which clearly deviate from the null distribution.

3.2. Two SNPs Are Associated with Serum IFN-α in SLE Patients. Regression models were also used to assess the association of each of the 11 SNPs with serum IFN-α activity in SLE patients. An association was observed between rs9568401 G and high serum IFN-α in both African and European Americans. In European-Americans, the rs1408806 G allele which was associated with anti-Sm antibodies was also associated with increased serum IFN-α. These associations are illustrated in Figure 3, which shows quantitative IFN-α by genotype category. The minor allele frequency of each SNP was relatively low, and thus minor allele homozygotes were rare and are combined with heterozygotes in this graph. Dominant or recessive models could not be assessed due to the rarity of homozygous minor allele subjects, and the graph is not meant to represent a dominant relationship. While the rs1048806 SNP is also associated with an autoantibody trait, the rs9568401 SNP was not associated with any autoantibodies and was exclusively associated with serum IFN-α activity.

3.3. Multiple Subphenotype Modeling Supports Complex Association Patterns between Genetic Variants, Autoantibodies, and Serum IFN-α Activity. With regard to the rs1408806 G allele which was associated with both serum IFN-α and anti-Sm in European ancestry, the association between these two phenotypes appeared to be independent (Figure 4(a)). Given the strong relationship between serum IFN-α and autoantibodies in SLE [4], we also examined serum IFN-α in the context of the other SNP-autoantibody relationships we had identified rs9521996/anti-RNP in African Americans and rs4894215/anti-Ro in European-Americans, (Figures 4(b) and 4(c) resp.). Both of these SNPs demonstrated evidence for a secondary association with serum IFN-α which was dependent upon the associated autoantibody. Summarizing the four SNPs which demonstrate significant associations following multiple comparison corrections, one SNP is associated with serum IFN-α alone, two are associated with autoantibody profiles which are associated with higher IFN-α, and one SNP is associated with both serum IFN-α and autoantibody profile independently. These relationships are depicted in Figure 5.

3.4. SCAN Database Search Results Predicted the Ancestral Background in Which the SLE Phenotype Association Was Observed. The SCAN database search examined both European- and African-derived populations, and the SNPs which were associated with SLE subphenotypes were associated with alternate gene expression in the SCAN database in only one ancestral background. In each of the autoantibody associations, the ancestral background in which the autoantibody association was observed in SLE patients was the same ancestral background in which differential gene expression was observed in the SCAN database (Table 2). The association between rs9568401 and serum IFN-α was observed in both ancestral backgrounds, but the SNP was only associated with alternate gene expression in the SCAN database in African ancestry subjects. Overall this general concordance in ancestral backgrounds between the SLE phenotype associations further supports the idea that the SNPs which impact gene expression in human cell lines are more likely to be associated with molecular phenotypes in human

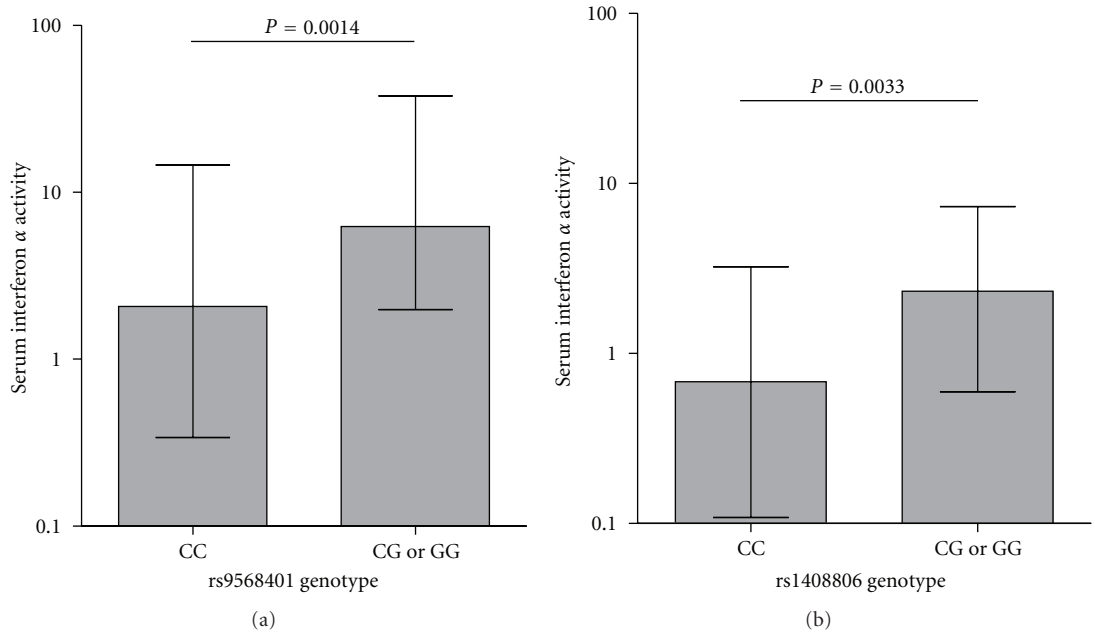

Figure 3: Serum IFN-α activity in SLE patients stratified by SNP genotype at rs9568401 (a) and rs1408806 (b). Minor allele homozygotes are combined with heterozygotes on the graph. Bars show the median error bars show the interquartile range. P value by Mann-Whitney U test.

disease. Representative transcripts that were differentially regulated by each associated SNP in the SCAN database are also shown in Table 2.

3.5. Case-Control Analysis Does Not Show Large Differences in Allele Frequencies When Comparing All SLE Patients to Controls. As shown in Table 3, we did not observe significant case-control associations for any of the 11 studied SNPs which would withstand statistical correction for multiple comparisons (all $P > 4.5 \times 10^{-3}$). The initial GWAS was designed to detect associations with either autoantibodies or serum IFN-α, and the SNPs we followed up were most strongly associated with these traits. The lack of strong case-control associations at the same SNPs supports the idea that the genetic effects we observe are relevant to patient subsets, and that the power to discover these SNPs would be more limited in a standard case-control study design.

4. Conclusions

In this study, we identify novel genetic variants associated with molecular phenotypes in SLE in two different ancestral backgrounds, using gene expression data as a guide for rational candidate gene selection from a previous GWAS study. In published overall case-control studies of SLE to date, there are examples of both shared associations across ancestral backgrounds [29], and associations which are particular to one or a few ancestral backgrounds [20, 30, 31]. In our study, it is striking that we did not find many associations which were shared between ancestral backgrounds and the majority were distinct to one ancestral background, despite studying molecular phenotypes which are shared across ancestral backgrounds. This would support the hypothesis that while similar molecular pathways may be dysregulated in SLE patients of different ancestral backgrounds, the particular steps in that pathway which are dysregulated may differ by ancestry. These differences would be important to appreciate as we envision personalized therapy using agents which target these pathways, such as the category of anti-IFN-α drugs which are being developed for SLE currently. Presumably many autoimmune disease risk alleles which are common in the population have been maintained due to some benefit in increasing immune responses in response to pathogens. Infectious disease has been a major selective force in human history, and it seems likely that different world populations may have developed and selected for different immune system polymorphisms which could result in a similar end pathway output. A striking example of this type of human convergent evolution has been shown in the case of the human lactase gene [32], in which lactase persistence in adulthood was conferred by a number of different polymorphisms that had arisen separately in different world populations, converging upon a similar end pathway result.

Heterogeneity is not unexpected in SLE, as clinically the syndrome is very diverse. Overall case-control genetic studies are likely to be significantly limited due to heterogeneity, as different polymorphisms will be more or less relevant in different patient groups. In the case of physical phenotypes, a number of studies support the idea that different genetic variants will be associated with particular clinical disease manifestations, such as rash, renal disease, and others [33–36]. Diversity in autoantibody and cytokine phenotypes

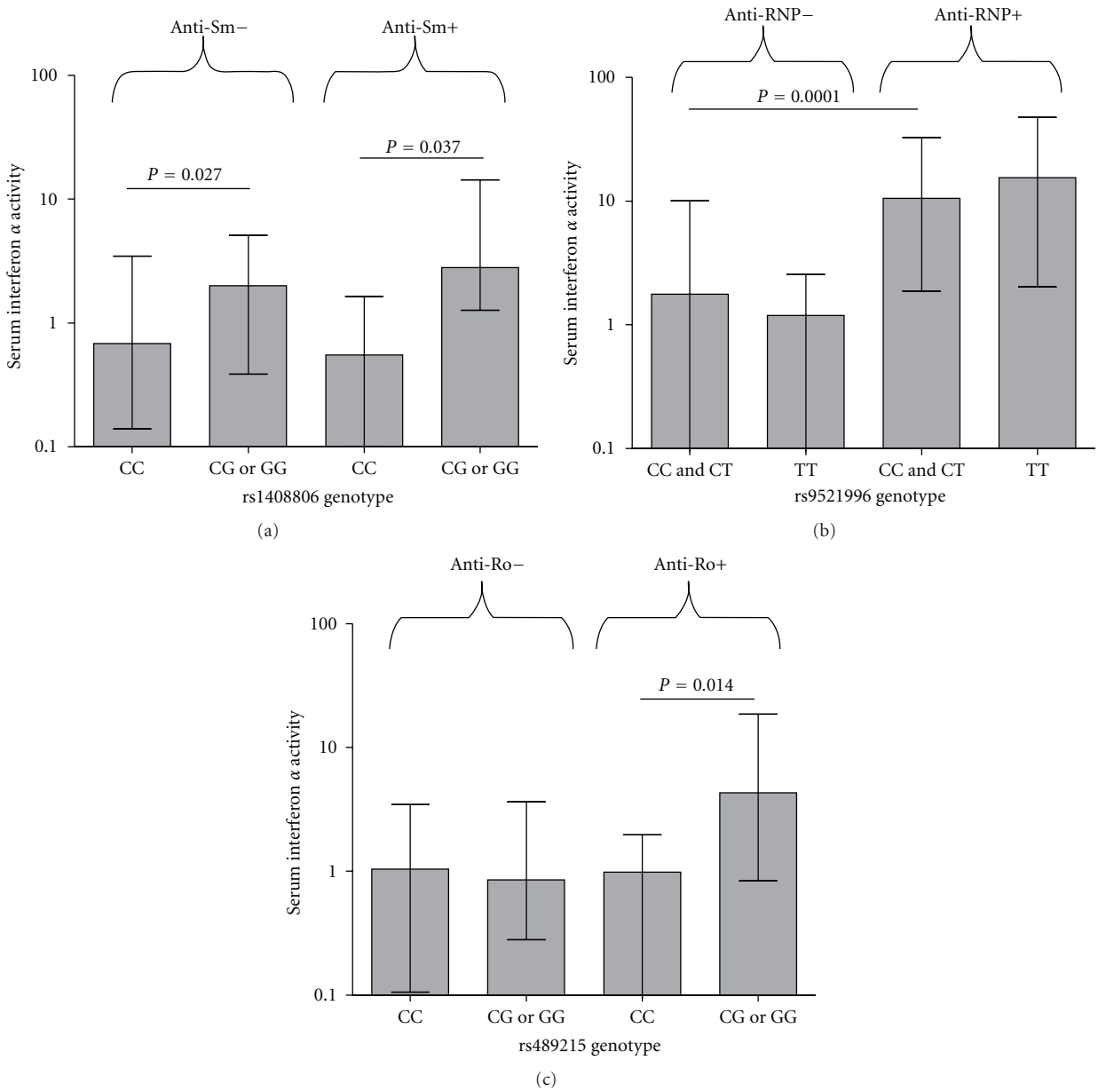

Figure 4: Serum IFN-α activity in SLE patients stratified by SNP genotype and the autoantibody associated with that particular SNP. Minor allele homozygotes are combined with heterozygotes on the graph. Bars show the median error bars show the interquartile range. P value by Mann-Whitney U test.

