Immunogenetics of Asthma and Systemic Lupus Erythematosus

by

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DISSERTATION

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ABBREVIATIONS

ANA: anti-nuclear antibody

ANOVA: analysis of variance

APC: antigen presenting cell

B6: C57BL/6

ELISA: enzyme-linked immunosorbent assay

EU: ELISA Unit

GN: glomerulonephritis

GWAS: genome-wide association study

H&E: haematoxylin and eosin

ITSM: immuno-tyrosine switch motif

MBL: mannose-binding lectin

MCP-1: monocyte chemoattractant protein-1

RFLP: restriction fragment length polymorphism

SAP: SLAM-associated protein

SLAM: signaling lymphocytic activation molecule

SLAMF: signaling lymphocytic activation molecule family (human)

Slamf: signaling lymphocytic activation molecule family (mouse)

SLE: systemic lupus erythematosus

SNP: single-nucleotide polymorphism

TCR: T cell receptor

T_H2: T helper 2

UTR: untranslated region

WT: wild type

CHAPTER 1: INTRODUCTION

Unraveling the polygenic predisposition of multifactorial diseases is a major challenge facing modern medicine. Their prevalence has strikingly increased during the past century due to dramatic changes in the human environment such as dietary shifts, psychological stress, altered pathogen/microbe milieu and exposure of humankind to various environmental stresses (e.g. chemicals, drugs, radiation etc.). These changes then pose increased pressure on the homeostatic regulation of our bodies, revealing quiescent genetic errors or turning formerly advantageous genetic alterations into disease causing factors. Another hypothesis states that human migration and therefore interbreeding of formally isolated populations, also increased the incidence of polygenic diseases (Awdeh & Alper 2005). This theory assumes that in different isolated populations, evolutionary selection can act against different alleles that must be present together in order for the disease to occur. As a result, high frequencies of some of these disease associated alleles persist in these populations. Therefore, offspring of parents originating from two of these populations would have a dramatically increased risk for the polygenic disease.

In this thesis I discuss my observations about two multifactorial immunopathologies, atopic asthma and Systemic Lupus Erythematosus (SLE). These two apparently distinct conditions manifest with profoundly different symptoms and pathologies; in the case of allergic asthma, an external antigen triggers disease, while SLE is characterized by autoantibody production against internal (nuclear) epitopes. Despite these differences, they share a common feature: both pathomechanisms involve exaggerated humoral immune responses.

1.1 Asthma

Asthma is a pulmonary disease associated with airway hyper-responsiveness to a variety of stimuli and chronic airway inflammation. There is accumulating evidence in the literature, that asthma is not a single-disease entity, since sub-phenotypes of this disease show differing pathologies, clinical expressions, response to treatments and long-term outcomes (Holgate & Davies 2009). Because most asthmatic patients are atopic (genetically predisposed to produce excessive IgE), this disease has been long regarded as an allergic disorder, characterized by T_H2 polarized T-cell responses. These T cell responses include the production of the cytokines IL-4, IL-5 and IL-13 which drives IgE production by B cells and the recruitment and activation of eosinophils. However, recent studies suggest that atopy is just one component of the pathology and that the structural and functional integrity of the airway epithelium is key to asthma susceptibility (Holgate & Davies 2009;Moffatt, Gut, Demenais, Strachan, Bouzigon, Heath, von Mutius, Farrall, Lathrop, & Cookson 2010). Moreover, high-throughput sequencing technology allowed for the precise characterization of the bronchial microbiome, which was shown to be altered in asthmatic lungs (Hilty et al. 2010). This suggests that host-microbe interaction is also a crucial element in asthma pathogenesis (Couzin-Frankel 2010).

Asthma has a strong genetic predisposition with an estimated 60% heritability (Duffy et al. 1990). Association studies of candidate genes and linkage analyses identified over forty asthma candidates (Weiss, Raby, & Rogers 2009), while a recent report of a consortium conducted, large scale, Genome Wide Association Study (GWAS) found ten significantly associated genes (Moffatt et al. 2010). Odds ratios associated to the polymorphisms of these genes are between 0.5 and 1.5, indicating that none of these variants have a major individual contribution to asthma heritability. Since the overall effect of these genes do not explain the high heritability of asthma, one can assume that these genes act together or with other genes in epistatic interactions (Weiss, Raby, & Rogers 2009).

Candidate gene analysis in childhood asthma

In contrast to linkage analysis and GWAS, genetic association studies of candidate genes is based on prior information about the pathogenesis of the disease.

We chose to examine the modifying effect of mannose-binding lectin (MBL), RANTES, TNFα, and MCP-1 genetic variants on childhood asthma. Mannose-binding lectin (MBL) is an essential effector molecule of the immune system which is involved in host-pathogen interactions, while RANTES, TNFα, and MCP-1 are cytokines (chemokines) which are involved in coordinating chronic inflammation. Cytokines play a crucial role in cell migration, activation and survival (Barnes 2008) in the asthmatic lung. Alterations in individual cytokine levels can lead to a cascade of events by affecting the secretion of other cytokines in a complex regulatory network.

Mannose-Binding Lectin (MBL)

MBL is a multimeric serum protein which contains up to six subunits, each of which is a trimer of an identical polypeptide chain. It is a soluble pattern recognition receptor, member of the collectin family and can bind mannose, N-acetyl-D-glucosamine, N-acetyl-mannosamine, fucose and glucose sugar residues on the surface of various microorganisms, which in turn initiates the activation of the complement cascade (Turner 2003). The serum concentration of mannose-binding lectin is highly reduced in heterozygous individuals with MBL allelelic variants which are unable to trimerize and therefore cannot assemble into functional proteins. Such allelic variants have been found to be associated with an increased risk of childhood respiratory infections (Garred et al. 2002).

RANTES

RANTES (CCL5) is a chemotactic protein which belongs to the CC chemokine family. It is primarily produced by T cells and the endothelium and attracts various leukocytes (e.g. monocytes, NK cells, T cells, basophils and eosinophils) through binding to the chemokine receptors CCR3, CCR5 and CCR1 (Kenneth M.Murphy, Paul Travers, & Mark Walport 2008). Promoter region (-403) of the RANTES gene carries a (G/A) functional polymorphism which affects transcription and has been shown to be associated with atopic dermatitis (Nickel et al. 2000).

Tumor Necrosis Factor alpha (TNFα)

TNFα belongs to the TNF family of cytokines (Bazzoni & Beutler 1996). The active protein is a homotrimer which is released as a soluble mediator from its membrane-bound precursor by proteolytic cleavage (Black et al. 1997) and it interacts with TNFR-I and TNFR-II receptors. TNFα is produced predominantly, but not exclusively by macrophages and serves as an important regulator of leukocyte activation and migration, acute-phase response, cell proliferation, differentiation, and apoptosis (Kenneth M.Murphy, Paul Travers, & Mark Walport 2008).
The -308 G/A polymorphism in the promoter region of human TNFα has been shown to be associated with a worse prognosis in cerebral malaria (McGuire et al. 1994) and in leishmaniasis (Cabrera et al. 1995). Furthermore, the disease associated "A" allele has been demonstrated to mediate stronger transcriptional activation than the common "G" allele (Wilson et al. 1997).

Monocyte chemoattractant protein-1 (MCP-1)

MCP-1, also known as CCL2, 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 (Daly & Rollins 2003). Surprisingly, considerable variability has been found in the ability of normal human PBMCs to produce MCP-1 in response to IL-1β. The -2518 A/G polymorphism in the distal promoter of MCP-1 is thought to be a contributor to this variability (Rovin, Lu, & Saxena 1999). This 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 (Rovin, Lu, & Saxena 1999).

Earlier reports indicate that the MCP-1 -2518 G allele is associated with the development of asthma and that the severity of the disease correlates with the homozygosity of the G allele (Szalai et al. 2001). In this thesis, genetic association studies were carried out to verify this finding on an independent cohort of asthmatic and control children. Additionally, 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.

Chlamydophila pneumoniae is a common respiratory pathogen that is thought to be associated with the development of asthma in susceptible individuals (Lemanske, Jr. 2003). Furthermore, *C. pneumoniae* infection causes MCP-1 secretion in human endothelial cells (Molestina et al. 1999). It is plausible that MCP-1 secretion, fine-tuned by the -2518 A/G polymorphism, modifies the outcome of infection with *C. pneumoniae* and creates a genetic milieu where host-pathogen interactions determine asthma susceptibility. Therefore, we asked whether the MCP-1 -2518 polymorphisms are associated with asthma development in *C. pneumoniae* infected children and whether there was any correlation between MCP-1 serum concentration and the status of *Chlamydophila pneumoniae* infection in asthmatic children.

1.2 Systemic Lupus Erythematosus (SLE)

SLE is a chronic, autoimmune disease that affects various organs (e.g. skin, central nervous system, lungs, kidneys and heart). Since lupus manifestations are most pronounced in connective tissues in the joints and around the organs, it is classified as a rheumatological disorder. Pathogenesis of lupus depends in part on T cell dependent autoantibodies that are mainly directed against self-antigens in the cell nucleus. The SLE autoantibodies may be a consequence of defective fine-tuning of various immuno-regulatory pathways that are involved in APC-T cell or T-B cell interactions (Schraml & Peng 2005), impaired censoring of autoreactive T and B cells in central lymphoid organs (Rubin 2005) or removal of immune complexes and apoptotic debris (Manderson, Botto, & Walport 2004). These processes are delicately mediated by cytokines, cell-surface receptors and the connected intracellular signaling molecules, all which have been implicated as SLE susceptibility genes (Graham et al. 2009;Magnusson et al. 2001).

Role of the SLAM-family (SLAMF) genes and their isoforms in the pathogenesis of SLE

The human chromosome locus 1q23, which includes the signaling lymphocyte activation molecule (SLAM)-family genes, was implicated in lupus susceptibility by several independent genome-wide linkage scans of SLE families (Gray-McGuire et al. 2000;Johanneson et al. 2002;Moser et al. 1999;Shai et al. 1999;Tsao et al. 1997;Tsao et al. 2002;Wakeland et al. 2001). In mice, the region syntenic to human 1q23 has been found to be associated in three different models of spontaneous lupus: the (NZB x NZW) F2 intercross, the NZM/Aeg2410 New Zealand mouse, and the BXSB mouse (Hogarth et al. 1998;Kono et al. 1994;Rozzo et al. 1996). These mice, like lupus patients, develop autoantibodies and severe nephritis.

In order to dissect the individual effect of this locus on SLE development, Wakeland and colleagues (Morel et al. 1997) generated the *Sle1* congenic mouse strain by transferring an NZM2410 genomic segment onto the non-autoimmune *C57BL/6* (*B6*) genetic background, using microsatellite marker-assisted breeding. *Sle1* mice develop anti-nuclear antibodies (ANAs), splenomegaly and have elevated percentages of activated (CD69+) CD4+ T cells and (B7.2+) B cells at 7-9 months of age (Morel et al. 2001b). However, these mice do not develop fatal glomerulonephritis (GN) unless they are bred with other SLE susceptibility loci (e.g. Sle1, Sle2, Sle3, Yaa etc.) on the *B6* background.

