Immunogenetics of Asthma and Systemic Lupus Erythematosus

Doctoral thesis

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INTRODUCTION

Complex or multifactorial genetic diseases are major causes of mortality globally and are caused by the interactions between several disease susceptibility loci and enviromental factors.

My studies focused on two multifactorial immunopathologies: the hypersensitivity reaction asthma and the autoimmune disorder Systemic Lupus Erythematosus (SLE). Allergic asthma is triggered by an external antigen, while SLE is characterized by autoantibody production against self (mainly nuclear) epitopes. Despite seeming like two entirely disparate conditions, both diseases involve exaggerated humoral immune responses in the host.

Our asthma studies were done at the Semmelweis University, Budapest. We were examining genetic association between polymorphisms of several candidate genes (MCP-1, RANTES, TNFα and MBL) with childhood asthma and we found, in accordance with a previous report, that among these polymorphisms, only the MCP-1 -2518A/G biallelic variant had a significant genetic association with the disease.

Monocyte chemoattractant protein-1 (MCP-1) belongs to the CC chemokine family. It is primarily secreted by dendritic cells, monocytes and macrophages, binds to CCR2 and acts as a chemoattractant on monocytes, basophils and memory T cells. Considerable variability has been found in the ability of normal human PBMCs to produce MCP-1 in response to IL-1β which is thought to be, at least partially, caused by the -2518A/G polymorphism in the distal promoter of MCP-1. This single nucleotide polymorphism (SNP) has been demonstrated to affect the transcriptional activity of the MCP-1 promoter and consequently, individuals who carry the G allele produce significantly more MCP-1 upon IL-1β treatment compared to individuals with the A/A genotype. In this thesis, we were trying to determine whether MCP-1 serum levels correlate with the presence or severity of asthma and whether there was an association between the MCP-1 -2518A/G polymorphism and MCP-1 serum levels. Since, host-microbe interactions are new exciting aspects of asthma research, we also tried to correlate the MCP-1 serum

level and the -2518A/G genotype with asthma susceptibility among *Chlamydophilia pneumoniae* infected children.

My subsequent work was carried out at the Harvard Medical School, Boston. Our studies were trying to elucidate the contribution of the SLAM family (*SLAMF*) genes in the pathogenesis of SLE using gene-targeted knockout and transgenic mouse models**.**

The SLAM family of homotypic co-stimulatory molecules consists of nine trans-membrane proteins (SLAMF1-9) and is expressed on a wide range of hematopoietic cells. Genome-wide linkage scans of human SLE patients and studies of murine congenic mouse strains consistently demonstrated the presence of a susceptibility locus on chromosome 1, and on the syntenic mouse region, which includes seven *SLAMF* (*Slamf* in mice) genes.

The *Slamf* genes are highly polymorphic between common non-autoimmune laboratory mouse strains such as *C57BL/6* (*B6*) (haplotype I SLAM locus) and *129/SvJ* (*129*) (haplotype 2 SLAM locus). Mice carrying a haplotype 2 SLAM locus (e.g. *129* or *NZW*) on the *B6* genetic background develop autoimmunity due to unidentified epistatic genetic interactions. The congenic *B6.129chr1b* (*129* segment embedded in the *B6* genetic background) and the *B6.Sle1b* (*NZW* segment embedded in the *B6* genetic background) mice spontaneously develop SLE-like symptoms (e.g. anti-nuclear autoantibodies, lymphocyte activation, mild nephritis) upon aging.

Among the human SLAM genes, SLAMF1, SLAMF3, SLAMF4 and SLAMF7 were implicated as lupus candidate genes. In mice studies, Ly108 (*Slamf6*) turned out to be the strongest candidate, because the ratio of transcripts encoding two distinct isoforms, Ly108-1 and Ly108-2, differs in *B6.Sle1b* and *B6* lymphocytes (Wandstrat et al., 2004). Ly108-1 and Ly108-2 are favored in the *B6.Sle1b* and *B6* genomes respectively, which is thought to influence early B cell development (Kumar et al., 2006). Because of the overlapping function and signaling of the Slam family genes, one can assume that their control over self-tolerance is not confined to only one receptor.