Table 2: Summary of the 4 SNPs associated with SLE phenotypes and the SCAN database results regarding ancestral background and representative associated transcripts.

SNP	Chr.	Nearby Gene	SLE association ancestry	Associated phenotype	SCAN ancestry	Representative SCAN transcripts
rs9521996 C	13	ANKRD10	AA	Anti-RNP	YRI	IRF3, MIF
rs1408806 G	9	TYRP1	EA	Anti-Sm	CEPH	CASP3, RIPK1
rs4894215 G	2	None within 200kb	EA	Anti-Ro	CEPH	HLADRB1, HLADQB1
rs9568401 G	13	DLEU2	EA, AA	IFN	YRI	IRAK2, NOD2

SNP: single nucleotide polymorphism, chr.: chromosome, SLE association ancestry: the ancestral background in which the SNP was associated with an SLE phenotype, SCAN ancestry: the ancestral background in which that SNP was associated with alternate gene expression, representative SCAN transcripts: genes which differentially expressed due to genotype at that SNP in the SCAN database; two transcripts of the >15 were chosen for inclusion in this table, with an emphasis on those transcripts with immune system relevance.

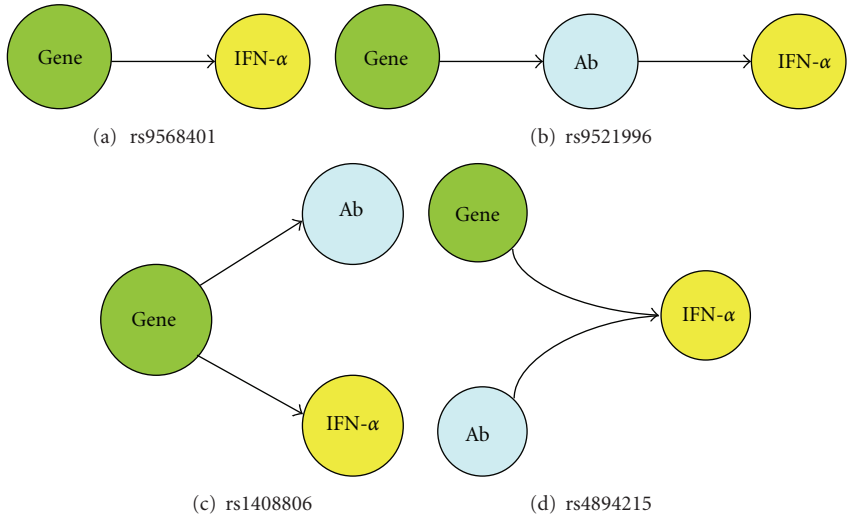

Figure 5: Diagrams depicting patterns of association observed between SNP genotype, autoantibodies, and serum IFN-α. Gene = SNP genotype at the indicated SNP, Ab = the particular autoantibody associated with that SNP, and arrows indicate the associations observed in the study.

Table 3: Case control analysis of 11 SNPs tested in this study in each ancestral background.

SNP	African Americans			European Americans		
	MAF	OR	*P* value	MAF	OR	*P*-value
rs9521996 C	0.285	1.02	0.86	0.136	1.46	0.12
rs11199974 G	0.258	0.89	0.44	0.482	1.11	0.56
rs7785392 T	0.473	0.80	0.084	0.612	0.78	0.16
rs9568401 G	0.122	0.74	0.12	0.085	1.04	0.90
rs4892122 G	0.279	1.14	0.32	0.295	1.19	0.39
rs4778708 T	0.407	0.95	0.68	0.268	1.10	0.64
rs1340981 A	0.161	0.92	0.61	0.397	0.88	0.47
rs1408806 G	0.174	0.80	0.19	0.246	0.85	0.44
rs4894215 G	0.358	0.94	0.64	0.430	1.08	0.67
rs1569428 G	0.341	0.70	0.0070	0.430	0.92	0.68
rs1159916 C	0.405	0.74	0.018	0.333	0.88	0.49

SNP: single nucleotide polymorphism, MAF: minor allele frequency in controls, OR: odds ratio, as calculated from the logistic regression model.

between SLE patients is also well recognized [4, 37, 38]. In this study we examine these two molecular phenotypes and find genetic associations which are relevant to subgroups of patients defined by these molecular characteristics. We have previously demonstrated strong subsetting of genetic risk related to molecular phenotypes in SLE in the case of the IRF5 gene. The majority of the genetic risk of SLE related to IRF5 was found within a particular serologic subgroup which constituted 40% of the overall SLE patient group studied [14]. This gene had been well validated as an SLE-risk gene in previous overall case-control studies [39, 40], but was later shown to have a very strong subgroup effect [14]. It seems likely that this phenomenon will be more widespread, and that many genetic loci could be very difficult to discover in an overall unstratified case-control study. Other autoimmune diseases such as rheumatoid arthritis have already set a strong precedent for the importance of serologic subsets in genetic analysis. The anti-CCP antibody positive versus negative groups of rheumatoid arthritis patients demonstrate large differences in genetic association, including the HLA region [41]. The genes we report in this study have not been previously identified in case-control studies, and in our case-control analysis of these loci support we do not see large overall allele frequency differences. This does not mean that the loci are irrelevant, as they clearly impact important pathogenic subphenotypes in SLE. Instead, this supports the idea that "all cases versus all controls" study designs will have limits, and it is unlikely that we will be able to fully map the genetic architecture of complex diseases fully using only case-control designs, even if very large and well-powered cohorts are used. In summary, it seems likely that both physical or clinical phenotypes as well as molecular phenotypes will need to be incorporated in genetic study designs to address disease heterogeneity and enable continued genetic discovery in autoimmune disease.

Another benefit of including molecular subphenotypes and gene expression into genetic association studies is that the genetic loci discovered in this manner are immediately linked to some biological alteration. This is especially useful when genes which have not been previously studied are implicated, or if a particular associated genetic variant is not within or near a known gene. If these variants are found in an overall case-control analysis, it can be difficult to plan follow-up functional experiments if little is known about the function of that gene. In our study, we found SNPs which were not in obviously relevant genetic regions, but nonetheless impacted upon important molecular phenotypes and altered expression of immune system molecules. While we cannot know the mechanism by which the genetic variant impacts upon gene expression via our current study, these questions can be followed up and validated in functional experiments.

Conflict of Interest

The authors report no conflict of interests.

Acknowledgments

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Research Article

Tolerogenic versus Inflammatory Activity of Peripheral Blood Monocytes and Dendritic Cells Subpopulations in Systemic Lupus Erythematosus

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Abnormalities in monocytes and in peripheral blood dendritic cells (DC) subsets have been reported in systemic lupus erythematosus (SLE). We aim to clarify the tolerogenic or inflammatory role of these cells based on ICOSL or IFN-α and chemokine mRNA expression, respectively, after cell purification. The study included 18 SLE patients with active disease (ASLE), 25 with inactive disease (ISLE), and 30 healthy controls (HG). In purified plasmacytoid DC (pDC) was observed a lower ICOSL mRNA expression in ASLE and an increase in ISLE; similarly, a lower ICOSL mRNA expression in monocytes of ALSE patients was found. However, a higher ICOSL mRNA expression was observed in ASLE compared to HG in myeloid DCs. Interestingly, clinical parameters seem to be related with ICOSL mRNA expression. Regarding the inflammatory activity it was observed in purified monocytes and $CD14^{-/low}$ $CD16^{+}$ DCs an increase of CCL2, CXCL9, and CXCL10 mRNA expression in ASLE compared to HG. In myeloid DC no differences were observed regarding chemokines, and IFN-α mRNA expression. In pDC, a higher IFN-α mRNA expression was observed in ASLE. Deviations in ICOSL, chemokine, and IFN-α mRNA expression in peripheral blood monocytes and dendritic cells subpopulations in SLE appear to be related to disease activity.

1. Introduction

Systemic lupus erythematosus (SLE) is a multisystemic disease resulting from an abnormal immunological function that affects several organ systems characterized by a broad spectrum of clinical manifestations and a multitude of cellular abnormalities. The primary pathological findings in SLE patients are inflammation, vasculitis, immune complex deposition, and vasculopathy [1–3]. The exact etiology still remains unclear; however defective clearance of apoptotic material and/or aberrant apoptosis, in combination with susceptible genetic background have been suggested to be involved in SLE development and progression [4–6].

SLE patients exhibit numerous aberrations in the immune system, comprising B cells, T cells, monocytes, and dendritic cells, resulting in B and T cell activation and consequent autoantibodies production against a large variety of autoantigens [2].

Abnormalities in monocyte phenotype and function have been identified in several autoimmune disorders, including SLE, which could contribute to disease pathogenesis [7, 8]. Likewise, dendritic cells (DCs) subsets are also implicated in SLE pathogenesis and progression [4, 9]. Recent studies have described alterations in the number of peripheral blood (PB) DCs, namely myeloid (mDC) and $CD14^{-/low}CD16^{+}$ subsets, in their ability to produce

inflammatory cytokines, activation status, and chemokine receptors expression [10, 11].

The immunologic self-tolerance breakdown, particularly in the control of self- and non-self-discrimination, results in the development of autoimmune diseases. Therefore, elucidate the mechanisms that regulate self-tolerance is important to understand self-directed immune responses and the mechanisms underlying autoimmune diseases [12, 13]. The notable functional plasticity of DCs, their lineage and maturational status, stimulation by pathogen-derived products, the net effect of antigen dose, and cytokine milieu determine whether an immunogenic or tolerogenic response will be developed [14].

One important mediator of DCs tolerogenic activity is ICOSL (inducible costimulator ligand), which is mainly expressed in pDC, mDCs, immature B cells, and monocytes and appears to be involved in the induction of a suppressive effect in T cells under an inflammatory environment as seen in SLE [15]. ICOS is a costimulator molecule expressed on CD4+ T cells, which was associated with secretion of interleukin 10 (IL-10) [15–17]. IL-10 is produced by T cells and induces tolerance and anergy in effector T cell [18]. ICOS is expressed at high levels in Th2 and at low levels in Th1 cells and the expression of this molecule inhibits the secretion of IL-2 [16]. The activation of ICOS/ICOSL pathway induces a differentiation of effector T cells in regulatory T cell and a sustained Th2 response [19, 20].

SLE is characterized by an inflammatory immune response mediated, in part, by cytokines and chemokines produced by antigen presenting cells (APC) and other immune cells, contributing for disease development and progression.

Multiple links of evidence support the involvement of IFN-α in the primary pathogenesis of SLE; high levels of serum IFN-α have been detected in SLE patients and have long been related with SLE pathogenesis [21]. Plasmacytoid DC (pDC) subpopulation is an important mediator of antiviral immunity through their extraordinary ability to secrete high levels of IFN-α in response to many DNA and RNA viruses and, in this sense, has been closely related to SLE physiopathology [22, 23].