Analysis of recombinant strains within the Sle1 locus resulted in the generation of the *B6.Sle1a*, *B6.Sle1b*, *B6.Sle1c* sub-congenics. These mice exhibit SLE-related phenotypes (i.e. ANA, spontaneous lymphocyte activation) to various extents and carry congenic loci with a relatively small number of lupus candidate genes (Morel, Blenman, Croker, & Wakeland 2001b). Sle1b is the most potent locus out of the three sub-congenic loci and these *B6.Sle1b* mice develop high titers of ANA with more than 90% penetrance at 12 months of age (Wandstrat et al. 2004). This locus carries 24 expressed genes and 2 pseudogenes originating from the NZW genome, 7 of which belong to the SLAM-family of receptors (**Figure 1**).

The SLAM genes are the most viable lupus susceptibility candidates out of the 24 possible targets because they are highly expressed in all lymphoid organs and are highly polymorphic. Most laboratory mouse strains can be classified into SLAM haplotype I or haplotype II loci based upon SNP, sequence and transcript analyses (Wandstrat, Nguyen, Limaye, Chan, Subramanian, Tian, Yim, Pertsemlidis, Garner, Jr., Morel, & Wakeland 2004). The haplotype I SLAM locus associated strains include *C57BL/6* (*B6*) and *MOLF*, while *BALB/c*, *129*, *MRL*, *NOD* and *NZW* contain the haplotype II locus (Wandstrat, Nguyen, Limaye, Chan, Subramanian, Tian, Yim, Pertsemlidis, Garner, Jr., Morel, & Wakeland 2004). Wakeland and colleagues proposed that autoimmunity develops when the haplotype II SLAM locus is introduced into the *B6* genome (Morel et al. 1999a;Wandstrat, Nguyen, Limaye, Chan, Subramanian, Tian, Yim, Pertsemlidis, Garner, Jr., Morel, & Wakeland 2004). This hypothesis was supported by the work of Marina Botto and her

group, who demonstrated that the hybrid of two non-autoimmune strains, *B6* (haplotype I SLAMs) and *129* (haplotype II SLAMs) develops a lupus like syndrome (Bygrave et al. 2004;Carlucci et al. 2010). They were able to segregate several lupus susceptibility loci by generating *B6.129* congenic strains. One of these strains, *B6.129chr1b*, contains a *129* genomic insert which entirely covers the SLAM locus. In conclusion, a lupus phenotype occurs in the congenic *B6.Sle1b* and *B6.129chr1b* mice because of unidentified epistatic genetic interactions between the haplotype 2 *Slamf* locus and the *B6* background genes.

SLAM genes belong to the subfamily of the immunoglobulin superfamily (Calpe et al. 2008b). They are differentially expressed on the cell-surface of various hematopoietic cells and most of them are involved in homotypic interaction. They transmit signals during cell-cell interactions in various immunological synapses (,i.e. T and B cell (Cannons et al. 2010;Morra et al. 2005;Punnonen et al. 1997;Qi et al. 2008;Romero et al. 2005), APC and T cell (Bleharski et al. 2001;Rethi et al. 2006;Wang et al. 2004), NK cell and its target cell (Bottino et al. 2001;Garni-Wagner et al. 1993;Wang et al. 2010) etc.) and some members have been shown to recognize bacterial cell wall components (Berger et al. 2010) (Malaviya et al. 1999). Upon ligand engagement, SLAM receptors are phosphorylated on a

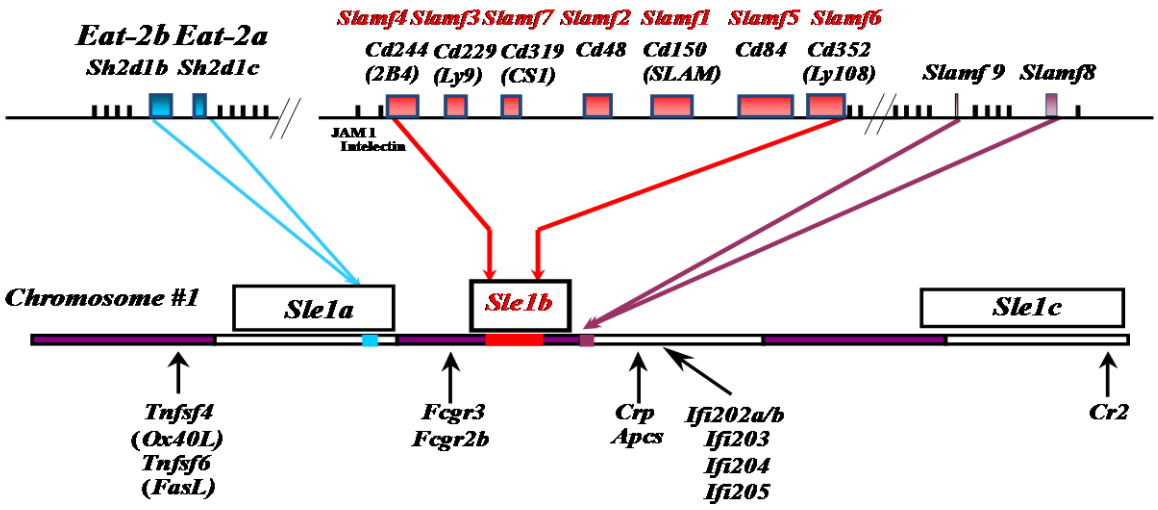

Figure 1. Genomic organization of the murine SLAM genes and the Sle1a, Sle1b, Sle1c lupus susceptibility loci. (Figure is adapted from Calpe et. al., 2008)

unique tyrosine containing motif, termed an immuno tyrosine switch motif (ITSM). Phosphorylated ITSM recruits the adaptor SLAM Associated Protein (SAP) in T cells and EAT2A or EAT2B in antigen-presenting cells (APCs) (Bottino, Falco, Parolini, Marcenaro, Augugliaro, Sivori, Landi, Biassoni, Notarangelo, Moretta, & Moretta 2001;Eissmann et al. 2005;Fraser et al. 2002;Morra et al. 2001;Roncagalli et al. 2005;Sayos et al. 1998;Tangye et al. 1999;Tovar et al. 2002). These adaptor proteins link the extracellular signals (cell-cell interaction, bacteria) to the intracellular signaling network.

Among the human SLAM genes, SLAMF1 (You et al. 2010), SLAMF3 (Cunninghame Graham et al. 2008), SLAMF4 (Kim et al. 2010) and SLAMF7 (Kim, Mathew, Patel, Pertusi, & Mathew 2010) were implicated as lupus candidate genes. In mice, Ly108 (*Slamf6*) is 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, Nguyen, Limaye, Chan, Subramanian, Tian, Yim, Pertsemlidis, Garner, Jr., Morel, & Wakeland 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. The goal of this work was to dissect the individual influence of *Slamf* members on SLE development, using knock-out and transgenic animal models.

CHAPTER 2: AIMS

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

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

2. To investigate the correlation of serum MCP-1 levels with asthma.

   2.1 To examine whether there is a difference between MCP-1 serum levels in control and asthmatic children.

   2.2 To assess whether there is a difference between MCP-1 serum levels in subgroups of asthmatic children (e.g. atopic vs. non-atopic, girls vs. boys, severity score etc.).

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

3. To determine whether the MCP-1 –2518A/G genotype modifies asthma susceptibility among *Chlamydophilia pneumoniae* infected children.

   3.1 Test the hypothesis that *Chlamydophilia pneumoniae* infection correlates with MCP-1 serum levels in asthmatic children.

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

1. To examine the individual effect of distinct *Slamf* members and their adaptor proteins on the pathogenesis of murine lupus using gene-targeted knockout mice:

   1.1 Comparing and characterizing the SLE-like syndrome in *Slamf1*$^{-/-}$, *Slamf2*$^{-/-}$, *Slamf3*$^{-/-}$, *Slamf6*$^{-/-}$ *[B6.129]* congenic knock-out strains.

   1.2 Test the hypothesis that *Slamf4*$^{-/-}$ *[B6]* develops autoimmunity.

   1.3 Test whether null mutations of the *SLAMF* adaptor proteins, SAP, EAT2A and EAT2B leads to SLE.

2. To dissect the role of the *Slamf6* (*Ly108*) isoforms in the pathogenesis of murine lupus.

   2.1 To test the hypothesis that *B6.Sle1b* peripheral CD4+ T cells have an intrinsic lupus related phenotype.

   2.2 Test the hypothesis that the over-expression of the putative pathogenic isoform, *Ly108-1* in T cells, causes autoimmunity.

   2.3 Test the hypothesis that the novel *Ly108* isoform, *Ly108-H1*, is protective against SLE when introduced into the *B6.Sle1b* genetic background by transgenesis.

   2.4 Determine the influence of *Ly108-H1* on CD4+ T cells.

CHAPTER 3: METHODS

Subjects

The asthmatic children attended the Allergic Outpatient Consultation of the Budai Children's Hospital and they had specialist physician-diagnosed asthma with the following characteristics: (1) recurrent breathlessness and expiratory dyspnea requiring treatment; (2) physician-diagnosed wheeze; (3) reversibility of the wheezing and dyspnea by bronchodilator treatment measured as forced expiratory volume in 1 second (FEV1) by a spirometer (Piston).

The control children were randomly selected from outpatients from the Orthopaedic Department in the Budai Children's Hospital. Subjects in the control group had mild musculoskeletal alterations such as pes planus or scoliosis but showed no symptoms of asthma or infection and required no medication. Stratification by age and sex was used to approximately match the age and sex distribution of the control subjects with that of the asthmatic cases.

Laboratory analysis

Total genomic DNA was extracted from white blood cells using the method of Miller (Miller, Dykes, & Polesky 1988) or by using QIAamp DNA Blood Midi Kit (QIAGEN GmbH).

All genotypes were determined by PCR-RFLP method. We used the following primers for PCR amplifications: MCP-1FOR: 5'-TCT CTC ACG CCA GC ACT GACC-3' and MCP-1REV: 5'-GAG TGT TCA CAT AGG CTTCTG-3'; RANTES_FOR: 5'-CAC AAG AGG ACT CAT TCC AAC TCA-3' and RANTES _REV: 5'-GTT CCT GCT TAT TCA TTA CAG ATC GTA-3'; TNF_FOR: 5'- ATC TGG AGG AAG CGG TAG TG-3' and TNF_REV: 5'-AAT AGG TTT TGA GGG CCA TG-3'. We applied restriction enzyme digestion on the PCR products to be able to detect the different SNP alleles (RFLP). MCP-

1 PCR products were digested with PvuII, while RANTES and TNFα PCR fragments were digested with RsoI and NcoI restriction endonucleases respectively.

MBL PCR-RFLP assay was performed as described by (Ten et al. 1999).

The serum levels of MCP-1 were measured in duplicate with the Quantikine colorimetric sandwich ELISA kit (R&D Systems).

C. pneumoniae–specific IgA and IgG were determined from serum using Sero CP-IgA, IgG protein ELISA (Savyon Diagnostics) according to the manufacturer's protocol.

Total serum immunoglobulin E (IgE) levels and specific IgE levels to more than 100 allergens were determined by the Pharmacia CAP System (Uppsala, Sweden).

Statistical Methods

Allele frequencies were calculated by allele counting. 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) programs."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).

Mice.