AIMS

1. **Genetic association of candidate genes in childhood asthma and correlation studies between MCP-1 serum levels and other asthmatic parameters.**

   1.1. To evaluate the association of genetic variants of MCP-1, RANTES, TNFα and MBL with childhood asthma.

   1.2. To examine whether there is a difference between MCP-1 serum levels in control and asthmatic children or within subgroups of asthmatic children (e.g. atopic vs. non-atopic, girls vs. boys, severity scores etc.).

   1.3. Test the hypothesis that there is a correlation between the MCP-1 –2518A/G genotype and the concentration of MCP-1 in the serum.

   1.4. To determine whether the MCP-1 –2518A/G genotype modifies asthma susceptibility among *Chlamydophilia pneumoniae* infected children and whether *Chlamydophilia pneumoniae* infection correlates with MCP-1 serum levels in asthmatic patients.

2. Elucidation of the interplay of the SLAM family (SLAMF) genes and their isoforms in the pathogenesis of Systemic Lupus Erythematosus (SLE)

2.1. To examine the individual effect of distinct *Slamf* members on the pathogenesis of murine lupus by comparing and characterizing the lupus-like syndrome in *Slamf1*$^{-/-}$, *Slamf2*$^{-/-}$, *Slamf3*$^{-/-}$ and *Slamf6*$^{-/-}$*[B6.129]* congenic knock-out strains.

2.2. To test the hypothesis that *B6.Sle1b* peripheral CD4^{+} T cells have an intrinsic lupus-related phenotype and dissect the role of *Slamf6* (*Ly108*) isoforms in the regulation of T cell intrinsic lupus phenotypes.

2.3. To test the hypothesis that the over-expression of the putative pathogenic isoform *Ly108-1* in T cells causes autoimmunity.

2.4. To test the hypothesis that the novel *Ly108* isoform *Ly108-H1* is protective against lupus when introduced into the *B6.Sle1b* genetic background by transgenesis and determine the influence of *Ly108-H1* on CD4^{+} T cells.

METHODS

Our asthma studies were carried out on DNA and serum samples which were obtained from asthmatic and non-asthmatic children attended the Budai Children's Hospital.

Total genomic DNA was extracted from white blood cells using the method of Miller (Miller et al., 1988) or by using QIAamp DNA Blood

Midi Kit (QIAGEN GmbH). All SNP genotypes were determined by PCR-RFLP (restriction fragment length polymorphism) method.

Serum samples were analyzed by ELISA (enzyme-linked immunosorbent assay) for MCP-1 levels (R&D Systems), total and allergen-specific IgE levels and anti-*C. pneumoniae* antibodies (Savyon Diagnostics).

Data were analyzed using MedCalc 5.0 (MedCalc Software, Belgium), SPSS 11.0 (SPSS Inc.) and Arlequin 1.1 (Genetics and Biometry Lab, University of Geneva) softwares. "Hardy-Weinberg" equilibrium was tested by using a $\chi 2$ goodness-of-fit test. Fisher's exact test was used to test for differences in allele distribution between the groups. Confidence intervals were calculated at the 95% level. Two-tailed *t* tests were considered significant at *p* value <0.05. Statistical comparisons among groups were performed by analysis of variance (ANOVA).

For the SLE studies, we kept mice under specific pathogen free (SPF) conditions. Common laboratory mouse strains were obtained from The Jackson Laboratory and from Taconic. Gene-targeted knock-outs of *Slamf1, Slamf4* and *Slamf6* were generated by the Terhorst lab. *Slamf2*$^{-/-}$ mice were generated by the Sharpe lab and *Slamf3*$^{-/-}$ mice were obtained from Dr. DJ. McKean. The congenic *B6.129chr1b* (Carlucci *et. al*., 2007) mice were generously donated by Dr. M. Botto (Imperial College London). *B6.Sle1b* (*Sle1b*) (Morel *et al.*, 2001) mice were provided by Dr. L. Morel.

Titer of anti-nucleosome, anti-chromatin, anti-double-stranded DNA and anti-single-stranded DNA autoantibodies were determined by ELISA.

Flow cytometry analyses were performed on single cell suspensions of spleens and thymuses. Cells were stained with directly-conjugated monoclonal antibodies (eBioscience, BD Pharmingen or BioLegend) and analyzed with LSRII cytometer (BD Pharmingen).

Histopathology scores were determined from hematoxylin- and eosin-stained paraffin kidney sections by a board-certified pathologist with subspecialty expertise in kidney pathology.