There is a growing evidence suggesting that infiltration of T lymphocytes and other leukocytes into the sites of inflammation plays a critical role in organ involvement in SLE [24]. Chemokines have an important role in the migration and homing, necessary for the initiation of a cellular immune response in the sites of inflammation, and are able to regulate a differential recruitment of T helper (Th1 and Th2) lymphocytes [25].

Alterations in the cytokine and chemokine profile in SLE patients compared to normal controls have been described and reflect alterations in the inflammatory environment [2, 26, 27]. Chemokines like CCL2, CXCL10, CXCL9, CCL4, and CCL5 present raised levels in SLE patients serum and may be related to disease activity, contributing to the inflammatory disorder [28, 29].

In this context, we evaluated the regulatory function of peripheral blood monocytes, mDCs, $CD14^{-/low}CD16^{+}$ DCs, and pDCs subsets by the ICOSL mRNA expression and, on the other hand, we assessed the inflammatory role of these cells by the mRNA expression of IFN-α and the chemokines CCL2, CXCL9, CXCL10, CCL4, and CCL5.

2. Methods

2.1. Patients and Samples. Forty-three SLE patients were enrolled in the study, eighteen with active disease (ASLE) (100% female, mean age 33 ± 11 years) and twenty-five with inactive disease (ISLE) (84% female, mean age 33 ± 10 years). Patients were recruited fulfilling the 1997 American College of Rheumatology (ACR) classification criteria for SLE [30]. All patients are followed at the Lupus Clinic, Rheumatology Department of the University Hospital of Coimbra. After assessing disease activity at the time of evaluation, according to the SLE Disease Activity Index 2000 (SLEDAI 2k) [30, 31], SLE patients were divided into two groups, one with active (SLEDAI $2k \geq 5$; $n = 18$) and the other with inactive (SLEDAI $2k < 5$; $n = 25$) SLE [32]. The patients medication, at time of evaluation and additional clinical and therapeutic regimen, was recorded at the time of analysis (Table 1).

The healthy control group (HG) consisted of 30 healthy individuals (90% female; mean age 30 ± 6 years). These participants were required to complete a brief questionnaire regarding previous or current medical conditions. All were free from autoimmune disease, active inflammatory condition and were not undergoing treatment with any immunomodulatory drugs.

K3-EDTA-anticoagulated peripheral blood samples were collected from each participant and FACS-sorted within 18 hours after collection.

2.2. Ethics. The study protocol was approved by the local ethics committee. All participants gave and signed informed consent and the principles of Helsinki Declaration were respected.

2.3. Cell Sorting of Monocytes, $CD14^{-/low}CD16^{+}$ DC, mDCs, and pDCs. For the cell sorting of monocytes, $CD14^{-/low}$ $CD16^{+}$ DC, mDCs, and pDCs, 3 mL of each K3-EDTA PB sample were added to 10 mL of NH_4Cl solution (Sigma, St. Louis, MO, USA) in order to lyse red blood cells. After 20 minutes of incubation, samples were centrifuged (5 minutes, at 540 ×g) and the cell pellet was stained with the following monoclonal antibodies (mAb): anti-CD16 fluorescein isothiocyanate (FITC) (Sanquin–Pelicluster, Amsterdam, The Netherlands), anti-CD33 phycoerythrin (PE), anti-CD45 peridinin chlorophyll protein (PerCP) (BDB, San Jose, CA, USA), anti-HLA-DR phycoerythrin cyanine 7 tandem (PECy7) (BDB), and anti-CD123 allophycocyanin (APC) (Macs Miltenyi Biotec, Bergisch Gladbach, Germany). Once incubated for 20 minutes at room temperature in the darkness, the cells were washed and resuspended in phosphate-buffered saline (PBS) (Gibco BRL-life Technologies, Vienna, Austria).

Cell sorting and purification were performed in FACSAria II cell sorter (BDB) using the FACSDiva software (BDB). Monocytes were identified and sorted by

Table 1: Clinical findings in 43 patients with systemic lupus erythematosus (SLE).

	ASLE ($n = 18$)	ISLE ($n = 25$)
Mean SLEDAI scores	9.7 ± 3.2	1.6 ± 0.9
Mean time since diagnosis	7.6 ± 7.4	9.0 ± 6.0
Lupus nephritis	44.4%	61.3%
Neurolupus	0%	19.4%
Lupus arthritis	66.7%	58.1%
Haematological involvement	100%	87.1%
Lupus cutaneous involvement	77.8%	74.2%
Severe Lupus*	44.4%	71%
Anti-dsDNA antibodies**		
Low positive	11.1%	32.3%
Moderately positive	22.2%	22.6%
High positive	55.6%	6.5%
Treatment		
Hydroxychloroquine	94.4%	87.1%
Immunossupressants***	66.7%	32.3%
Steroids****	83.4%	12.9%
Low dose	46.6%	100%
Moderate dose	33.3%	0%
High dose	20.1%	0%

ASLE: Active disease group.
ISLE: Inactive disease group.
*Lupus severity in accordance with cumulative major organ involvement.
**Anti-dsDNA antibodies: low positive (<20 IU); moderately positive (20–50 IU); high positive (>50 IU).
***Azathioprine, mycophenolate mo1etil, cyclosporine, tacrolimus, methotrexate, cyclophosphamide, or rituximab.
****Low dose, upto 10 mg/day; moderate dose, 10–30 mg/day; high dose, more than 30 mg/ day; n = sample investigated.

HLA-DR$^+$/CD33^{high}/CD45^{high} phenotype, and the three DCs subpopulations, characterized by intermediate forward (FSC) and side scatter (SSC) between those of lymphocytes and monocytes, were purified according to the following immunophenotype features: myeloidDCs (mDCs) present HLA-DR^{high}/CD33^{high}/CD16^{neg}/CD123^{dim} immunophenotype, CD14$^{-/low}$CD16$^+$ DC subset are HLA-DR^{inter}/CD33^{inter}/CD123^{inter}, and plasmacytoid DCs (pDC) are HLA-DR^{high}/CD123^{high}CD33$^{neg/\,dim}$/CD16^{neg} (Figure 1) [33, 34]. The number of cells obtained of each cell population after FACSAria cell sorting is described in Table 2.

After cell sorting, the purity of the isolated cell populations was evaluated in the FACSCanto II flow cytometer (BDB) using the FACSDiva software (BDB) and acquiring a representative number of sorted cells, and it was consistently greater than 90%.

2.4. Gene Expression Analysis after Sorting of Monocytes, Dendritic Cells Subsets. Sorted cell populations were centrifuged for 5 minutes at 300 g and the pellet was resuspended in 350 μL of RLT Lysis Buffer (Qiagen, Hilden, Germany) and the total RNA extraction was performed with the RNeasy Micro kit (Qiagen) according to the supplier's instructions. Total RNA was eluted in a 14 μL volume of RNase-free water. In order to quantify the amount of total RNA extracted and verify RNA integrity, samples were analyzed using a 6000 Nano Chip kit, in an Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany) and 2100 expert software, according to the manufacturer's instructions. RNA was reverse transcribed with SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Relative quantification of gene expression by real-time PCR was performed in the LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland). Real-time PCR reactions were carried out using 1X QuantiTect SYBR Green PCR Master Mix (Qiagen), 1X QuantiTect Primer Assay (IFNA1 QT00201964, ICOSLG QT00004669, CCL2 QT00212730, CCL4 QT01008070, CCL5 QT00090083, CXCL9 QT00013461, and CXCL10 QT01003065) (Qiagen), and 20 ng of cDNA sample, in a total volume of 10 μL. The reactions were performed using the following thermal profile: 15 min at 95°C, 50 cycles of 15 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C. Melting point analysis was done to ensure amplification of the specific product. Real-time PCR results were analyzed with the LightCycler software (Roche Diagnostics). GeNorm Reference Gene Selection kit (Primer Design Ltd., Southampton, UK) in conjunction with the geNorm software (Primer Design Ltd.) were used to select the reference genes to normalize data. The reference genes used for gene expression analysis in monocytes were ATP synthase (ATP5B) and the beta-2-microglobulin (B2 M); in mDC and CD14$^{-/low}$CD16$^+$ DC were the B2 M and ubiquitin-c (UBC); in pDC were the B2 M and ATP5B. The normalized gene of interest expression levels were calculated by using the delta-Ct method [35].

2.5. Statistical Analyses. Statistical evaluation of data was analyzed using the nonparametric Mann-Whitney U test between the studied groups. All statistical analyses were performed using IBM SPSS statistics 20 software (Armonk, NY, USA) and differences were considered as statistically significant when the P value was less than 0.05.

3. Results

3.1. Frequency of Peripheral Blood Monocytes, CD14$^{-/low}$ CD16$^+$ DCs, mDCs, and pDCs in SLE Patients and Healthy Control Group. As shown in Table 3, frequency of peripheral blood mDCs and pDCs was lower in ASLE group than in control group, particularly pDCs. A lower pDC frequency was also observed in ISLE group compared to HG. In contrast, no significant differences were found in the frequency of circulating monocytes and CD14$^{-/low}$CD16$^+$ DCs. We also verified a lower absolute number of monocytes in ASLE compared to HG as well as a lower number of peripheral blood pDCs in SLE patients, especially in ALSE group.

Since the number of dendritic cells obtained after cell sorting was significantly lower than those of monocytes, we only evaluated the mRNA expression of IFN-α, ICOSL,