B6, *NOD/LtJ*, *MOLF/EiJ*, *BALB/c* and *B6.C-H-2bm12/KhEg* (*bm12*), *129/SvEvTac* (*129*) mice were obtained from The Jackson Laboratory and from Taconic. *B6.129chr1b* (Carlucci *et. al*., 2007) mice generously donated by Dr. M. Botto (Imperial College London). *B6.Sle1b* (*Sle1b*) (Morel *et al.*, 2001) mice were provided by Dr. L. Morel. Ly108-/- [*129xB6*] (Howie et al. 2005) was backcrossed six times with *B6* and breeders were selected for the smallest congenic interval. *Slamf2*$^{-/-}$ mice (Gonzalez-Cabrero 1999) were generated with J1 *129* ES cells and backcrossed onto the *C57BL/6* background for 12 and onto the *BALB/c* background for 10 generations. *Slamf1*$^{-/-}$ mice (Wang, Satoskar,

Faubion, Howie, Okamoto, Feske, Gullo, Clarke, Sosa, Sharpe, & Terhorst 2004) were also generated with J1 *129* ES cells and backcrossed for 9 generations onto the *C57BL/6* and the *BALB/c* background.

Autoantibody ELISA.

Titer of anti-nucleosome (anti-histone / DNA complex) antibodies in mouse sera were determined by ELISA as described (Mohan et al. 1998). Anti-chromatin and anti-dsDNA assays are performed according to a standardized protocol (Cohen & Maldonado 2003). Anti-ssDNA ELISA was explained in (Walter et al. 2010). Autoantibody titer was expressed as ELISA units (EU), comparing OD values of samples with a standard curve prepared with serial dilutions of ANA-positive NZM2410 serum pool.

ANA

Specificity of autoantibodies was determined by indirect immunofluorescence using permeabilized Hep-2 cells (Antibodies Incorporated). After incubation with various dilutions of mouse sera, Hep-2 slides were developed with anti–mouse IgG F(ab')2 (Invitrogen). Quantitative analysis was performed by acquiring fluorescent images (Zeiss AxioImager M1) and determining main fluorescent intensity of Hep-2 nuclei (Volocity-Perkin Elmer).

Flow cytometry.

Single cell suspensions of spleens and thymuses were stained with directly conjugated monoclonal antibodies (eBioscience, BD Pharmingen or BioLegend) after blocking non-specific binding with CD16/32 and 20% rabbit-serum or 10% rabbit-serum. Data was acquired with LSRII cytometer (BD Pharmingen) and analyzed using FlowJo software (Treestar).

Histopathology.

Five-micron paraffin kidney sections were stained with haematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) for microscopic analysis. Kidneys were scored by a board-certified pathologist with subspeciality expertise in kidney pathology using the International Society of Nephrology / Renal Pathology Society (ISN/RPS) classification of lupus nephritis (Weening et al. 2004) for general classification into kidneys with active ("proliferative") glomerulonephritis (classes III and IV) and inactive ("non-proliferative") glomerulonephritis (classes I and II). The NIH lupus criteria (Austin, III et al. 1983) were also determined for each kidney in order to semiquantitatively assess the degree of lupus-like inflammatory activity (activity index) and chronic parenchymal damage (chronicity index).

Murine transfer model of lupus.

As described by Eisenberg and Cohen (Morris, Cohen, & Eisenberg 1990) 7-9 wks old naïve female *bm12* mice were injected *i.p.* with splenocytes, purified CD4+ T cells or purified CD62L+ CD4+ naïve T cells (Miltenyi, magnetic bead cell separation) from age and sex matched donors.

Genotypic analysis.

Polymorphic genetic markers were typed by PCR. Primer sequences were obtained from Mouse Genome Informatics database (http://www.informatics.jax.org/).

Metaphase Fluorescence In Situ Hybridization (FISH).

Metaphase chromosome preparations were derived from LPS (10μg/ml, Sigma) activated splenocytes. RP23-77A8 (*Slamf1*, *Slamf5*, Ly108) *B6* BAC clone was labeled with biotin-nick translation method (Roche) and hybridized overnight with the metaphase preparations. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated streptavidin followed by DAPI counter-staining.

RT-PCR.

RNA was isolated from cells using the RNeasy kit (Qiagen) or Trizol (Invitrogen). Reverse transcription was performed with the Protoscript cDNA Kit (New England Biolabs). Individual Ly108 isoforms were amplified with the following primers: Ly108commonForward (in Exon 5): 5'-TTTGACTAGCCAACATCC-3'; Ly108-1reverse: 5'-TTAAGAGTATTCGGCCTC-3'; Ly108-2/H1reverse: 5'-TCAGGAGTTATAGTTG AT-3'. RT-PCR for selective semi-quantitative amplification of Ly108-H1 was performed with the following primers: Ly108-H1forward: 5'-CCTACTCCCTGCAAATCAGC-3'; Ly108-H1reverse: 5'-CCGGTTAAAGCCACTGTTTCCTG-3'. GAPDH specific primers were obtained from New England Biolabs.

Immunoprecipitation and Western blotting.

Anti-Ly108 (13G3) monoclonal antibody, R1 and R4 rabbit polyclonal antibodies were generated by S. Rietdijk and X. Romero. R1 and R4 are directed against the peptides Cys-KNDSMTIYSIVNHSRE and Cys-ALTGYNQPITLKVNTLINYNS peptides respectively. Ly108 was precipitated from cell lysates using anti-Ly108 (13G3) coated protein-G agarose beads (Invitrogen) and deglycosylated using immobilized Carbohydrate Binding Domain-PNGase-F fusion protein (CBM-PNGaseF), generously donated by Dr. Anthony Warren (UBC, Canada). SDS-PAGE gel-electrophoresis (with 12% continuous gel and MOPS running buffer) and blotting was performed using the Invtitrogen XCell SureLock system.

CHAPTER 4: RESULTS

4.1 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 of developing asthma in children

We compared the genotype of the MCP-1 –2518A/G polymorphism in one hundred and sixty asthmatic Hungarian children (95 boys; 65 girls; 3–18 years of age, mean 10.5 [SD: 4.3] years) with 158 healthy children (91 boys; 67 girls; age 3–18 years of age, mean 11.2 years [SD: 4.8]). In agreement with a previous study (Szalai, Kozma, Nagy, Bojszko, Krikovszky, Szabo, & Falus 2001) we found that the more inducible MCP-1 –2518G gene variant is present at a significantly higher frequency in asthmatic children than in healthy controls (**Table 1**). $\chi 2$ goodness-of-fit test verified that the allele frequencies followed the Hardy-Weinberg principle. The odds ratio, an estimate for the relative risk associated with

Polymorphism	Population	Genotypes			Total	Allele G frequencies
		AA	AG	GG		
MCP-1	Asthmatic	68 (42.5%)	76 (47.5%)	16 (10.0%)	160	33.8%*
-2518 A/G	Controls	96 (60.8%)	48 (30.4%)	10 (6.3%)	158	22.8%*

* P<0.01; odds ratio = 1.73 (1.22-2.45) asthmatic vs. control children

Table 1. Genotype and allele frequencies for MCP-1 -2518 polymorphism in asthmatic and control children.

this polymorphism was 1.73 (95% CI 1.22-2.45). In conclusion, our study verified the previous independent result, stating that the MCP-1 –2518G allele is a risk factor for childhood asthma in the Hungarian population.

No significant association between non-functional MBL variants, RANTES -403G/A or TNFα -308G/A polymorphisms and childhood asthma.

In order to assess whether non-functional mannose binding lectin polymorphisms were associated with asthma, we examined the allele frequency of four MBL variants in 139 asthmatic children (79 boys and 60 girls; age range, 3-18 years; mean age, 10.5 years [SD 4.1]) and 174 healthy children (95 boys and 79 girls; age range, 3-18 years; mean age, 11.5 years [SD 3.9]). Allele *A* was the normal allele, allele *B* was a G54D mutation, allele *C* was a E57G mutation and allele *D* was an R52C mutation (Madsen et al. 1994;Sumiya et al. 1991). The B, C and D alleles are located in the collagenous Gly-Xaa-Yaa repeated structure of MBL, which is essential for trimerization of the protein. There was no significant difference between the prevalence of these genetic variants between control and asthmatic children (data not shown).

The frequency of polymorphisms in the promoters of RANTES -403G/A and TNFα -308G/A were determined in 139 asthmatic and 174 control children. Allele frequency of the minor "A" allele of RANTES -403 SNP was 13.9% in asthmatic and 13.2% in control children, whereas allele frequency of the minor "A" allele of TNFα -308 SNP was 17.0% in asthmatic and 16.4% in control children. Allele frequencies in the case of both polymorphisms corresponded to the Hardy-Weinberg equilibrium, but no statistically significant association have been found between these variants and childhood asthma.

MCP-1 serum levels are significantly higher in control than in asthmatic children.

MCP-1 serum concentrations in asthmatic patients and healthy control groups were measured by ELISA (**Table 2**). Surprisingly, we found that the serum MCP-1 levels were significantly higher in control than in asthmatic children (315 ± 106 pg/mL *vs*. 189 ± 85 pg/mL, respectively; $p < 0.0001$). Since glucocorticoids inhibit MCP-1 expression

(Miyamasu et al. 1998), we stratified the asthmatic population according to their inhaled steroid usage (patients with oral glucocordicoid therapy were excluded from the study). No significant difference was found in serum MCP-1 levels between patients who used inhaled corticosteroids and the non-users (**Table 3**).

Population	n	SeMCP-1 (pg/ml)
Asthmatic patients	66	189 ± 85*
Controls	46	315 ±106*

* P< 0.0001

Table 2. Mean serum MCP-1 levels in control and asthmatic children (mean+/- SD).

Patients with atopic asthma or high serum levels of IgE have significantly lower levels of serum MCP-1.

Asthma patients were stratified according to their atopic status (positive skin prick test to at least one allergen) or their serum IgE level (>100kU/L is considered high IgE level). Both patients with atopic asthma and high serum levels of IgE had significantly lower levels of serum MCP-1 as compared to controls (**Table 3**).

Patients with severe asthma have significantly lower serum levels of MCP-1compared to other patients.

In order to examine whether the asthma severity score correlated with serum MCP-1 concentration in patients, we stratified our samples according to the Global Initiative for Asthma guidelines (NHLBI/WHOWorkshop Report 1995.). We did not find a significant difference in MCP-1 serum levels between score 1, 2 or 3 patients [there was no score 4 (severe persistent asthmatic patients) in the study] judged by ANOVA (Table 3), however we did find significantly higher serum MCP-1 in the combined score 1+2 groups compared to score 3 patients (150 ± 40 pg/mL and 199 ± 85 pg/mL in patients with severity scores = 3, and severity scores = 1 +2, respectively; $p < 0.05$) most likely indicating a weak, non-linear correlation between the severity score and MCP-1 serum levels.

Population	n	SeMCP-1 (pg/ml)	ANOVA	
			F	p
Inhaled steroids			With vs. without inhaled steroids	
Yes	51	185 ± 89	0.45	0.5
No	15	202 ± 71		
			High vs. low IgE responders	
High IgE responders (>100kU/l)	39	178 ± 78		
Low IgE responders (<100kU/l)	22	224 ± 90	4.3	0.04
			Atopic vs. non-atopic	
Atopic*	52	178 ± 81	3.8	0.05
Non-atopic*	14	230 ± 93		
			Girls vs. boys	
Girls	22	224 ± 88	5.5	0.02
Boys	44	172 ± 80		
Asthma severity scores			1 vs. 2 vs. 3	
1 (intermittent)	24	193 ± 79		
2 (mild)	29	203 ± 100	1.8	0.18
3 (moderate)	13	150 ± 40†		

* Positive skin prick test to at least one allergen and/or positive total or specific IgE levels.