Chronic graft vs. host disease, the murine transfer model of lupus (Morris et al., 1990) was induced in B6 coisogenic, MHCII disparate bm12 mice by injection of splenocytes or purified $CD4^+$ T cells.

RT-PCR, microsatellite marker analysis and Fluorescent in Situ Hybridization were carried out according to standard protocols.

Anti-Ly108 antibodies were generated by the Terhorst lab. Immunoprecipitation was performed using protein-G agarose beads (Invitrogen). SDS-PAGE gel-electrophoresis and blotting were performed using the Invitrogen XCell SureLock system.

RESULTS

1. Genetic association of candidate genes in childhood asthma and correlation studies between MCP-1 serum levels and other asthmatic parameters.

We examined genetic associations between polymorphisms of four immunoregulatory candidate genes (MCP-1, RANTES, TNFα and MBL) with childhood asthma. Our studies demonstrated that in concert with previous findings, only the MCP-1 -2518A/G biallelic variant had a significant genetic association with asthma. Since the disease-associated "G" allele was also described to be transcriptionally more active and associated with higher protein secretion, we expected to find elevated MCP-1 levels in asthmatic patients as compared to healthy controls. Surprisingly, we found that serum MCP-1 levels were significantly lower in asthmatic children and that atopy or elevated serum IgE was associated with a further reduction in MCP-1 levels compared to non-atopic patients. Furthermore, patients with a high severity score (score 3) had significantly lower serum MCP-1 levels compared to patients with lesser scores (scores 1-2). Additionally,

serum MCP-1 levels were 1.3 times higher in asthmatic girls than in asthmatic boys.

Since these findings regarding the concentration of serum MCP-1 were the opposite of what was expected, we also tried to correlate the MCP-1 -2518 genotype with serum levels in asthmatic and non-asthmatic children. There was no association between these parameters, indicating that serum levels were independently regulated from this polymorphism.

There is an increased interest in the role of the epithelial barrier and the impact of the microbiome in recent studies of asthma (Couzin-Frankel, 2010). Our results show that children infected with a common respiratory pathogen *Chlamydophila pneumoniae* are 2.84 times more susceptible to the onset of asthma if they carry the minor MCP-1 -2518 "G" allele than the major "A" allele. Unfortunately, since carrying the -2518G allele alone increases the susceptibility to asthma we were not able to clearly separate the contribution of *C. pneumoniae* from the affect of the allele in this study. There was no significant association between the MCP-1 -2518 genotype or the *Chlamydophila pneumoniae* infection status and MCP-1 serum levels in asthmatic children.

2. Elucidation of the interplay of the SLAM family (SLAMF) genes and their isoforms in the pathogenesis of Systemic Lupus Erythematosus (SLE)

In order to examine the role of *Slamf* genes in the development of murine lupus, we utilized gene targeted knock-out mice which were generated in *129*-derived embryonic stem cells (ES cells) and subsequently crossed into *B6* or *BALB/c* mice.

By comparing spontaneous SLE development in *B6.129* and *BALB/c.129* congenic knock-out strains, we found that the 10-13 month-old, aged *Slamf1*$^{-/-}$, *Slamf2*$^{-/-}$ and *Slamf3*$^{-/-}$*[B6.129]* strains developed autoantibodies, while the *Slamf1*$^{-/-}$ and *Slamf2*$^{-/-}$*[BALB/c.129]* mice did not.

The phenotype of *Slamf2*$^{-/-}$*[B6.129]* mice was similar to the phenotype of *Slamf1*$^{-/-}$*[B6.129]* and *B6.129chr1b* or *B6.Sle1b* control mice with respect to the development of significantly increased titers of antinuclear antibodies, splenomegaly and $CD4^+$ T cell activation at 1 year of age. *Slamf2*$^{-/-}$*[B6.129]* mice, however, already exhibited autoantibodies at 3 months of age and severe proliferative glomerulonephritis as early as 6 months of age. On the *B6* background, a null mutation of the *Slamf4* gene, which is the ligand for *Slamf2*, caused mild autoimmunity, indicating that the interaction between *Slamf2* and *Slamf4* plays a role in maintaining self tolerance.