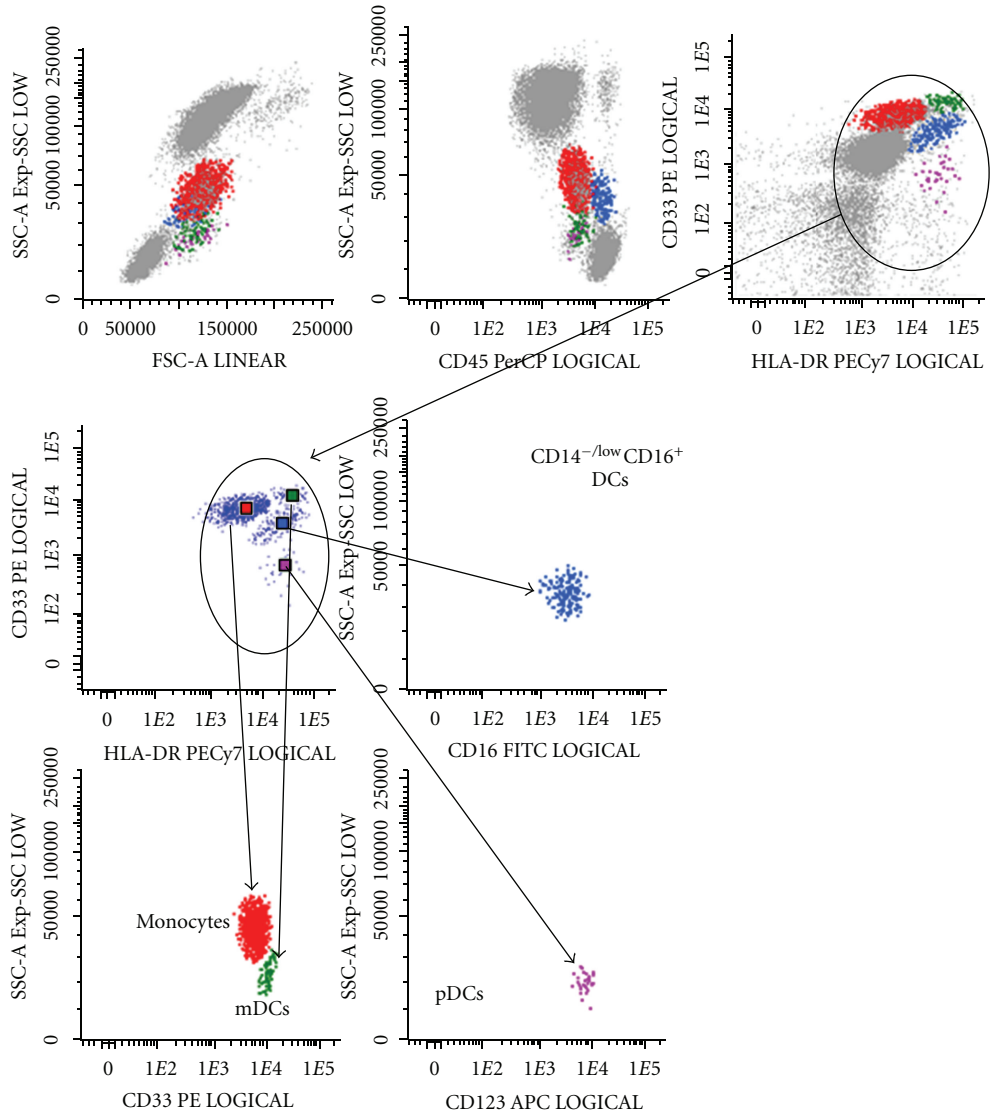


Figure 1: Flow cytometry gate strategy to obtain purified monocytes and peripheral blood dendritic cells by cell sorting.

CXCL9, and CXCL10 on mDCs and CD14$^{-/low}$CD16^{+} dendritic cells and of IFN-α and ICOSL on pDCs (Table 2).

3.2. Tolerogenic Role of Monocytes, CD14$^{-/low}$CD16^{+} DCs, mDCs, and pDCs Based on ICOSL mRNA Expression. Concerning the tolerogenic function of monocytes and DCs subsets, a lower mRNA expression of ICOSL was observed in ASLE compared to HG in monocytes (Figure 2(b)) and, on the other hand, an increased ICOSL mRNA expression in mDCs from both SLE groups compared to HG, was found (Figure 4(a)).

No significant differences were observed in CD14$^{-/low}$ CD16^{+}DC subset between the studied groups (Figure 3(a)).

Moreover, in pDC subpopulation, a lower ICOSL mRNA expression in ASLE and higher in ISLE compared to HG was observed (Figure 5(b)).

3.3. Inflammatory Role of Monocytes, CD14$^{-/low}$CD16^{+} DCs, mDCs, and pDCs Based on Chemokines and IFN-α mRNA Expression. In purified monocytes was observed a significant increase of CXCL9 and CXCL10 mRNA expression in both SLE groups compared to HG (Figures 2(d) to 2(e)). Similarly

Table 2: Number of sorted monocytes and peripheral blood dendritic cells in the three studied groups (HG, ASLE, and ISLE).

	HG ($n = 30$)	ASLE ($n = 18$)	ISLE ($n = 25$)
Number of sorted cells			
Monocytes	143701 ± 110950	91029 ± 83915	115407 ± 10558
$CD14^{-/low}CD16^{+}$ DCs	15393 ± 18486	9667 ± 11976	7251 ± 3903
mDCs	8709 ± 7107	4365 ± 3228	3771 ± 3076
pDCs	5281 ± 3894	1363 ± 1291	3416 ± 2655

HG: Healthy control group.
ASLE: Active disease group.
ISLE: Inactive disease group.

Table 3: Frequency and absolute value of monocytes and peripheral blood dendritic cells in the three studied groups (HG, ASLE, and ISLE).

	HG ($n = 30$)	ASLE ($n = 18$)	ISLE ($n = 25$)
Frequency (%)			
Monocytes	3.9 ± 0.97	3.02 ± 1.61	3.56 ± 1.32
$CD14^{-/low}CD16^{+}$ DCs	0.54 ± 0.29	0.45 ± 0.30	0.55 ± 0.33
mDCs	0.29 ± 0.18*	0.21 ± 0.15**	0.29 ± 0.32
pDCs	0.10 ± 0.07*	0.02 ± 0.03	0.07 ± 0.07***
Absolute Value (cells/μL)			
Monocytes	284,6 ± 84,2*	193,3 ± 97,5	228,4 ± 87,1
$CD14^{-/low}CD16^{+}$ DCs	39,2 ± 23,2	28,1 ± 20	34,1 ± 19,1
mDCs	21,4 ± 15,1	13,9 ± 11,1	18,2 ± 14
pDCs	7.08 ± 5.16*	1.24 ± 1.28**	3.82 ± 3.51***

Note: results are expressed as mean ± standard deviation.
Statistically significant differences were considered when $P < 0.05$ (Mann-Whitney U test): *HG versus ASLE; **ASLE versus ISLE., ***HG versus ISLE.
HG: healthy control group.
ASLE: active disease group.
ISLE: inactive disease group.

a higher mRNA CCL2 expression was observed in ASLE compared to HG and ISLE (Figure 2(c)). Moreover CCL4 mRNA expression was higher in ISLE, reaching statistical significance when compared with ASLE group (Figure 2(f)). Regarding IFN-α and CCL5 mRNA expression, no differences were found between the studied groups (Figures 2(a) and 2 (g)).

In $CD14^{-/low}CD16^{+}$ DC subset a higher CXCL10 and CXCL9 mRNA expression in ASLE was noted, when compared with HG, and in the latter chemokine, when compared with ISLE (Figures 3(c) to 3(d)). The evaluation of the IFN-α mRNA expression did not present significant differences between the studied groups (Figure 3(b)).

Regarding the mDCs subpopulation, we did not found statistical significant differences for IFN-α, CXCL9, and CXCL10 mRNA expression between the studied groups (Figures 4(b) to 4(d)).

IFN-α mRNA expression evaluated on pDC subset revealed a significant increase in both SLE groups when compared with HG, particularly in ALSE (Figure 5(a)).

3.4. ICOSL mRNA Expression and Clinical Parameters. When we grouped SLE patients based on the amount of anti-dsDNA antibodies in negative, low (<20 IU), moderate (20–50 IU), and high positive (>50 IU), we found, in pDC, an increase on ICOSL mRNA expression in the groups without anti-dsDNA antibodies and lower positive, when compared with moderate and high positive groups. Inline with this observation, we also detected a significant increase of ICOSL expression in mDC on negative group and in a lower extension in high positive group, when compared with lower and moderate positive groups. Moreover, in $CD14^{-/low}CD16^{+}$DC, we found a decrease on ICOSL expression on moderate-positive group when compared with high-positive and negative groups (Figure 6).

Concerning cutaneous involvement, we found, in SLE patients without this clinical feature, an increase on ICOSL mRNA expression in pDC. Also, an increase of its expression was observed in mDC in patients with this clinical parameter (Figure 7).

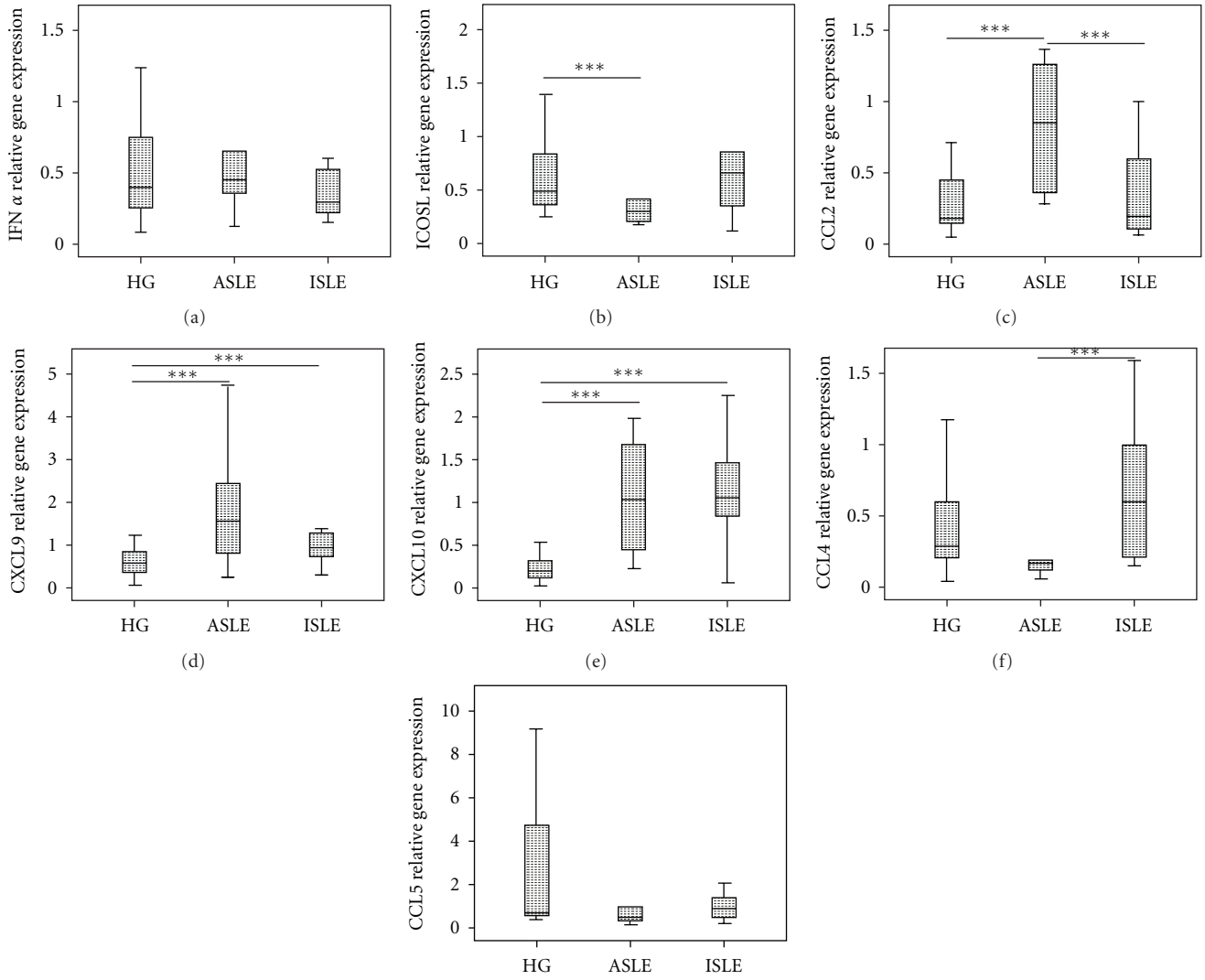


Figure 2: IFN-α, ICOSL, CCL2, CXCL9, CXCL10, CCL4, and CCL5 relative gene expression in cell-sorted monocytes in the three studied groups (HG, ASLE, and ISLE).