† $P < 0.05$ (1+2 vs. 3)

Table 3. Comparison of the mean serum MCP-1 levels of asthmatic children according to their inhaled steroid usage, genders, total IgE levels and atopic status (mean+/- SD).

Significant gender bias in serum MCP-1 levels among asthmatic children.

We found that serum MCP-1 levels were 1.3 times higher in asthmatic girls than in asthmatic boys (**Table 3**).

There is no correlation between the MCP-1 –2518A/G genotype and the concentration of MCP-1 in the serum.

Since the MCP-1 –2518 polymorphism affects the secretion of this cytokine, we examined if MCP-1 serum levels in asthmatic and non-asthmatic individuals vary according to their

MCP-1 –2518 genotypes	Asthmatic patients		ANOVA*			
			AA vs. AG vs. GG		AA vs. AG + GG	
	SeMCP-1 (pg/ml)	n	F	p	F	p
AA	211 ± 96	28				
AG	175 ± 76	28	1.5	0.24	2.8	0.1
GG	170 ± 76	10				
	Control subjects					
AA	328 ± 107	23				
AG	295 ± 105	16	0.8	0.46	1.2	0.28
GG	318 ± 62	7				

*Analysis of variance (ANOVA) with additive as well as dominant models of MCP-1 genotype was performed.

Table 4. MCP-1 serum levels in control and asthmatic children according to their MCP-1 genotypes (mean +/- SD).

MCP-1 –2518G/A genotype.

We stratified asthmatic and healthy control patients according to their MCP-1 genotype and compared MCP-1 serum levels by ANOVA. No significant associations were found between the MCP-1 genotype and serum MCP-1 levels according to ANOVA analysis using either the additive (AA vs. AG vs. GG) or the dominant (AA vs. AG + GG) models (**Table 4**).

Asthmatics infected with *Chlamydophila pneumoniae* are more frequent carriers of the MCP-1 -2518 G allele as compared to infected non-asthmatics.

Current or recent infection with *C. pneumoniae* was determined by measuring specific antibodies in sera against this pathogen by ELISA. To judge whether anti-*C. pneumoniae* seropositive children, carrying MCP-1 -2518 G variants were more susceptible to asthma we studied 139 asthmatic and 174 control children, using logistic regression analysis.

There was a significantly higher percentage of anti-*C. pneumoniae* IgG seropositive, MCP-1 -2518G allele carriers among asthmatic children than in the control group (65.8% asthmatic vs. 37.8% controls). As shown in **Table 5**, MCP-1 -2518G allele carriers that were anti-*C. pneumoniae* IgG seropositive had a 2.84 fold increase in asthma susceptibility as compared to seropositive A/A homozygotes (95% CI = 1.43-5.87; p=0.001). However, carrying the MCP-1 -2518G allele alone was already a significant risk factor [OR (95% CI)= 1.88 (1.32–2.69) in this study] for development of the disease. As these two results were not significantly different, the data are only suggestive that *C. pneumoniae* status contributes to an added risk in the onset of asthma. A larger cohort of subjects is required to further address this issue.

	MCP-1 -2518 G carriers vs. A/A homozygotes	
C.pneumoniae status	OR	p Value
IgG positive (n = 148)	2.84 (1.43-5.87)	0.001
IgA positive (n=98)	1.99 (0.97-4.86)	0.06
IgG +IgA positive (n=66)	2.91 (1.18-7.56)	0.03

Table 5. Logistic regression analysis for the association between the prevalence of the MCP-1 -2158 genotypes and asthma in children positive for C. pneumoniae specific antibodies. Data are adjusted for age and sex. The dependent variable in the multiple logistic regression analysis was asthma; independent variables were carrier status, age, and sex. Only those subjects were considered who were positive for the given *C. pneumoniae* antibodies. (Table is adapted from Tolgyesi et. al., Pediatric Research, 2006)

No correlation was found between MCP-1 serum levels and *Chlamydophila pneumoniae* infection among asthmatic children.

There was no association between MCP-1 serum concentration and *Chlamydophila pneumoniae* infection status in asthmatic children (n=31 infected; 185+/-81pg/ml vs n=33 noninfected 191+/-90 pg/ml; p=0.8) .

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

4.2.1 Examining the individual effect of distinct *Slamf* members and their adaptor proteins on the pathogenesis of murine lupus using gene-targeted knockout mice

Slamf1$^{-/-}$*[B6.129]* and *Slamf3*$^{-/-}$*[B6.129]* mice, but not *Slamf1*$^{-/-}$ *[BALB/c.129]* develop lupus

In order to examine the role of Slamf1 and Slamf3 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. Female *Slamf1*$^{-/-}$ *[B6.129]*, *Slamf3*$^{-/-}$*[B6.129]* and *Slamf1*$^{-/-}$ *[BALB/c.129]* mice were kept under specific pathogen free (SPF) conditions during a period of 10 to 13 months until they were sacrificed for evaluation of spontaneous autoimmunity. Sera from aged *Slamf1*$^{-/-}$ *[B6.129]* and *Slamf3*$^{-/-}$*[B6.129]* mice but not from *Slamf1*$^{-/-}$ *[BALB/c.129]* animals contained high levels of anti-nuclear antibodies, as judged by ELISA (**Figure 2A**). To further characterize the spontaneous humoral autoimmunity which we found in these *B6.129* congenic knock-outs, we examined spleens and kidneys of the aged *Slamf1*$^{-/-}$ *[B6.129]* mice. Aside from the apparent splenomegaly in *Slamf1*$^{-/-}$ *[B6.129]* mice (**Figure 2B**), we found an increased percentage of effector/memory (CD44+CD62L-) CD4+ T cells (**Figure 2C**) compared to *B6* controls, indicating spontaneous activation of T lymphocytes in these mice. Although we did not observe any mortality among the *Slamf1*$^{-/-}$*[B6.129]* congenics, a significant fraction of these mice developed active glomerulonephritis (**Figure 2D, 2E**).

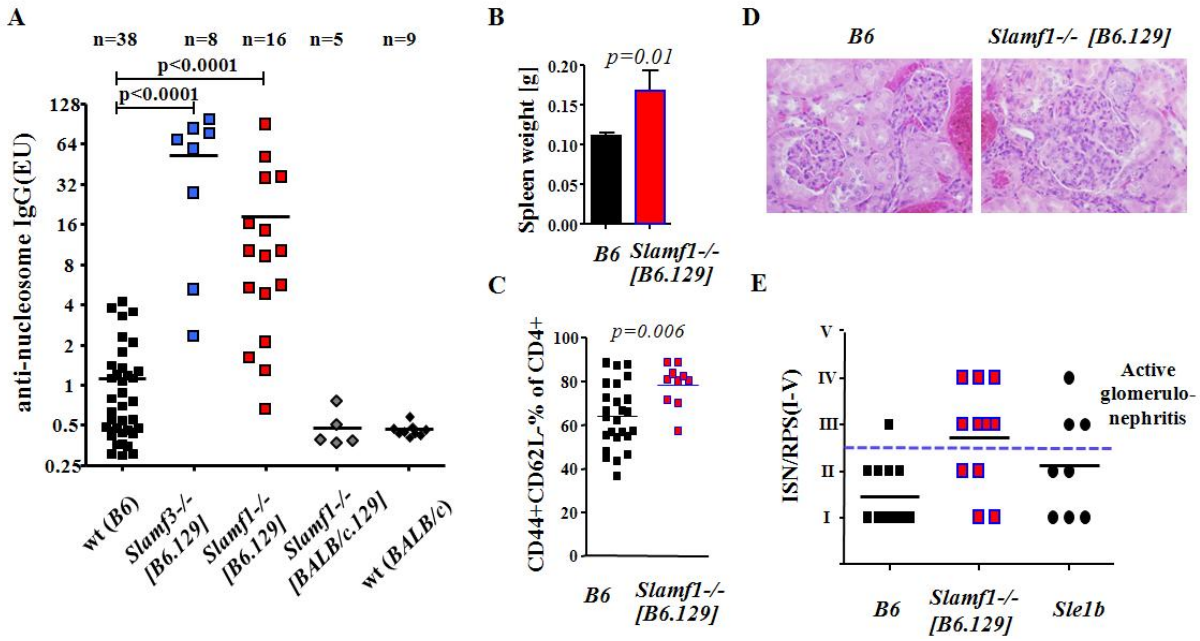

Figure 2. *Slamf1*$^{-/-}$*[B6.129]* and *Slamf3*$^{-/-}$*[B6.129]* mice, but not *Slamf1*$^{-/-}$ *[BALB/c.129]* develop lupus. (A) Anti-nucleosome autoantibodies in aged *Slamf1*$^{-/-}$ *[B6.129]*, *Slamf3*$^{-/-}$ *[B6.129]* and *Slamf1*$^{-/-}$ *[BALB/c.129]* mice determined by ELISA. Sera of 10 to13 month old individual females were used for the analysis. (B) Mild splenomegaly in aged *Slamf1*$^{-/-}$ *[B6.129]* mice. (C) Increased percentage of effector/memory CD4+ T cells in *Slamf1-/-[B6.129]* mice as judged by flow cytometry. Splenocytes were taken from 10-13 month old female *B6* (n=25) and *Slamf1-/- [B6.129]* mice (n=10). (D) Glomerulonephritis in *Slamf1*$^{-/-}$*[B6.129]* mice. Kidney sections from 10-13 month old female mice were processed and stained with haematoxylin and eosin. Glomerulonephritis manifesting as hypercellular, enlarged glomeruli with accumulation of leukocytes in the glomerular capillaries. (E) Quantitative evaluation of renal histology (done by Vijay K. Vanguri). ISN/RPS scores were determined as described in Methods. Scores III-IV (above dashed line) indicates active proliferative glomerulonephritis. (*B6* n=13; *Slamf1-/- [B6.129]* n=11; *Sle1b* n=8)

Neither *Ly108*$^{-/-}$*[B6.129]* nor *Ly108*$^{-/-}$*[B6]* mice develop lupus

In order to examine whether disruption of the lupus candidate gene, *Slamf6* (Ly108) would affect the development of SLE, we backcrossed our congenic *Ly108*$^{-/-}$ *[B6.129]* mouse, which had been generated with *129*-derived ES cells (Howie, Laroux, Morra, Satoskar,

Rosas, Faubion, Julien, Rietdijk, Coyle, Fraser, & Terhorst 2005), with the *wt B6* mouse. In addition, we generated a new *B6.Ly108*$^{-/-}$ mouse strain using *B6* ES cells (**Figure 3B**).

We found that anti-nucleosome IgG levels in aged *Ly108*$^{-/-}$ *[B6.129]* and *B6.Ly108*$^{-/-}$ were similar to the negative control *B6*. By contrast, high titers of anti-nucleosome IgG autoantibodies were detected in the age matched *Ly108* sufficient *B6.129chr1b* and *B6.Sle1b* controls (**Figure 3A**). Examining the expansion of splenic effector/memory CD4+ T cells, which is characteristic to this SLE model, produced similar results (data not shown).