In contrast to *Slamf1*$^{-/-}$ and *Slamf2*$^{-/-}$, *Slamf6 (Ly108)*$^{-/-}$*[B6.129]* mice did not develop lupus. This observation marks *Ly108* as a major lupus candidate gene in the SLAM locus and corresponds to other publications that demonstrate that the expression level of the Ly108-1 and Ly108-2 regulates SLE development. Interestingly, our protein expression studies revealed the presence of a previously unrecognized Ly108 isoform, termed Ly108-H1 (Ly108-Haplotype I), which is expressed in *B6* but not *B6.Sle1b* or *B6.129chr1b* strains. We examined the role of this newly identified isoform in murine lupus by introducing a B6 BAC transgene onto the *B6.Sle1b* genome which was modified to only express Ly108-H1 by recombineering. While *B6.Sle1b* mice develop marked spontaneous autoimmunity upon ageing (characterized by autoantibody production, spontaneous activation of $CD4^+$ T cells and spontaneous germinal center formation) the *Sle1b*.BACLy108-H1 hemizygous transgenic littermates had a markedly reduced disease.

Since we found that peripheral T cells from *B6.Sle1b* mice were able to induce aggravated autoimmunity in the chronic graft-versus-host-induced disease model, we focused our studies on the role Ly108 in these cells. We demonstrated that transgenic overexpression of Ly108-1 into *B6* T cells was able to induce spontaneous lupus. In contrast, the *Sle1b*.BACLy108-H1 $CD4^+$ T cells suppressed the autoimmune phenotype induced by the chronic graft-versus-host disease model that is normally observed when using *B6.Sle1b* peripheral T cells. Taken together, we demonstrate here for the first time that the Ly108-1 and Ly108-H1 isoforms are able to regulate T cell intrinsic SLE phenotypes in a reciprocal manner.

CONCLUSIONS

1. Genetic association of candidate genes in childhood asthma and correlation studies between MCP-1 serum levels and other asthmatic parameters.

- The MCP-1 –2518G polymorphism is associated with an increased risk for the development of asthma in a cohort of Hungarian children.
- No significant association is found between non-functional MBL variants, RANTES -403G/A or TNFα -308G/A polymorphisms and childhood asthma.
- MCP-1 serum levels are significantly higher in non-asthmatic than in asthmatic children.
- Atopic asthma patients and patients with high serum IgE levels have significantly lower levels of serum MCP-1 than the other patients.
- Patients with a high asthma severity score (score 3) have significantly lower serum MCP-1 levels than patients with less severe forms of the disease.
- There is a significant gender bias in serum MCP-1 levels among asthmatic children in favor of females.
- There is no correlation between the MCP-1 –2518A/G genotype or *Chlamydophila pneumoniae* infection status and MCP-1 serum concentration.

2. Elucidation of the interplay of the SLAM family (SLAMF) genes and their isoforms in the pathogenesis of Systemic Lupus Erythematosus (SLE)

- The *Slamf1*$^{-/-}$, *Slamf2*$^{-/-}$ and *Slamf3*$^{-/-}$ congenic knock-out mouse strains, which were generated in *129* ES cells and crossed onto the C57*BL/6* genetic background, develop spontaneous lupus-like autoimmunity.

- *Slamf1*$^{-/-}$ and *Slamf2*$^{-/-}$ mice do not develop autoimmunity on the *BALB/c* genetic background, indicating that lupus is the result of epistatic genetic interactions between the haplotype 2 (129) SLAM locus and the *B6* background genes.

- Expression of the Ly108 gene is crucial for the development of SLE in the *B6.129chr1b* congenic lupus model, since neither *Ly108*$^{-/-}$*[B6.129]* nor *Ly108*$^{-/-}$*[B6]* mice develop lupus.

- Disruption of *Slamf2*/*Slamf4* heterotypic interaction induces autoimmunity.

- *B6.Sle1b* lupus prone congenic mice have an intrinsic peripheral CD4^{+} T cell phenotype.

- Transgenic overexpression of Ly108-1 in the T cell compartment causes humoral autoimmunity.

- Ly108-H1 is a novel protein isoform which is co-expressed with Ly108-1 and Ly108-2 in Slamf haplotype 1 mice.

- The BAC Ly108-H1 transgene ameliorates the spontaneous SLE phenotype in *B6.Sle1b* mice.

- T cell-dependent autoimmunity is ameliorated by Ly108-H1 in the bm12 chronic graft-versus-host disease transfer model of murine lupus.

LIST OF CANDIDATE'S PUBLICATIONS

Related to the thesis

Asthma

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