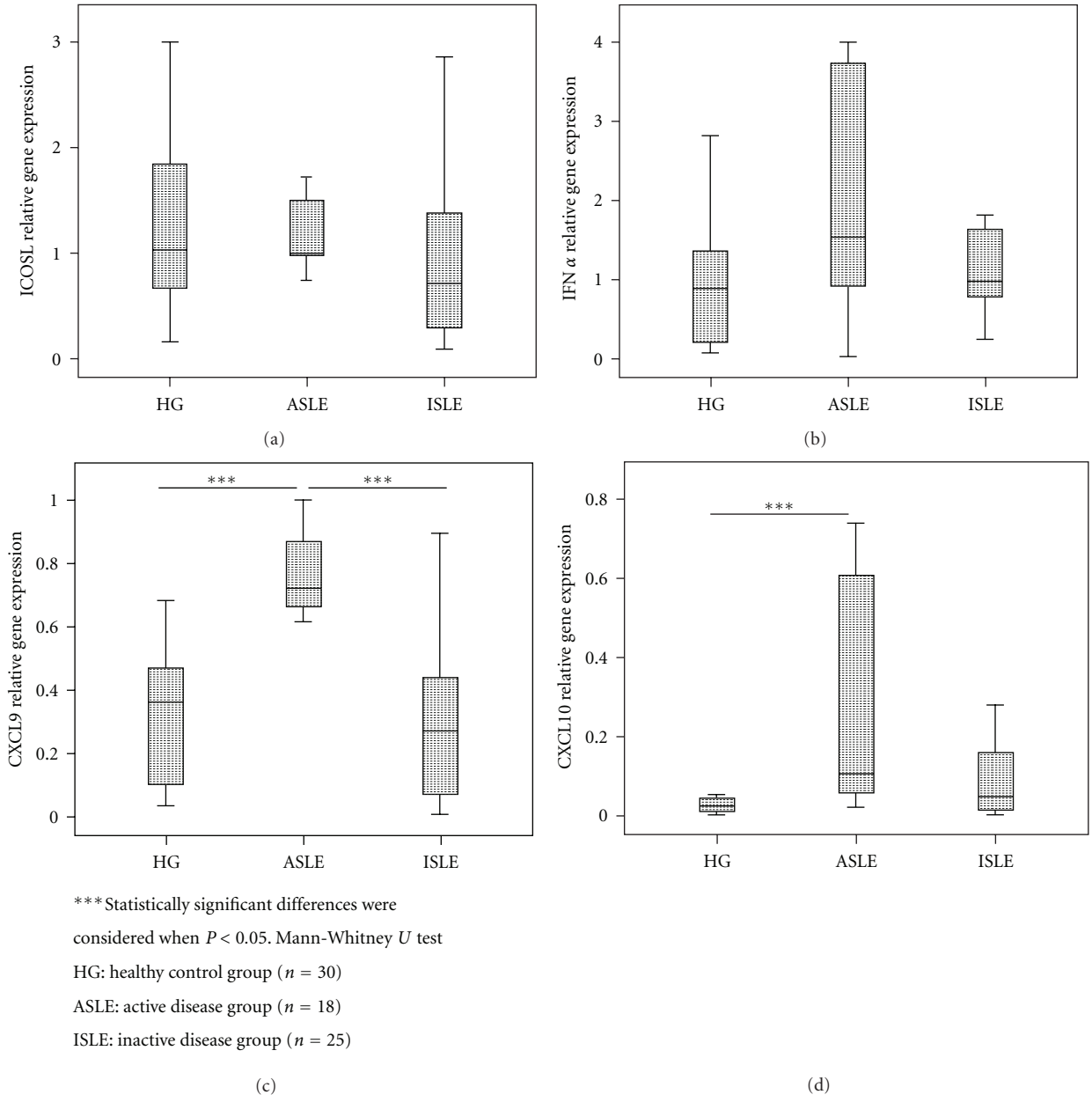
No more statistical significant differences were found relating other clinical parameters and/or other studied molecules.

4. Discussion

Monocytes and DCs are involved in the host defense and regulation of inflammation, playing a critical role in both adaptive and innate immune responses and in tolerance development. SLE is a variable autoimmune inflammatory condition, associated to tissue destruction wherein several abnormalities and disturbances have been attributed to these cells in SLE [8, 26, 36].

The tolerogenic function mainly attributed to pDC is, in part, mediated by the expression of ICOSL which has the ability to generate anergy in T cells and induce differentiation of *naive* T cells into regulatory T cells [32, 37, 38].

The lower levels of ICOSL mRNA expression observed in pDC from ASLE patients could be related to the higher inflammatory peripheral environment, due to increased



*** Statistically significant differences were considered when $P < 0.05$. Mann-Whitney U test

HG: healthy control group ($n = 30$)

ASLE: active disease group ($n = 18$)

ISLE: inactive disease group ($n = 25$)

Figure 3: IFN-α, ICOSL, CXCL9, and CXCL10 relative gene expression in cell-sorted $CD14^{-/low}CD16^{+}$ DCs subset in the three studied groups (HG, ASLE, and ISLE).

levels of proinflammatory cytokines and the presence of circulating immune complexes, which is inline with the higher levels of IFN-α mRNA found in these cells. The opposite was observed in ISLE, namely, higher mRNA expression of ICOSL and lower of IFN-α. This pattern of ICOSL expression in pDC was also observed in SLE patients without anti-dsDNA antibodies or with lower levels, as well as in the group of patients without skin involvement.

In fact, the lower mRNA expression of ICOSL and the mechanisms involved in ICOS/ICOSL pathway are related to loss of tolerance to self-antigens that occur in SLE, especially in patients in active phase [32, 37, 38]. It is described that the absence of interaction of ICOS with its ligand overrides the induction of anergy in T cells, considered the first step in the differentiation of T helper cells into T suppressor cells [15]. The reduction of ICOSL expression may also be explained, at least in part, by a negative feedback mechanism by which high levels of ICOS lead to the decrease of ICOSL expression. Since it was reported that active SLE patients have an increased expression of ICOS on CD4+ and CD8+ T cells, thus, apparently, exists a negative correlation between these two molecules [16, 39]. Results of Yang et al. showed

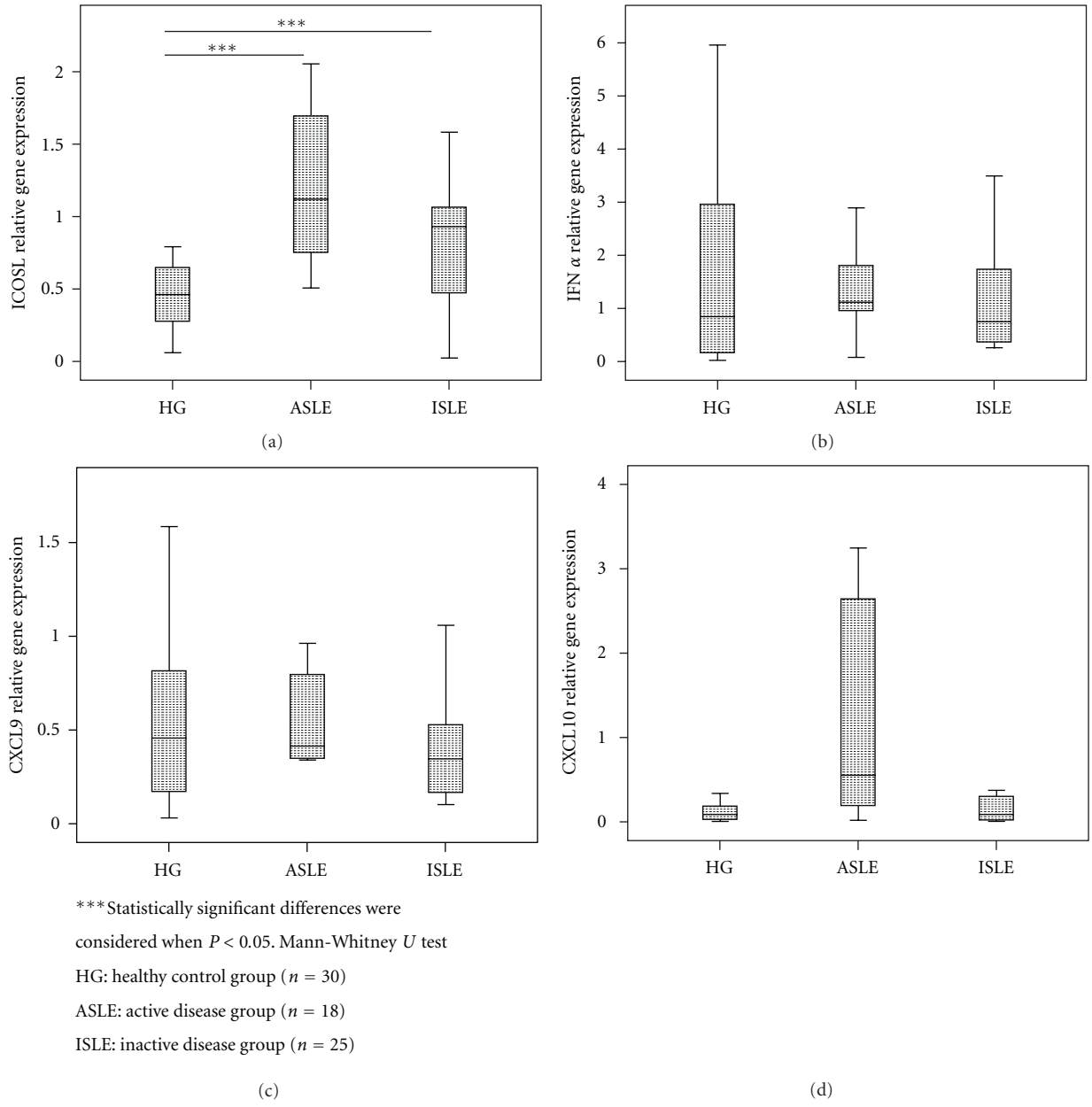
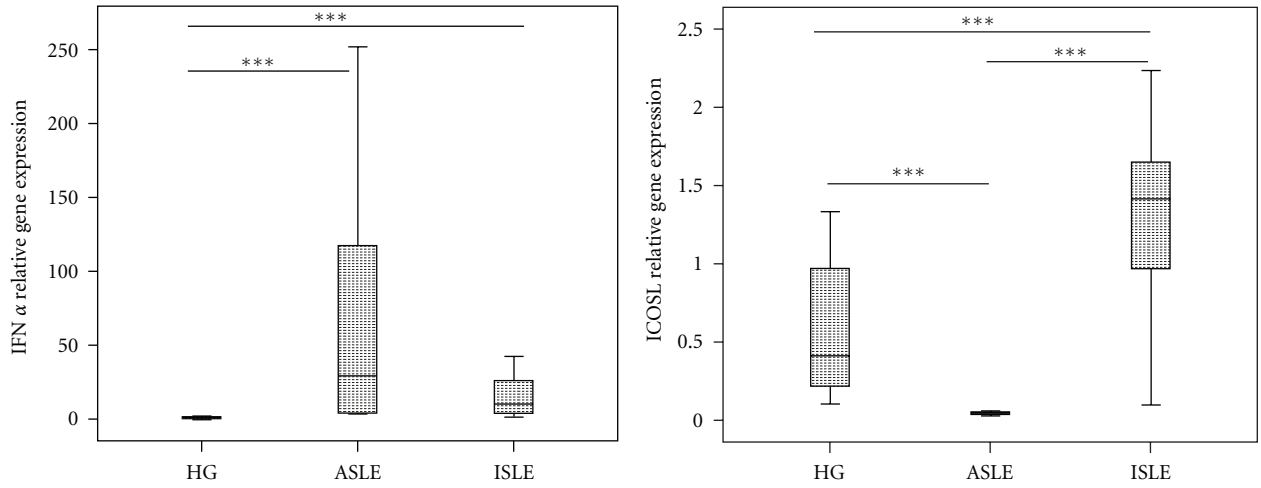


Figure 4: ICOSL, IFN-α, CXCL9, and CXCL10 relative gene expression in cell-sorted mDCs subset in the three studied groups (HG, ASLE, and ISLE).

a decreased expression of ICOS on CD4+ and CD8+ T cells from ISLE patients when compared with ASLE, resulting in a possible increase of ICOSL in these patients [16].