In summary, we did not detect any sign of humoral autoimmunity in the aged *Ly108*$^{-/-}$ *[B6.129]* or the *Ly108*$^{-/-}$*[B6]* mice. Importantly, low resolution microsatellite analysis of the *129*-derived genomic segment in *Ly108*$^{-/-}$*[B6.129]* and the *Ly108+ B6.129chr1b* mice showed that they were similar (**Figure 3B**). Further mapping of the congenic breakpoint by high resolution SNP microarray (The Jackson Laboratory) showed that the *129*-derived segment is 2Mb longer in *Ly108*$^{-/-}$*[B6.129]* than in *B6.129chr1b*

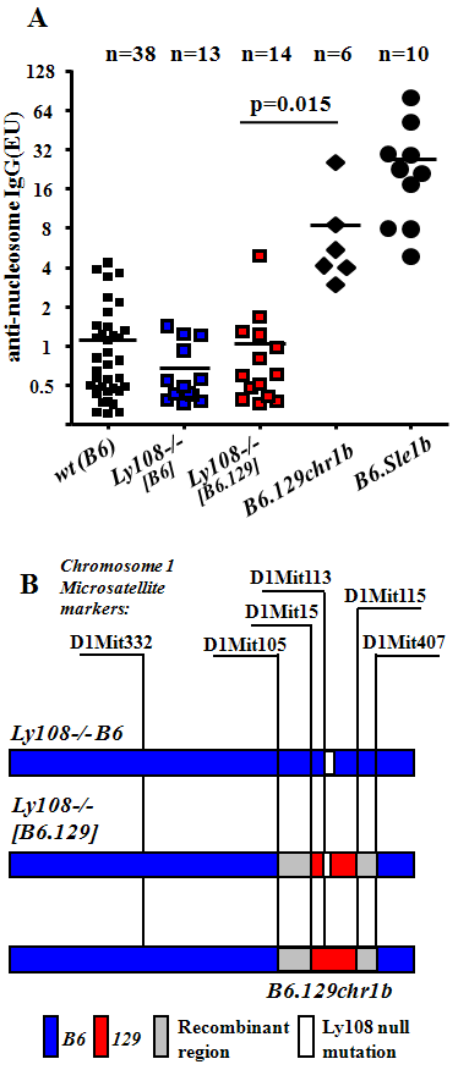

Figure 3. Neither *Ly108*$^{-/-}$*[B6.129]* nor *Ly108*$^{-/-}$*[B6]* mice develop lupus. (A) Absence of anti-nucleosome autoantibody production in aged Ly108-/- *[B6.129]* in comparison to lupus-prone *B6.129chr1b* mice determined by ELISA. Sera of 10-13 month old individual females were used for the analysis. (B) The *129* segments in chromosome 1 of *Ly108-/- [B6.129]* and of *B6.129chr1b* mice have similar boundaries determined by PCR analyses of microsatellite markers.

which overhang contains 18 RefSeq genes (data not shown). None of the possible differences between the *Ly108*$^{-/-}$*[B6.129]* and *B6.129chr1b* genomic sequence overlaps with any of the previously described SLE suppressor regions in 129 or NZW (Morel et al. 1999b;Subramanian et al. 2005).

Disruption of *Slamf2- Slamf4* heterotypic interaction induces autoimmunity

Slamf2$^{-/-}$ knock-out mice were generated in *129*-derived embryonic stem cells (ES cells) and subsequently crossed into *B6* and *BALB/c* mice. As expected, we did not find autoantibodies in the sera of aged *Slamf2*$^{-/-}$*[BALB/c.129]* mice, but did find elevated anti-nucleosome antibodies in *Slamf2*$^{-/-}$*[B6.129]* sera as early as 3 months of age (**Figure 4A**). By 6 months of age, two thirds of these mice had active glomerulonephritis which was marked by hypercellular glomeruli with leukocyte infiltration in glomerular capillaries along with expansion of mesangial areas (**Figure 4B**).

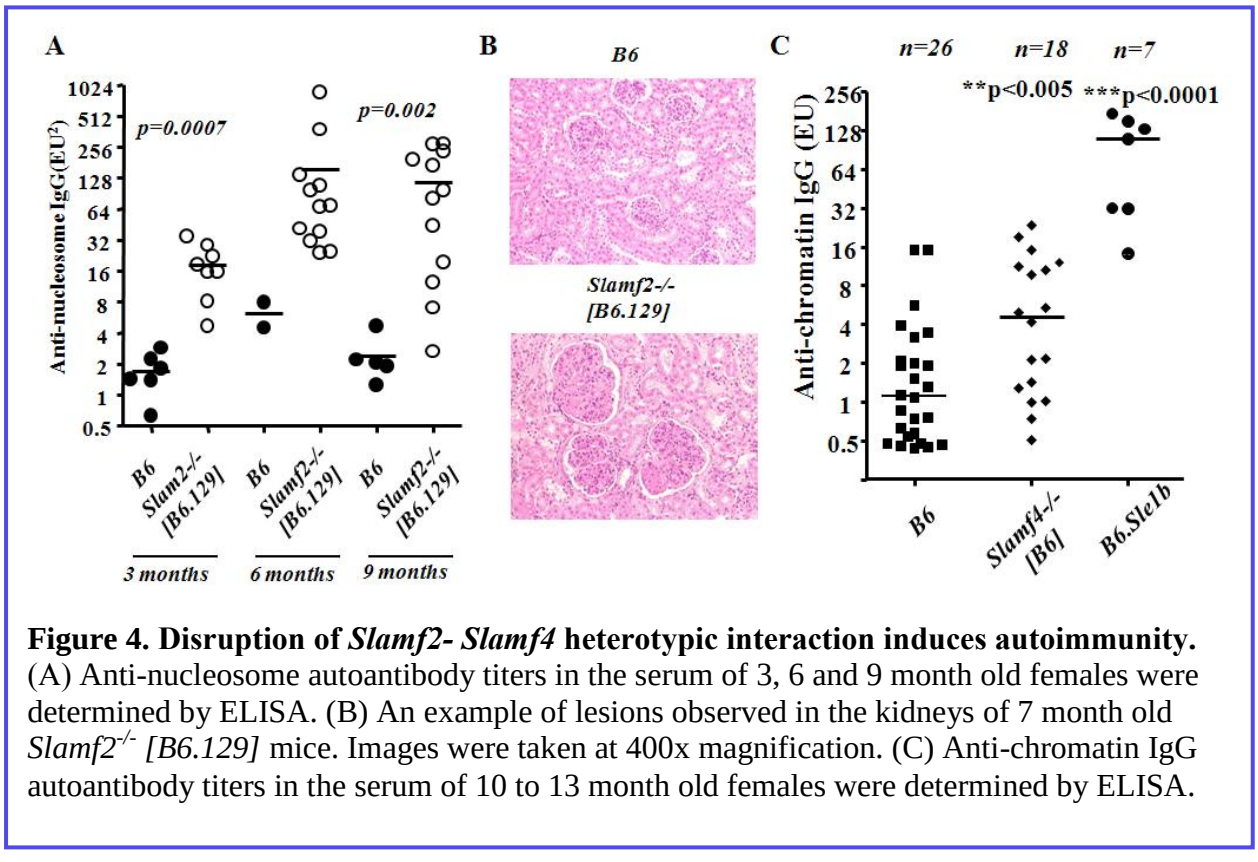

Figure 4. Disruption of *Slamf2- Slamf4* heterotypic interaction induces autoimmunity. (A) Anti-nucleosome autoantibody titers in the serum of 3, 6 and 9 month old females were determined by ELISA. (B) An example of lesions observed in the kidneys of 7 month old *Slamf2*$^{-/-}$ *[B6.129]* mice. Images were taken at 400x magnification. (C) Anti-chromatin IgG autoantibody titers in the serum of 10 to 13 month old females were determined by ELISA.

Furthermore, the *Slamf2*$^{-/-}$*[B6.129]* mice had an expansion of activated/memory T cells and activated B cells at 6 months of age (data not shown). These phenotypes occurred earlier than the similar ones described in the *Slamf1*$^{-/-}$*[B6.129]* or *B6.129chr1b* animals. Other work by our collaborator Arlene Sharpe has shown with the D011.10 adoptive transfer model (Kearney et al. 1994), that *Slamf2*$^{-/-}$ DO11.10 T cells were resistant to tolerance induction following adoptive transfer into *Slamf2*$^{-/-}$ recipients *in vivo*. These findings demonstrate that Slamf2 has critical role in peripheral T cell tolerance and suggest that a defect in peripheral T cell tolerance may be key to the development of SLE-like phenotype in *Slamf2*$^{-/-}$ *[B6.129]* mice. Based on all of these data, we hypothesized that disruption of *Slamf2* signals contributes to autoimmunity independently of the presence of the hybrid *129xB6* genetic background.

Unique among *Slamf* members, *Slamf2* is not engaged in homotypic interaction, but binds to *Slamf4* (and CD2 in humans). Therefore, according to our hypothesis, *Slamf2* and *Slamf4* knock-outs generated in B6 ES cells should develop spontaneous autoimmunity. As shown in **Figure 4C**, aged *Slamf4-/- [B6]* does indeed develop mild spontaneous autoimmunity, characterized by low titer serum anti-chromatin autoantibodies.

Knocking out the adaptor proteins of *Slamf* does not induce autoimmunity

Slamf members signal through the adaptor proteins SAP, Eat2A and Eat2B in mice and these proteins utilize identical anchoring ITSM motifs for initiating signal transduction (**Figure 5A**). We therefore examined if causing an imbalance in adaptor protein expression by targeted mutation of SAP, EAT-2A or EAT-2A+B could trigger SLE.

There was no sign of humoral autoimmunity in the absence of any of the adaptor proteins (**Figure 5B**). Moreover, the SAP null mutation even disrupted the low basal level of anti-nucleosome IgG antibody titers present in aged *B6* mice.

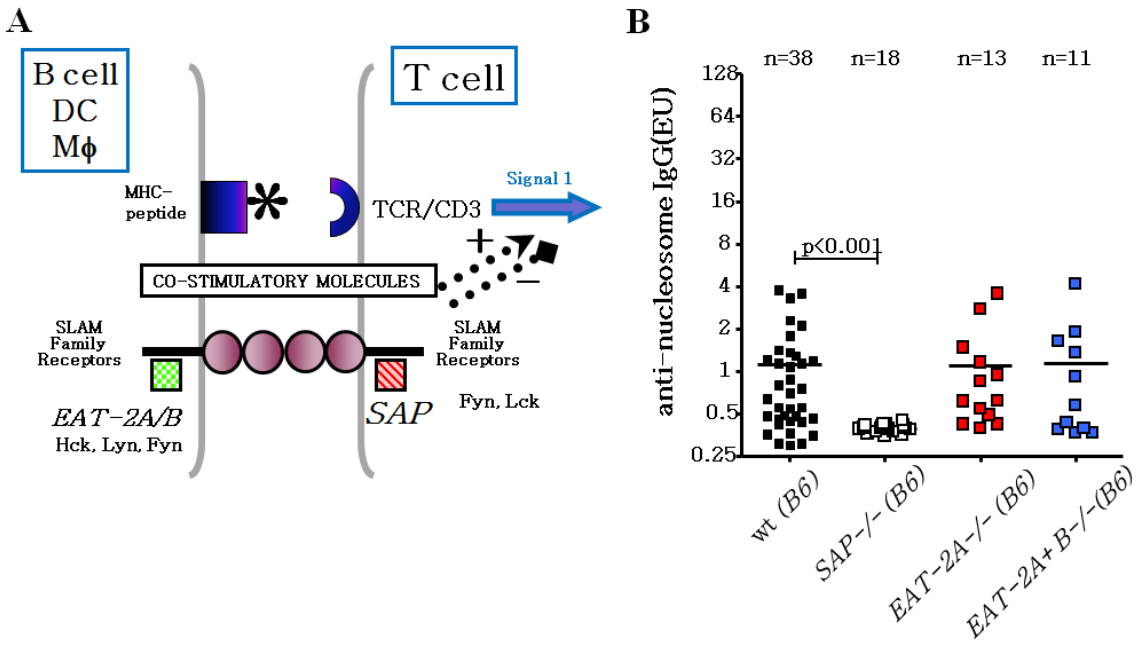

Figure 5. Knocking out the adaptor proteins of *Slamf* does not induce autoimmunity. (A) Binding of SLAM receptors to their ligands induces recruitment of SAP and Eat2A or Eat2B to their cytoplasmic ITSM motif. These adaptor proteins transmit SLAMF receptor mediated signals through Src kinases (e.g. Fyn) to the intracellular signal transduction network which modulate other activator signals (e.g. TCR crosslinking). (Figure is adapted from Calpe et. al., 2008) (B) Anti-nucleosome antibody titers were determined by ELISA. Sera of 10 to13 month old individual females were used for the analysis.