As observed in pDC, ICOSL mRNA expression in monocytes is reduced when compared to the HG, probably due to the same mechanisms observed in pDC. On contrary, high mRNA expression of this molecule was observed in mDC from ASLE and, in a lower extent, for ISLE patients when compared with control group, which could mean that this subpopulation of dendritic cells is less sensitive to the peripheral inflammatory environment, probably due to the fact that the majority of peripheral blood mDCs are recent immigrates from bone marrow with an immature phenotype, which could be particularly true in SLE patients, where an increase migration of these cells to peripheral tissues could induce an increase in the hematopoiesis of this cell lineage [40, 41]. In line with this explanation is the fact that no statistical significant differences were observed in this cells for IFN-α and chemokines mRNA expression among the studied groups. Furthermore the more immature status of mDC could be also the explanation for the higher mRNA expression of ICOSL found in patients with skin involvement, to where occurs an increased mDC migration.

Previous data have reported elevated levels of IFN-α in the SLE patient's serum [42, 43], which is in agreement with the higher mRNA expression of this cytokine in pDC



*** Statistically significant differences were considered when $P < 0.05$. Mann-Whitney U test

HG: healthy control group ($n = 30$)

ASLE: active disease group ($n = 18$)

ISLE: inactive disease group ($n = 25$)

(a) (b)

Figure 5: IFN-α and ICOSL relative gene expression in cell-sorted pDCs subset in the three studied groups (HG, ASLE, and ISLE).

from SLE patients, particularly in those with active disease. Dall'Era et al. and Kirou et al. related the serological levels of IFN-α with SLE clinical manifestations and disease activity [42, 44].

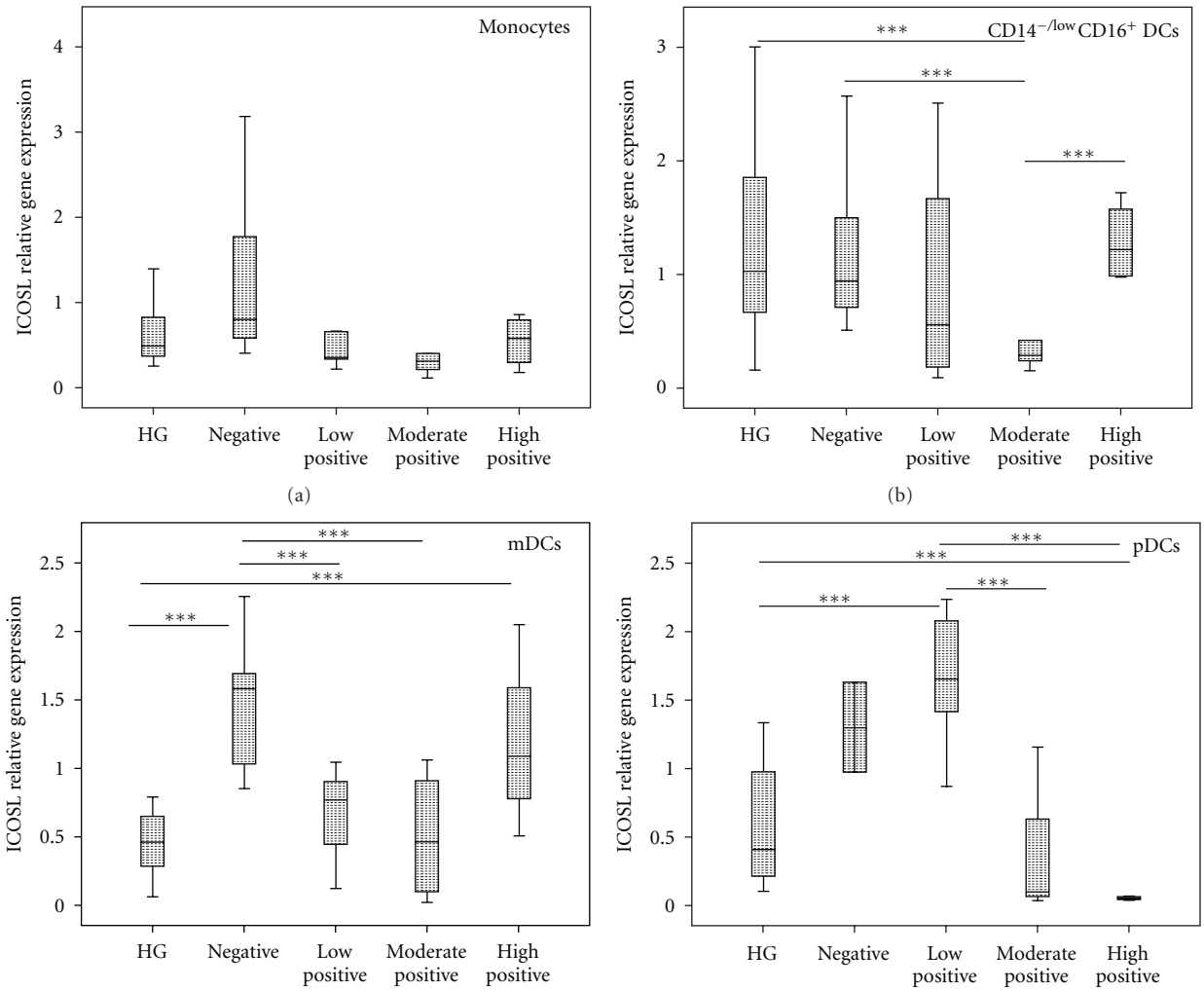
IFN-α is a pleiotropic cytokine, important in the immune regulation, that is produced by multiple cell types in response to viral infection. pDCs have a special role in the IFN-α production and are the most important sources of serum interferon [45]. IFN-α can affect multiple cell types involved in SLE and has the potential to influence the development, progression, and pathogenesis of SLE as it can control the function and activation states of most important immune cell subsets and function as a bridge between innate and adaptive immunity [46].

Some studies have demonstrated that the frequency of circulating pDCs is markedly reduced in SLE patients [47, 48]. However, functional studies revealed that pDCs, upon stimulation, have a normal IFN-α producing capacity, which means that aberrant pDC activation may be an important step in autoimmune diseases like SLE. In fact, an important finding was that the immune complexes present in SLE patients serum contain nucleic acids that are internalized via the FcγRIIa, reach the endosome, and stimulate TLR7 and/or TLR9, leading to subsequent activation of transcription factors and IFN-α production [49, 50].

Several studies have revealed the important role of chemokines and IFN-α in SLE activity. Many have reported high levels of those in the serum as well as of mRNA chemokine expression in peripheral blood leukocytes of these patients, particularly in active disease [29, 51, 52]. DCs subtypes have individual functions and appear to influence multiple processes that may activate or regulate autoreactive B cells. Part of their influence is dictated by their receptors and cytokines profiles and also by their location [9]. In the present study the use of purified peripheral blood monocytes and DCs subpopulations emphasizes the role of these cells in SLE pathophysiology, based on their chemokine expression.

The altered chemokines mRNA expression observed on monocytes in SLE patients, namely, in ASLE, is in accordance with the abnormalities already observed in these patients [8, 53]. The high levels of CCL2, CXCL9, and CCL4 mRNA expression observed on monocytes from SLE patients are consistent with other reports that have found increased levels in serum from these patients [52, 54]. These findings may be associated to the IFN-α pathway, since higher levels of IFN-α have been associated with increased levels of chemokines in SLE patients, suggesting an upregulation of this chemokine production according to Bauer et al. studies [28, 54]; likewise Quiong Fu has suggested the importance of type I IFN system in modulating chemokine expression, linking these two networks in the SLE pathogenesis [55].

Moreover, the inflammatory environment of SLE may lead to chemokine imbalance, including monocyte mobilization. CCL2 is involved in monocyte recruitment into focus of active inflammation and may act as a potent factor in the polarization of Th0 cells toward a Th2 phenotype [56]. In turn, there is increasing evidence that CXCL10 levels are elevated in serum and in tissues of SLE patients, contributing to a large variety of SLE manifestations [57].



***Statistically significant differences were considered when $P < 0.05$. Mann-Whitney U test

Note: HG: healthy control group

Anti-dsDNA antibodies:

low positive (< 20 IU); moderate positive (20–50 IU); high positive (> 50 IU).

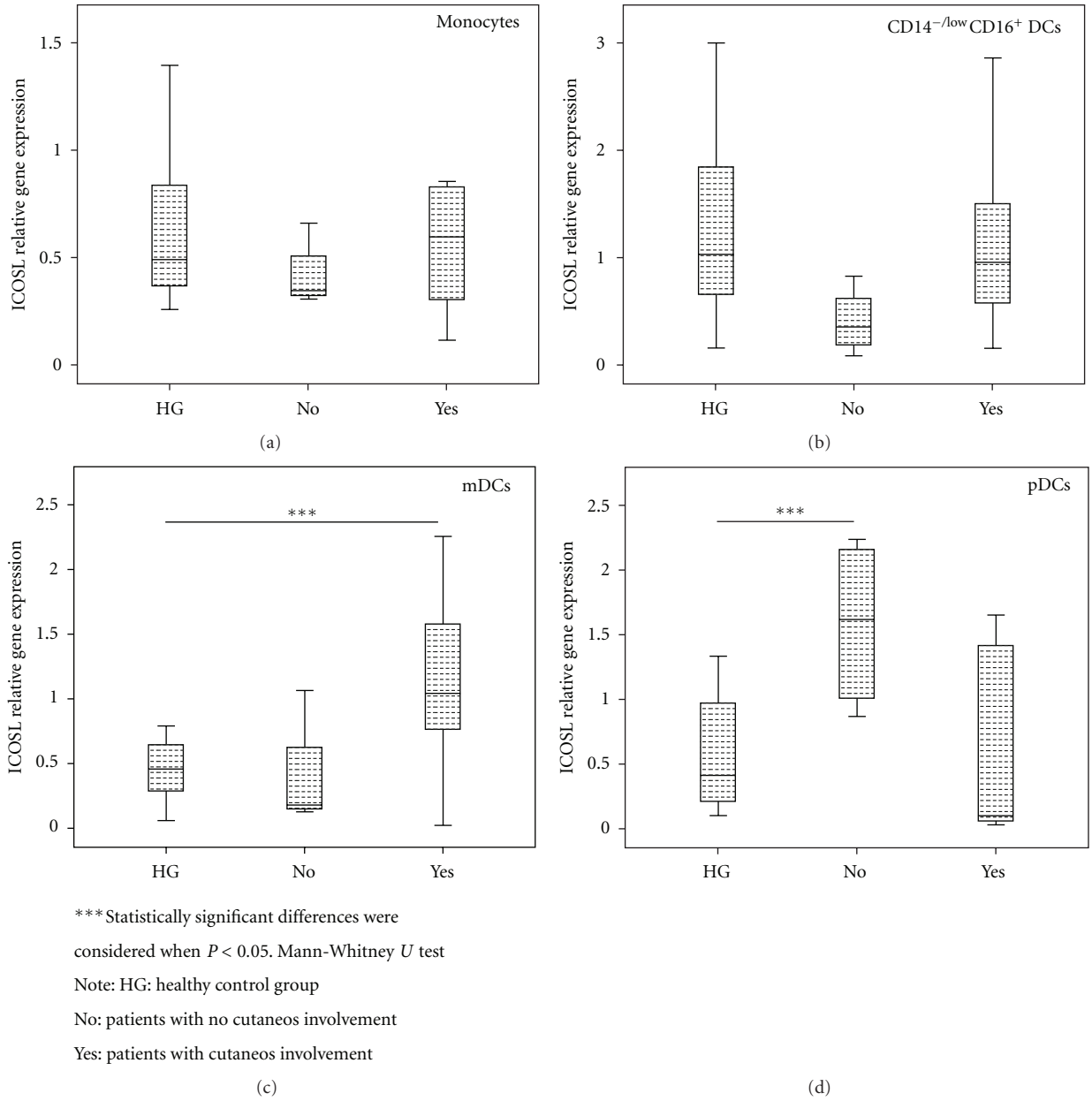
Figure 6: ICOSL relative gene expression in cell-sorted monocytes and DCs subsets, according to the amount of anti-dsDNA antibodies: negative; low, moderate, and high positive.

Furthermore, according to Kong et al. data, CXCL10 levels correlate positively with SLE disease activity and may represent a fair marker for monitoring disease activity [58]. As reported by Karonitsch et al., CXCL10 and CXCL9 mRNA expressions in monocytes were increased in SLE patients, associated with increased responsiveness of monocytes to IFN-γ, confirmed by mRNA levels of IFN-inducible STAT-1–dependent CXCL10 and CXCL9 genes [59].

Like monocytes, $CD14^{-/low}CD16^{+}$ DC subpopulation presented higher levels of CXCL9 and CXCL10 mRNA expression in ASLE group. This data point to a common role of these cells in SLE pathophysiology, as we previously reported [10].

Apparently less sensitive to microinflammatory changes than monocytes, $CD14^{-/low}CD16^{+}$ DC express FcγRII $CD16^{+}$ [60], which allow these cells to respond to peripheral activators motifs like circulating immune complexes. Moreover, these cells are tissue derivated, reentering in the peripheral circulation, as previously reported [61, 62], reflecting in the periphery the tissue injure.

As we previously described, no significantly differences on CXCL9 and CXCL10 mRNA expression in mDC were



*** Statistically significant differences were considered when $P < 0.05$. Mann-Whitney U test

Note: HG: healthy control group

No: patients with no cutaneos involvement

Yes: patients with cutaneos involvement

Figure 7: ICOSL relative gene expression in cell-sorted monocytes and DCs subsets, according to the cutaneos involvement of SLE patients.

observed in SLE patients, when compared with the control group. In agreement with our data, Gerl et al. reported no differences in the expression of CCR7, CCR1, and CCR5 chemokine receptors in mDC from SLE patients [11].

In conclusion our data clearly demonstrates a different role for monocytes and DCs subsets in SLE pathophysiology.

In active disease, peripheral blood monocytes and $CD14^{-/low}CD16^{+}$ DCs exhibit an upregulation of chemokine expression, probably due to a higher activation status in the periphery, contributing to the recruitment of neutrophils, monocytes/macrophages, and T and NK cells to peripheral tissues.

In turn, pDCs upregulate IFN-α and downregulate ICOSL mRNA expression in ASLE, exhibiting a pro-inflammatory profile and, conversely, in ISLE they seem to display a more tolerogenic activity.

Authors' Contribution

T. Carvalheiro, A. Rodrigues, and A. Lopes contributed equally to this paper.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Genetic Associations in Acquired Immune-Mediated Bone Marrow Failure Syndromes: Insights in Aplastic Anemia and Chronic Idiopathic Neutropenia

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Increasing interest on the field of autoimmune diseases has unveiled a plethora of genetic factors that predispose to these diseases. However, in immune-mediated bone marrow failure syndromes, such as acquired aplastic anemia and chronic idiopathic neutropenia, in which the pathophysiology results from a myelosuppressive bone marrow microenvironment mainly due to the presence of activated T lymphocytes, leading to the accelerated apoptotic death of the hematopoietic stem and progenitor cells, such genetic associations have been very limited. Various alleles and haplotypes of human leucocyte antigen (HLA) molecules have been implicated in the predisposition of developing the above diseases, as well as polymorphisms of inhibitory cytokines such as interferon-γ, tumor necrosis factor-α, and transforming growth factor-β1 along with polymorphisms on molecules of the immune system including the T-bet transcription factor and signal transducers and activators of transcription. In some cases, specific polymorphisms have been implicated in the outcome of treatment on those patients.

1. Introduction

Autoimmune diseases have been in the spotlight over the years and many of them seem to share similar underlying pathophysiology and immunogenetic mechanisms resulting from the interaction of multiple genetic and environmental factors [1–5]. Numerous genome-wide association studies have proven to be a useful tool in revealing the involvement of chromosomal loci that are associated with susceptibility to specific disorders [6, 7]. However, in the distinct but nonetheless related group of immunomediated bone marrow (BM) failure syndromes, there is an underexplored field of genetic associations. In this paper we will highlight such associations focusing on two diseases which share similar immunopathologic features, namely acquired aplastic anemia (AA) and chronic idiopathic neutropenia (CIN), both belonging to the group of BM failure syndromes.

2. Aplastic Anemia

2.1. Pathogenetic Features. Acquired AA is a disease characterized by a hypoplastic or aplastic BM and peripheral pancytopenia of a varying degree [8]. Although in some AA patients viral infection, drug, or chemical exposure can be linked to the disease pathogenesis, in most cases the underlying etiology remains elusive. However, numerous studies have unveiled the role of T lymphocytes in the pathogenesis of AA. Specifically, it has been shown that oligoclonally expanded self-reactive T cells [9–11] induce apoptosis of hematopoietic stem/progenitor cells [12]; this can be mediated either through an interaction via the Fas/Fas-ligand (FasL) pathway [13, 14] or by the production of proinflammatory and growth inhibitory cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) [15–19], thus resulting in a depletion of the hematopoietic stem cell pool in the BM. Other immune cells and molecules have been also implicated in the pathogenesis of acquired AA and have been reviewed elsewhere [20, 21]. The guidelines of treatment of AA suggest either hematopoietic stem cell transplantation (HSCT), which can cure the disease but it is not applicable to all patients, and/or immunosuppressive treatment (IST) with antithymocyte globulin (ATG) and/or cyclosporine A (CsA) [22–25]. The responsiveness of a

significant proportion of AA patients to immunosuppressive therapy gives further evidence for the underlying immune pathophysiology of the disease and classifies it in the wide spectra of autoimmune diseases [20, 26]. However, in several cases of IST-treated patients, the development of clonal disease has been the most serious complication, where the expansion of clones and clonal progression has been attributed to an immune selection and immunological escape [27, 28].

2.2. Genetic Factors in Aplastic Anemia

2.2.1. HLA Molecules. The human leukocyte antigen (HLA) is the most polymorphic genetic system. Its genes reside on chromosome 6 and determine HLA class I molecules encoded by HLA-A, HLA-B, and HLA-C loci, as well as HLA class II molecules encoded by HLA-DRA, -DRB1, -DRB3, -DRB4, -DRB5, -DQA1, -DQB1, -DPA1, and -DPB1 loci [29]. Given the fact that oligoclonally expanded T cells are involved in the pathophysiology of acquired AA [9–11] and since the interaction between $CD8^+$ or $CD4^+$ and their targets is mediated by HLA class I or II peptides, respectively, it has been suspected that polymorphic loci of these genes would be implicated in the susceptibility to the disease. Indeed, over the years various groups have tested this hypothesis in distinct populations and ethnicities.

By serological and molecular typing, it has been shown in a number of studies that HLA-DR2 was the gene associated with susceptibility to AA and in particular HLA-DRB1∗15 allele in Chinese, Japanese, and Caucasians of different ethnicities [30–34] although there have been some contradicting facts [35]. Furthermore, it has been found that the HLA-DRB1∗1501 and not the HLA-DRB1∗1502 allele was the one associated with an increased risk of developing AA [31, 36, 37] despite the fact that in a group of Japanese patients the DRB1∗1502 allele showed increased frequency, attributed mainly to the older age group [38]. However, only patients bearing the HLA-DRB1∗1501 allele and interestingly the DRB1∗1501-DQA1∗0102-DQB1∗0602 haplotype had a better response to CsA treatment [31, 38].

Other HLA alleles predisposing to development of AA have also been investigated. In a case report, the HLA-DRB1∗0405 allele was a candidate gene for susceptibility to AA [39], while in another study, high-resolution genotyping of HLA-DRB1 showed that the HLA-DRB1∗04 allele coding for alanine at position 74 (HLA-DR4-Ala74) predisposed to severe AA (SAA) independently from the DRB1∗1501 risk allele [40]. Furthermore, the DRB1∗04 alleles had a worse response to CsA and a tendency to a poor prognosis. The HLA-DRB1∗07 allele has been reported overexpressed in AA patients with no difference between adults and children, placing it as a susceptible allele for AA, at least in that cohort of Iranian subjects [35]. In addition, other candidate alleles predisposing to AA and SAA in children have been reported such as HLA-B∗48:01, HLA-DRB1∗09:01, and HLA-B14 [41, 42]. However, in the latter study [42], it was demonstrated that different HLA associations occur in children and adults; therefore any assumptions regarding HLA allele distribution between these groups should be made cautiously.

Beside risk alleles, there have been studies revealing the possible protective role of HLA variants in developing AA. The allele HLA-DRB1∗13 appeared to be protective in SAA children of Turkish origin [43], as well as the HLA-DRB1∗03:01, HLA-DRB1∗11:01, and HLA-B∗51:01 alleles in Chinese children with AA [41]. Likewise, in a small cohort of Pakistani AA patients, HLA-DRB1∗03 had higher frequency in controls suggesting a putative protective role [33]. In addition, in the Korean population, the DRB1∗1302 allele has been found significantly lower in the group of SAA patients compared to controls or non-SAA patients [36]. However, in the previously mentioned study, the haplotype A∗31-B∗51-DRB1∗13 was associated with predisposition to AA along with the A∗02-B∗40-DRB1∗15 and A∗33-B∗58-DRB1∗15 haplotypes [33].