4.2.2 Dissecting the role of *Slamf6* (*Ly108*) isoforms in the pathogenesis of murine lupus.

Sle1b has an intrinsic peripheral CD4+ T cell phenotype

While T cells in the *Sle1* congenic mice show a broad range of autoimmune phenotypes [e.g. spontaneous CD4+ T cell activation, decreased number of T regulatory cells, presence of histone-specific T cells, increased proliferation and cytokine production (Chen, Cuda, & Morel 2005;Morel et al. 2001a)], the *Sle1b* subcongenic mice carry only a fraction of these defects [i.e. increased percentage of activated T cells and elevated calcium influx after receptor cross-linking (Chen, Cuda, & Morel 2005;Wandstrat, Nguyen, Limaye, Chan, Subramanian, Tian, Yim, Pertsemlidis, Garner, Jr., Morel, & Wakeland 2004)].

In order to test the hypothesis that peripheral T cells from *Sle1b* carry an intrinsic lupus related phenotype, we utilized the *bm12* model of murine lupus. This model involves the transfer of MHCII disparate *B6* splenocytes or purified CD4+ T cells into the *bm12* (*B6* coisogenic) mice and results in a SLE-like Chronic Graft-vs-Host disease (CGVHD) (Morris et al.,1990). According to the studies of Eisenberg and others (Morris et al. 1990), the graft CD4+ T cells induce polyclonal B cell activation in the host, resulting in significant autoantibody production against various nuclear antigens (e.g. single-stranded DNA, double-stranded DNA, chromatin) as early as 4 weeks after the cell transfer.

As demonstrated in **Figure 6**, the *Sle1b* total splenocytes or purified CD4+ total T cells induced a much higher anti-double stranded DNA (dsDNA) and single-stranded DNA (ssDNA) response than the wild-type *B6* ones upon injection into the *bm12* host.

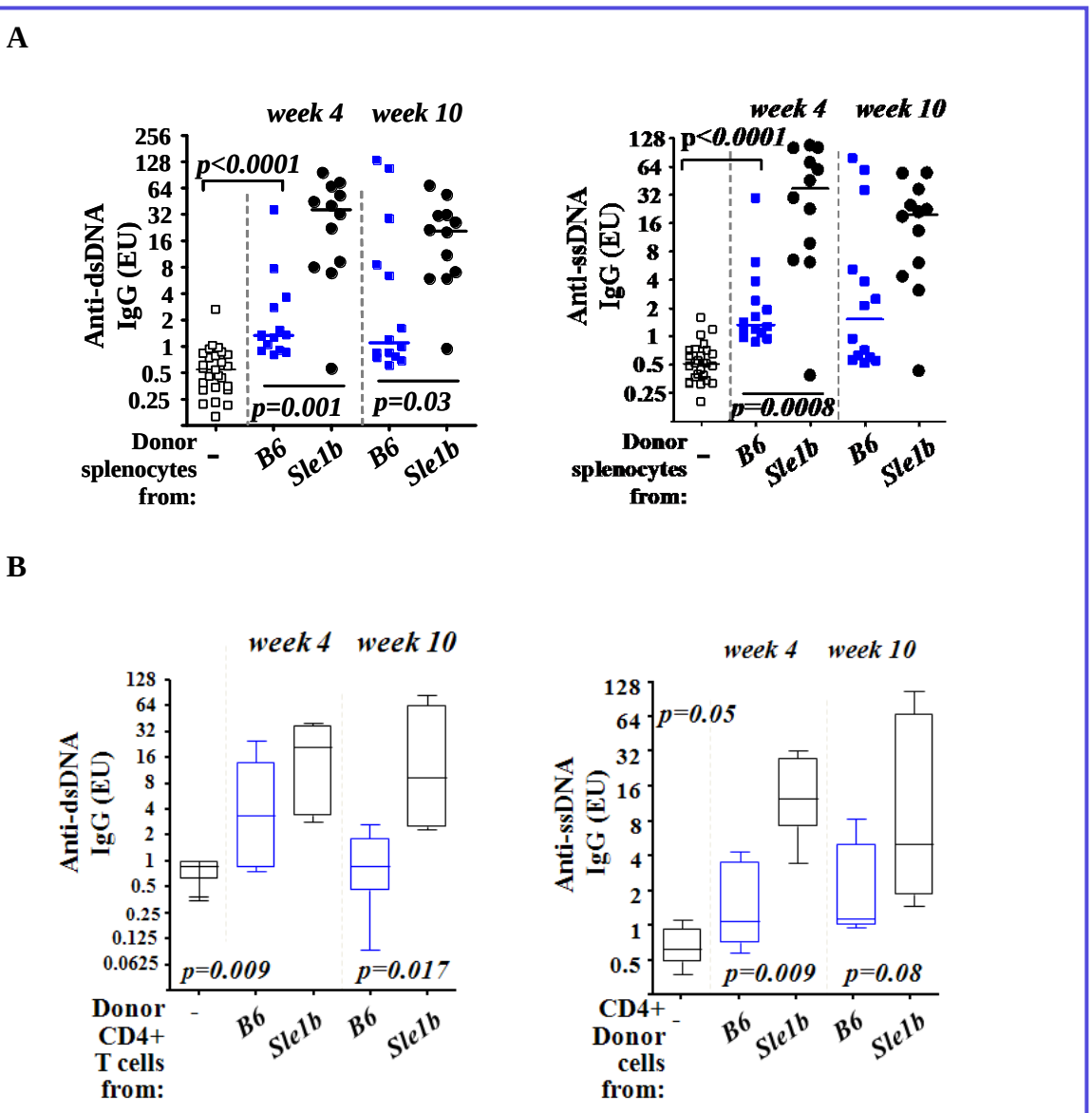

Figure 6. The transfer of *Sle1b* splenocytes or CD4+ T cells into *bm12* recipients induces higher autoantibody titers than the transfer of cells derived from *wt B6* mice. (A) Splenocytes ($5x10^7$) isolated from individual *B6* or *Sle1b* mice were transferred into *bm12* recipients by *i.p.* injection. After four or ten weeks, anti-dsDNA (left panel) and anit-ssDNA (right panel) antibodies in the serum were determined by ELISA and are expressed as ELISA Units (EU). (B) Purified CD4+ T cells ($7x10^6$) isolated from individual *B6* or *Sle1b* mice were transferred into *bm12* recipients by *i.p.* injection. After four or ten weeks, anti-dsDNA (left panel) and anti-ssDNA (right panel) antibodies in the serum were determined by ELISA and are expressed as ELISA Units (EU). *B6→bm12* (n=5); *Sle1b→bm12* (n=6).

Ly108 isoforms are differentially expressed in *B6* and *Sle1b* CD4+ T cells.

Differential gene expression in *B6.Sle1b* and *B6* anti-CD3 activated CD4+ T cells was compared by microarray hybridization.

Three probes recognizing different parts of the Ly108 transcripts (**Figure 7**) showed the most significant differences in gene expression between the *B6.Sle1b* and *B6* (1425086_PM_a_at $p_{adjusted}=5.6x10^{-6}$; 1420659_PM_at $p_{adjusted}=9.0x10^{-5}$; 1457773_PM_at $p_{adjusted}=7.5x10^{-6}$) 24 hours after activation.

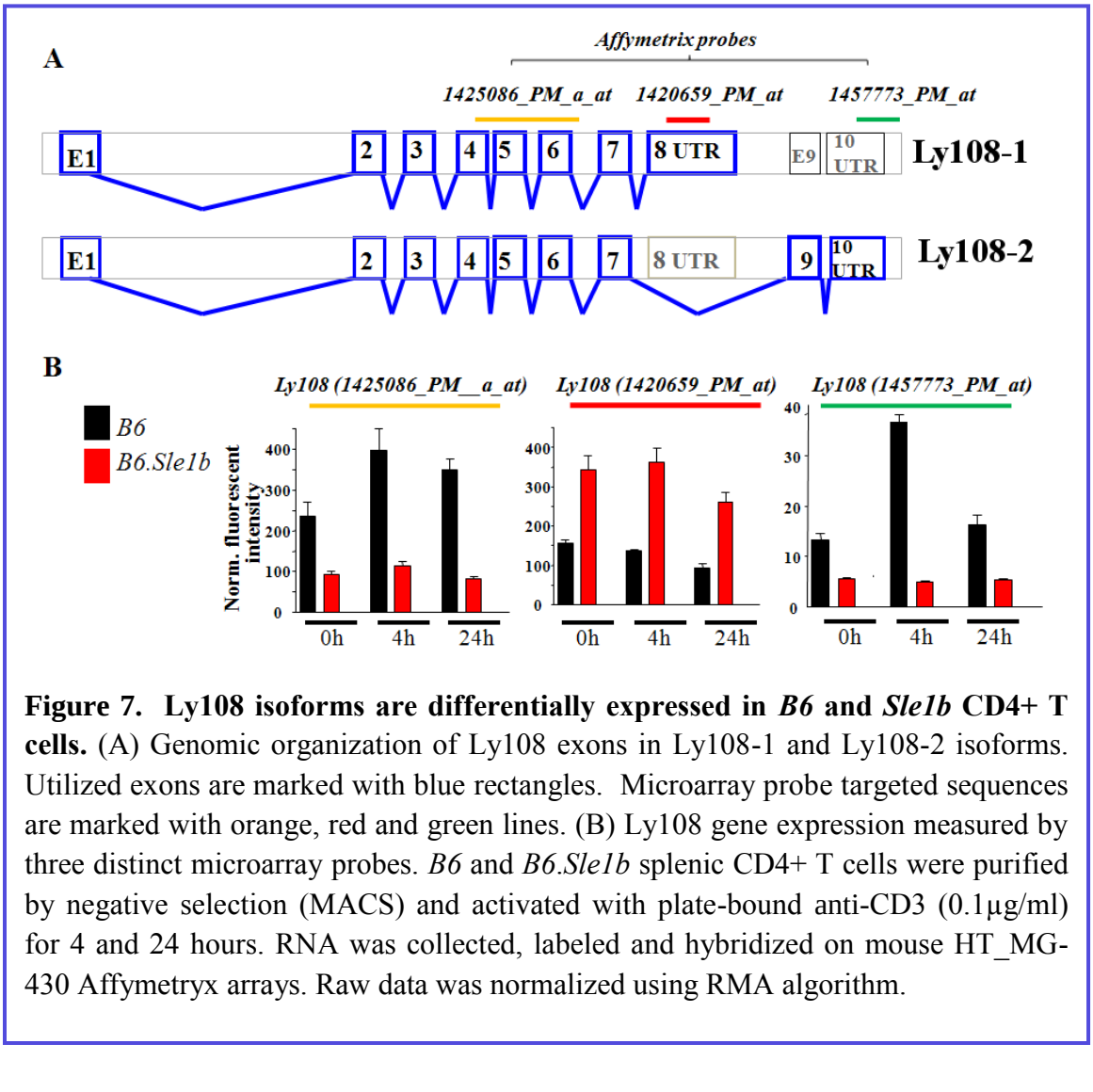

Figure 7. Ly108 isoforms are differentially expressed in *B6* and *Sle1b* CD4+ T cells. (A) Genomic organization of Ly108 exons in Ly108-1 and Ly108-2 isoforms. Utilized exons are marked with blue rectangles. Microarray probe targeted sequences are marked with orange, red and green lines. (B) Ly108 gene expression measured by three distinct microarray probes. *B6* and *B6.Sle1b* splenic CD4+ T cells were purified by negative selection (MACS) and activated with plate-bound anti-CD3 (0.1µg/ml) for 4 and 24 hours. RNA was collected, labeled and hybridized on mouse HT_MG-430 Affymetryx arrays. Raw data was normalized using RMA algorithm.