In a large-scale single-nucleotide polymorphism (SNP) array-based study concerning a Japanese AA patient cohort, copy number-neutral loss of heterozygosity (CNN-LOH) of the 6p arm (6pLOH) was detected in a substantial proportion of patients [44]. The HLA-A∗02:01, HLA-A∗02:06, HLA-A∗31:01, and HLA-B∗40:02 alleles were overrepresented in this population, and in the 6pLOH(+) clones, the missing HLA alleles were biased towards the four alleles mentioned above. This observation has led to the hypothesis that since cytotoxic T cells that presumably target antigen(s) present on hematopoietic stem cells through specific HLA class I molecules, 6pLOH(+) cell clones found in AA patients may have been derived by a progent that managed to escape the autoimmune attack by effectively deleting the risk of HLA species responsible for the immune insult. However, this escape mechanism from autoreactive cytotoxic T cells could not render 6pLOH(+) stem cells able to repopulate the BM effectively, unless immunosuppressive treatment was applied. The later observation was possibly due to the presence of inhibitory cytokines such as IFN-γ and TNF-α in the BM of AA patients [44]. Nevertheless, future studies should be designed to shed light into the origin of autoimmunity, predisposition to the disease, and outcome of treatment regarding the thousands of HLA variants and the different emerging haplotypes in distinct ethnic populations.

2.2.2. Myelosuppressive Cytokines and Molecules. Elevated levels of inhibitory cytokines such as IFN-γ and TNF-α as well as elevated levels of Fas antigen on $CD34^+$ progenitor cells in the BM of AA patients have been previously reported to play a key role in the pathophysiology of the disease [13, 45–47]. Polymorphisms in such cytokines have been investigated by various groups in order to expose a genetic predisposition to AA or the outcome of IST.

The TNF-α gene -308 promoter/enhancer polymorphism, and specifically the TNF2 allele (-308A), has been associated with elevated TNF-α levels [48] and has been shown to be overrepresented in a SAA Chinese population, contributing to the susceptibility to the disease in a DR3- and DR4-independent manner [49]. However, no susceptibility was demonstrated in milder forms of AA [49], which is consistent with other observations where the distribution of the TNF2 allele did not differ between AA patients

and controls [50, 51]. Nevertheless, there are contradicting observations where the specific −308 AA TNF-α genotype was overrepresented in the AA group of patients [52]. Likewise, although in a German group the response to immunosuppressive therapy due to this rare allele was better even after 3 months of treatment compared to noncarriers [50], in another group such association was not demonstrated [51]. The different outcomes of the studies might be attributed to the low number of subjects tested and to variations due to a nonhomogenous population with AA of varying degree and different ethnicities.

The IFN-γ +874 A/T gene polymorphism, and in particular the +874TT genotype, has been shown to result in elevated levels of IFN-γ production [53]. Many groups have demonstrated that the TT genotype is overrepresented in AA patients and correlates with susceptibility to the disease but not with the disease severity [52, 54–56]. Moreover, it has been shown that the above specific genotype might predict a good response to IST [55]. Other polymorphisms such as the −2,353 A/T rs7139169 and the −1,616 C/T rs2069705 have also been studied in AA, and it has been shown that the minor T allele of the former was protective and reduced the risk for AA, as well as the haplotype TCA regarding the polymorphisms in −2,353, −1,616, and +874 of IFNγ gene. In addition, the above-mentioned −2,353 T allele and TCA haplotype was shown to induce resistance to IST [51]. Polymorphisms of a CA repeat microsatellite sequence in the first intron of the IFN-γ gene have been also shown to affect the production of IFN-γ. Specifically, homozygosity for the 12 (CA) repeats in position 1349 of the gene results in production of higher levels of IFN-γ [53, 57]. The frequency of the 12-12 (CA) genotype as well as the single allele 12 in Caucasian and Chinese AA patients has been shown to be higher than controls, thus associating this variable number of dinucleotide repeat (VNDR) 1349 of IFN-γ to the risk of AA [57, 58].

Transforming growth factor-β1 (TGF-β1) is another cytokine playing a role in hematopoiesis with multifunctional effects. In patients with aplastic anemia, the frequency of genotypes associated with high production of TGF-β1 and in particular the −509 TT genotype was shown to be increased as opposed to controls [52, 56, 59]. This is in contrast to the fact that lower levels of TGF-β1 have been described in the serum and in vitro cultures of AA patients [60]. However, the expression levels in the periphery do not always reflect the levels of locally expressed cytokines in the BM. Other polymorphisms of TGF-β1 like −590 C/T rs1800469 as well as the P10L C/T rs1800470 have been reported to play no role in the susceptibility to AA. However, the T allele of the P10L C/T, along with the CT haplotype regarding the above two polymorphisms, has demonstrated higher response to IST even at the third month of treatment compared to patients lacking this haplotype [51].

Polymorphisms of molecules like FAS and various interleukins (IL) such as IL-1β, IL-2, IL-6, IL-10, and IL-12 that have a role in the pathogenesis of AA have been investigated by different groups, but no significant difference was observed between patient groups and controls [51, 52, 56, 59].

2.2.3. Other Immune Molecules. Increased expression of IFN-γ, TNF-α, and IL-2 from AA patients indicates that hematopoietic stem and progenitor cells are destroyed through a T-helper (Th)1 cell response [61]. T-bet or TBX21 belongs to the T-box family of transcription factors, it is the key regulator of Th1 development and function, and it is found in Th1 but not in Th2 cells [62, 63]. In patients with AA, T-bet is found elevated and transcribes actively the IFN-γ gene [64]. TBX21 has been suggested as a common risk gene for a variety of autoimmune disorders [65, 66]. Interestingly, the C allele of T-1993C, the TBX21 gene promoter, was associated with decreased risk in AA [67].

Signal transducer and activator of transcription 4 (STAT4) is a transcription factor binding to genes encoding T-bet and IFN-γ and plays a critical role in Th1 and Th17 cell differentiation [68]. Polymorphisms of the STAT4 gene have been associated with various autoimmune diseases [69–71]; among the polymorphisms tested, the rs7574865 was a candidate common risk polymorphism. In a cohort of Chinese population, the rs7574865 polymorphism was found to pose as a candidate susceptibility gene, with an increased frequency of the T allele and THE TT genotype [67]. However, no association between the-above mentioned polymorphism and the response to IST was established.

Molecules that are expressed on T cells affecting self-tolerance and autoreactivity have been extensively studied in autoimmune diseases. Cytotoxic T lymphocyte antigen 4 (CTLA4) is a molecule expressed on activated T cells that downregulates T cell autoreactivity [72]. Polymorphisms that result in a lower expression of CTLA4 [73, 74] have been associated with other autoimmune diseases [75]. Nonetheless, such associations were not observed in AA patients in a cohort of an Italian population [76].

Protein tyrosine phosphatase non-receptor-type 22 (PTPN22) gene encoding for a protein tyrosine phosphatase contributes to the modulation of negative T cell selection in the thymus and downregulation of autoreactive T cells in the periphery [77]. Although polymorphisms in this gene have been associated with autoimmune disorders [78], no contribution to the susceptibility in AA was observed, at least for the PTPN22 620W allele [79].

3. Chronic Idiopathic Neutropenia

3.1. Pathogenetic Features. Chronic idiopathic neutropenia (CIN) of adults is benign disorder of granulopoiesis representing the mild form of the spectrum of BM failure syndromes. It is an acquired form of neutropenia characterized by a prolonged and unexplained reduction in the number of circulating neutrophils below the lower limit of the normal distribution [80], although other forms of mild cytopenias might coexist [81, 82]. Similar to AA, the pathogenetic cause of neutropenia in CIN is attributed to impaired BM granulopoiesis due to an inhibitory effect of the BM microenvironment consisting of activated T lymphocytes [83, 84] and monocytes [85], proinflammatory mediators, and proapoptotic cytokines such as TNF-α, IL-1β, TGF-β1, IL-6 as well as IFN-γ, and FasL [80, 83, 84]. It has been documented that progenitor and precursor cells, especially

in the $CD34^+/CD33^+$ compartment, are depleted through an apoptotic mechanism implicating the FAS/FasL as well as the CD40/CD40L pathways in the presence of TNF-α [86, 87]. Treatment of CIN patients with G-CSF administration is only recommended in the rare cases of patients suffering from severe or frequent infectious episodes [88].

3.2. Genetic Factors in CIN

3.2.1. HLA Molecules. The major HLA alleles have been typed in a small cohort of a genetically homogenous population in Crete, Greece [89]. Of all the alleles tested (HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1), only the HLA-DRB1$*$1301 haplotype was significantly elevated in CIN patients as opposed to controls or other alleles. This was the first report of a genetic association to the predisposition of developing CIN. However, larger cohorts of patients need to be tested for a stronger association between HLA alleles and risk of CIN.

3.2.2. Myelosuppressive Cytokines and Molecules. The involvement of elevated proinflammatory and myelosuppressive cytokines such as TNF-α, IL-1β, IL-6, TGF-β1 and FasL in the pathophysiology of CIN is well established [83, 90, 91]. A possible association between the elevated levels of the above soluble mediators and the genetic predisposition for CIN has been investigated by two different groups. Although the -308G/A polymorphism of the TNF-α gene, especially the TNF2 allele, had been previously shown to contribute to increased serum levels of this cytokine in other disease entities [92, 93], no association was identified between this polymorphism with either the occurrence or the severity of neutropenia in CIN subjects [94, 95]. Likewise, no difference in frequency of the -511C/T IL1B and the +3953C/T IL1B SNP or the variable number tandem repeat (VNTR) in intron 2 of IL-1Ra gene (IL1RN) was detected in CIN patients, [95] although these polymorphisms have been associated with IL1B gene expression and increased IL-1β production [96, 97]. In the same cohort, the -174G/C SNP that has been associated with altered gene expression [98] failed to associate with CIN [95]. In the same context, the frequency of the -844T/C SNP of the FasL gene was not associated with CIN [94].

Interestingly though, out of three SNPs on the TGF-β1 gene, namely, the -509C/T, +869T/C, and the +915G/C, the -509C/T and specifically the T allele and the TT genotype occurred in a statistically higher frequency in CIN patients thus associating this genotype with the risk of development of CIN. However, it did not associate with the severity of neutropenia. Nonetheless, patients with the CT or the TT genotype displayed elevated levels of TGF-β1 in the serum or long-term BM cultures, indicating a contributory role of this cytokine in the pathophysiology of the disease [94].

4. Closing Remarks

Genetic factors that predispose to various disease states, including the acquired immune-mediated BM failure syndromes, may contribute to the pathophysiology of these disease entities. However, isolated SNPs are unlikely to be the only regulators of the complex mechanisms taking place in such autoimmune or immune-mediated conditions, but it is rather the specific combination of genotypes of cytokines and molecules on immune cells that predispose to AA and CIN and sustain the myelosuppressive BM microenvironment along with environmental factors. Wide-scale studies on different ethnic populations, with homogenous disease characteristics, would facilitate the systemic research for associations of genetic factors and disease risk.

Authors' Contribution

I. Mavroudi wrote the paper and H. A. Papadaki critically reviewed and revised the paper.

Conflict of Interests

No conflict of interest is declared by any of the authors.

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