As shown in **Figure 7**, probe 1425086_PM_a_at, which recognizes a sequence common to Ly108-1 and Ly108-2, indicates higher overall Ly108 expression in *B6* than in *B6.Sle1b* CD4+ T cells. This result corresponds to our Ly108 surface staining data with labeled monoclonal antibodies (data not shown). Probe 1420659_PM_at recognizes the 5' UTR region of Ly108-1 and indicates a higher expression level of this transcript in *B6.Sle1b*, while probe 1457773_PM_at recognizes the 5' UTR of Ly108-2 and shows that *B6* expresses this transcript at a higher level.

In conclusion, our data verifies the observation of others (Wandstrat, Nguyen, Limaye, Chan, Subramanian, Tian, Yim, Pertsemlidis, Garner, Jr., Morel, & Wakeland 2004) that expression of Ly108-1 and Ly108-2 (UTR sequences) in *B6* and *B6.Sle1b* CD4+ T cells are differentially regulated. However, these results do not exclude the contribution of other possible isoforms with the same UTR sequences.

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

Since peripheral T cells from *B6.Sle1b* mice were able to induce aggravated autoimmunity in a chronic graft-versus host disease model, we next tested whether transgenesis of the putative pathogenic Ly108-1 isoform into the *B6* T cell compartment would also induce disease.

Cloning cDNA into the hCD2 transgene vector has been shown to direct transgene expression to the T cell compartment (Zhumabekov, Corbella, Tolaini, & Kioussis 1995). We generated two independent CD2-Ly108-1 transgenic lines on the *B6* genetic background. As shown in **Figure 8**, these lines expressed the transgene in peripheral T cells but not in B cells. Cell surface Ly108 staining indicated that line 3144 had a 20% overexpression, while line 3162 had an overexpression of 200%. CD2-Ly108-1 transgenics developed humoral autoimmunity upon aging indicated by the presence of anti-chromatin autoantibodies in aged mice. This preliminary data confirms that Ly108 is important player in lupus development. Additionally, it suggests that Ly108-1 is indeed, the disease inducing isoform in *B6.Sle1b*. However, artificially maintained expression levels by an

unnatural promoter raises concerns about the relevance of the in vivo experiments with these mice.

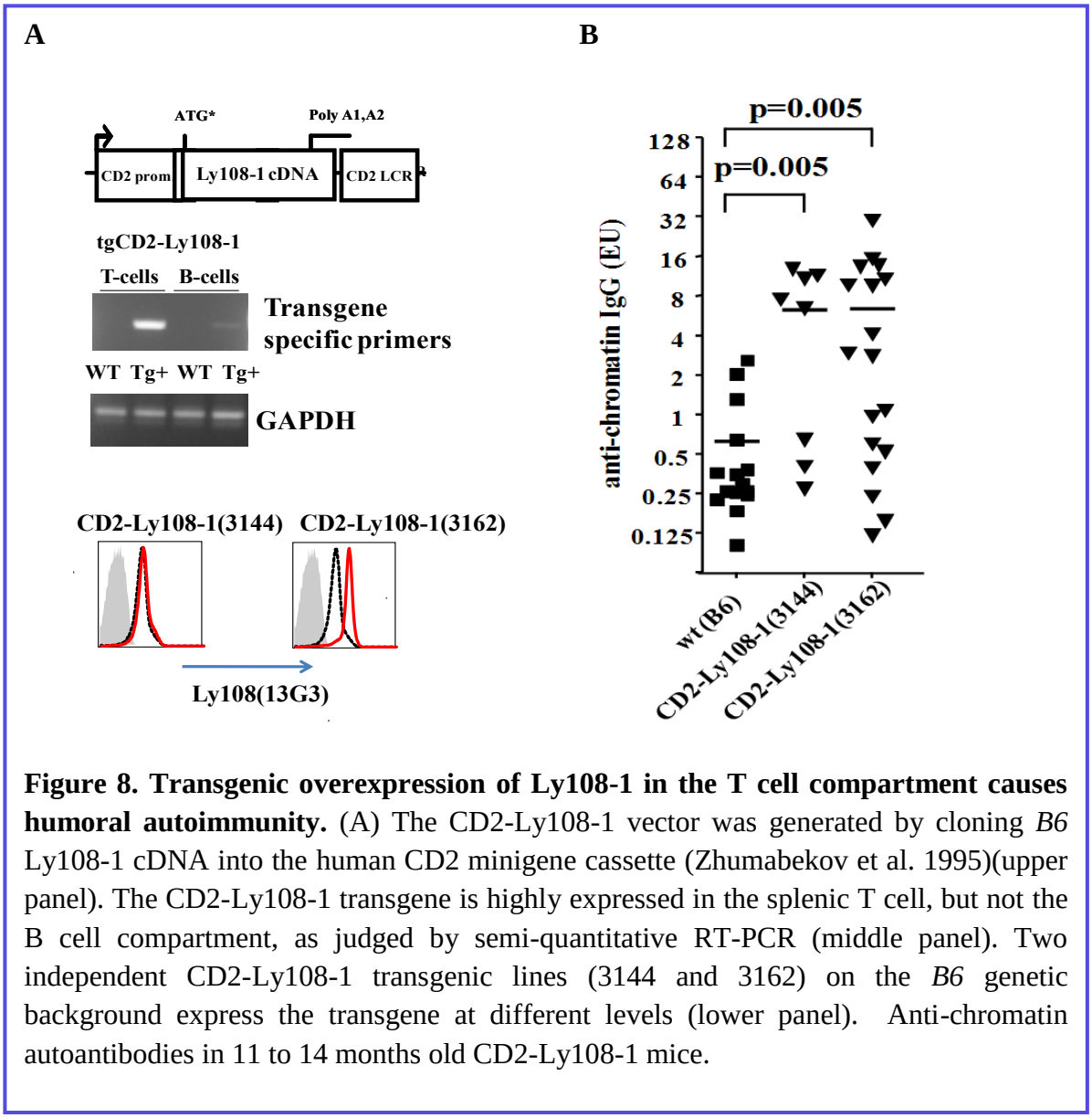

Figure 8. Transgenic overexpression of Ly108-1 in the T cell compartment causes humoral autoimmunity. (A) The CD2-Ly108-1 vector was generated by cloning *B6* Ly108-1 cDNA into the human CD2 minigene cassette (Zhumabekov et al. 1995)(upper panel). The CD2-Ly108-1 transgene is highly expressed in the splenic T cell, but not the B cell compartment, as judged by semi-quantitative RT-PCR (middle panel). Two independent CD2-Ly108-1 transgenic lines (3144 and 3162) on the *B6* genetic background express the transgene at different levels (lower panel). Anti-chromatin autoantibodies in 11 to 14 months old CD2-Ly108-1 mice.

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

In order to examine differences in the expression of protein isoforms between haplotype 1 (e.g. *B6*) and haplotype 2 (e.g. *129*) mouse strains, thymocyte lysates were immuno-precipitated with anti-Ly108 (13G3) monoclonal antibody. Ly108-1 and Ly108-2 isoforms were identified by Western blotting using two polyclonal antibodies. Antibody R4 was directed against a unique amino acid sequence in the C-terminus of the Ly108-2 cytoplasmic tail, while antibody R1 was specific for the segment of the cytoplasmic tail that is shared by the two isoforms (**Figure 9A**). As expected, there were clear bands corresponding to Ly108-1 (34kDa) and Ly108-2 (36kDa) after probing with the R1 antibody, in thymocyte lysates from *B6* and *129* mice (**Figure 9B left panel**). Surprisingly, R4 detected two bands in *B6* thymocytes, but only one band in *129* cell lysates (**Figure 9 right panel**). The upper band corresponded with Ly108-2 (36kDa), but the lower band indicated the presence of a yet unidentified Ly108 isoform. As this new isoform was only expressed by the haplotype I *Slam* locus, we decided to name it Ly108-H1 (Ly108 haplotype 1).

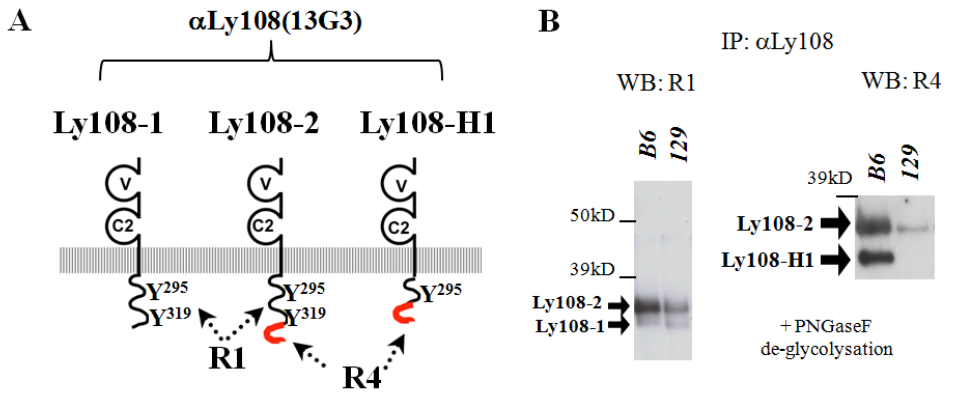

Figure 9. Ly108-H1 is a novel protein isoform. (A) Schematic illustration of the three Ly108 protein isoforms. Antibody R1 binds a segment shared by the isoforms Ly108-1 and Ly108-2. Antibody R4 is directed against the C-terminal amino acid sequence of isoforms Ly108-2 and Ly108-H1. (B) Polyclonal antibody R4 detects two forms of the Ly108 protein in immunoprecipitates (IP:αLy108) of thymocyte lysates from *wt B6* but not from *wt 129*. (WB performed by S. Rietdijk)

The Western blot results indicated that Ly108-H1 shared a common C-terminal sequence with Ly108-2, but lacked an ITSM containing the Y^{319} tyrosine. Using this information we were able to clone Ly108-H1 cDNA from *B6* thymocytes. Stable transfection of BI-141 T cell hybridoma cells with the pCI-NEO-Ly108-H1 expression vector confirmed that the cloned cDNA encoded for a protein identical to the one found in *B6* thymocytes (data not shown). Sequence analysis of Ly108-H1 and Ly108-2 cDNA revealed, that the two splicing isoforms utilized the same exons, however, the splicing machinery skips exon 7 during the pre- Ly108-H1 pre-mRNA processing (**Figure 10A**), most probably due to the presence of pyrimidine rich tandem repeat sequences in intron 6 of the *B6* Ly108 gene (data not shown).

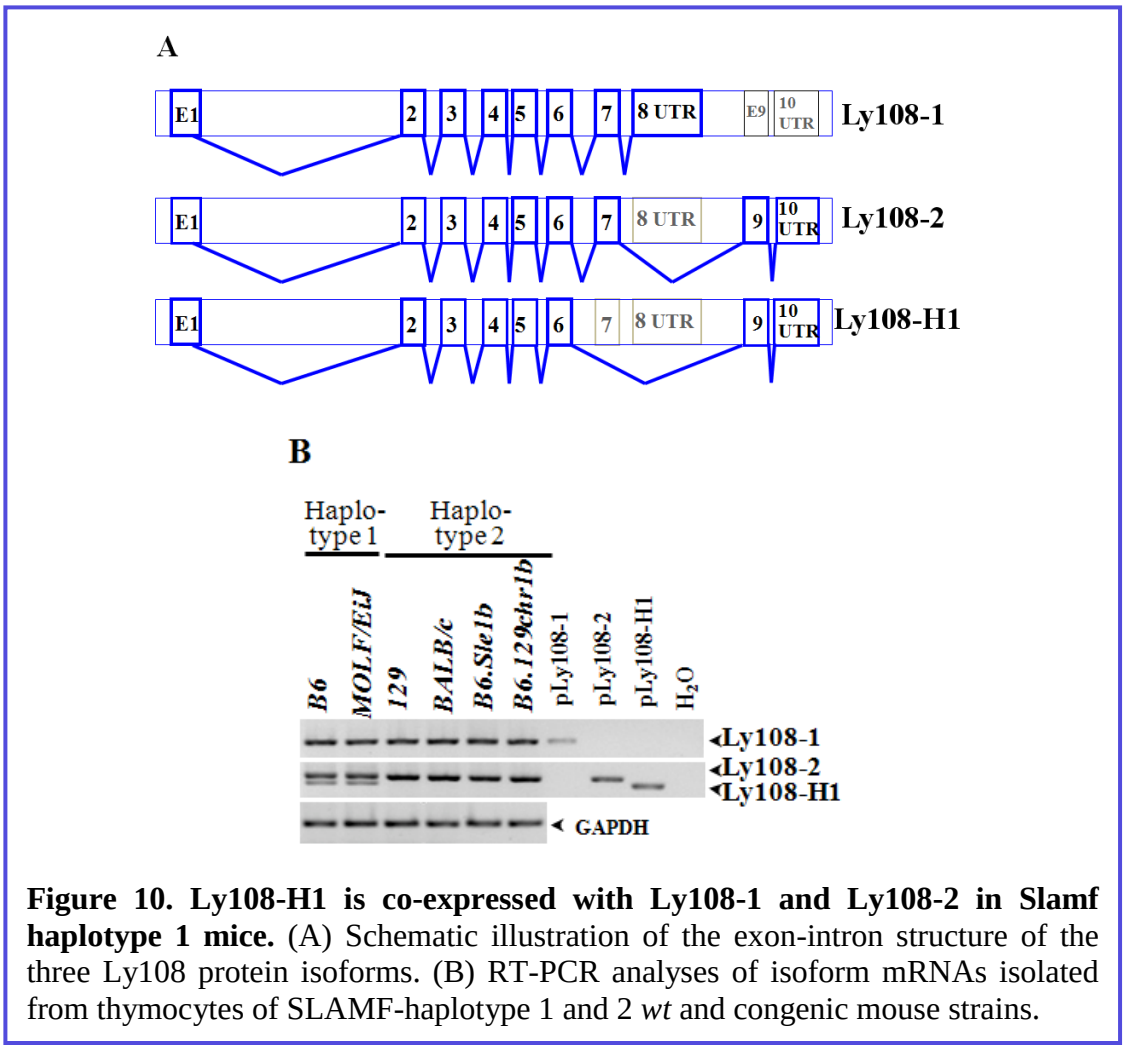

Figure 10. Ly108-H1 is co-expressed with Ly108-1 and Ly108-2 in Slamf haplotype 1 mice. (A) Schematic illustration of the exon-intron structure of the three Ly108 protein isoforms. (B) RT-PCR analyses of isoform mRNAs isolated from thymocytes of SLAMF-haplotype 1 and 2 *wt* and congenic mouse strains.

In order to prove that the expression of Ly108-H1 is haplotype dependent, we amplified cDNA from the lupus-prone congenic strains, *B6.Sle1b* and *B6.129chr1b* and from various *Slamf*-haplotype 1 and 2 mice by RT-PCR. To this end, oligonucleotide primers that either spanned exons 5-8 (detecting Ly108-1) or exons 5-9 (common to Ly108-2 and Ly108-H1) were used. Ly108-H1 was detectable in *Slamf*-haplotype 1, but not in any haplotype 2 thymocytes (**Figure 10B**). More importantly, Ly108-H1 is absent in the congenic mouse strains, *B6.Sle1b* and *B6.129chr1b*, suggesting that the presence of this novel protein isoform might affect the development of lupus. Since Ly108-H1 utilizes the same UTR as Ly108-2, earlier studies comparing Ly108 gene-expression between *B6* and *B6.Sle1b* mice overlooked the presence of Ly108-H1 (Kumar, Li, Yan, Bhaskarabhatla, Mobley, Nguyen, Mooney, Schatzle, Wakeland, & Mohan 2006). As a result of this oversight, the role of Ly108-1 and Ly108-2 in lupus development may have been overestimated.

Generating the *Sle1b*.BACLy108-H1 transgenic mice

In order to study the role of the *Ly108-H1* isoform in the pathogenesis of lupus, we produced a bacterial artificial chromosome (BAC) transgene vector that only expresses Ly108-H1 (**Figure 11A**) and introduced it into the *B6.Sle1b* genetic background. To generate the tgBACLy108-H1 vector, the BAC DNA was first modified using homologous recombination in bacteria by the Red/ET recombineering method in E.*coli* (Cotta-de-Almeida et al. 2003;Muyrers et al. 1999) to remove unnecessary coding sequences by several alterations of the *B6* BAC clone RP23-77A8, which contains full length *Slamf1*, *Slamf5* and *Ly108*.

Specifically, we deleted a DNA fragment containing *Ly108* exon 7 and exon 8 from the BAC DNA, thus preventing potential expression of Ly108-1 and Ly108-2 by the transgene. Next, the genomic sequences encoding *Slamf1* and *Slamf5* were removed by two subsequent recombineering steps. The vectors were then injected into *B6.Sle1b* oocytes.

To confirm the correct integration and function of the modified BAC clone, two methods were necessary: Fluorescent in Situ Hybridization (FISH) and Semi-quantitative PCR. As

shown for the Ly108-H1 transgenic mouse, the transgene integration was confirmed by FISH using fluorescently labeled RP23-77A8 BAC clone as a probe (**Figure 11B**). Semi-quantitative RT-PCR indicated that Ly108-H1 was expressed in thymocytes derived from hemizygous transgenic *Sle1b*.BACLy108-H1 mouse, whilst absent in *B6.Sle1b* thymocytes (**Figure 11C**). Cytofluorimetric analyses of *Ly108* expression on the surface of T-lineage cells isolated from *Sle1b*, *Sle1b*.BACLy108-H1 or *B6* mice (**Figure 11D**) supported the notion that Ly108 surface expression on T cells was slightly higher in hemizygous *Sle1b*.BACLy108 mice than in their *B6.Sle1b* transgene-negative littermates.

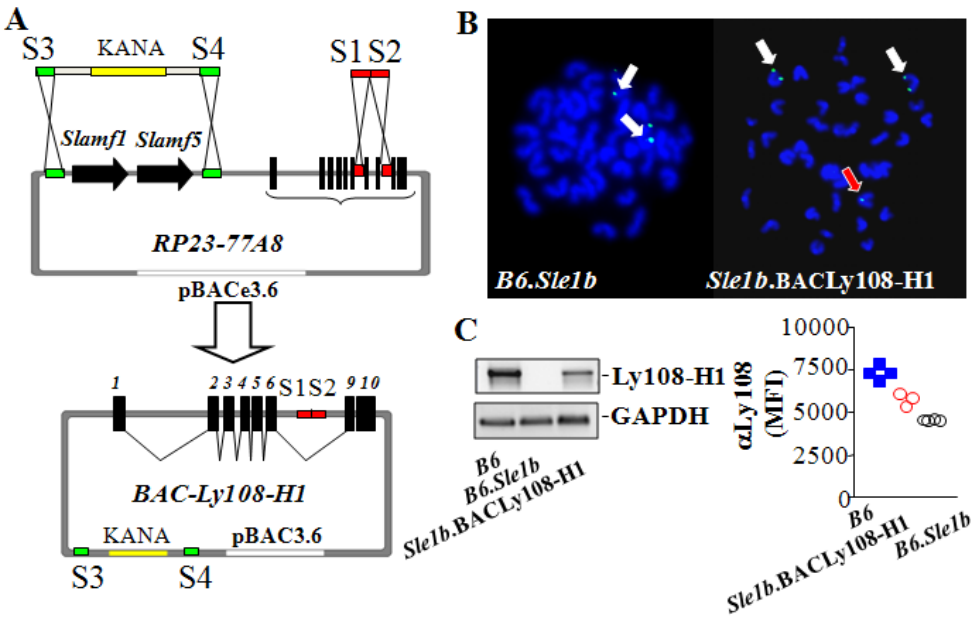

Figure 11. Generating the Sle1b.BACLy108-H1 transgenic mice. (A) The BACLy108-H1 transgene vector, encoding Ly108-H1, was generated by deleting exons 7 and 8 of Ly108 (which exons are not necessary for Ly108-H1 expression) and the Slamf1 and Slamf5 genes from the *B6* BAC clone RP23-77A8 by Red/ET recombineering . S1, S2, S3 and S4 are homology arms for Red/ET recombination. (B) As judged by Fluorescent In Situ Hybridization in metaphase chromosome preparations, the hemizygous transgenic *Sle1b*.BACLy108-H1 genome contains one additional Ly108 hybridization spot (red arrow), in comparison to *Sle1b.* (C) T cell lineage cells isolated from *Sle1b*.BACLy108-H1 mice express Ly108-H1. RT-PCR prepared with thymic mRNA using Ly108-H1 specific primers (left panel). Cell surface expression of Ly108 using DP thymocytes isolated from *B6*, *B6.Sle1b* or *Sle1b*. BACLy108-H1 mice using flow cytometry (right panel).

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

In order to assess whether Ly108-H1 affects spontaneous the lupus phenotype in *Sle1b*

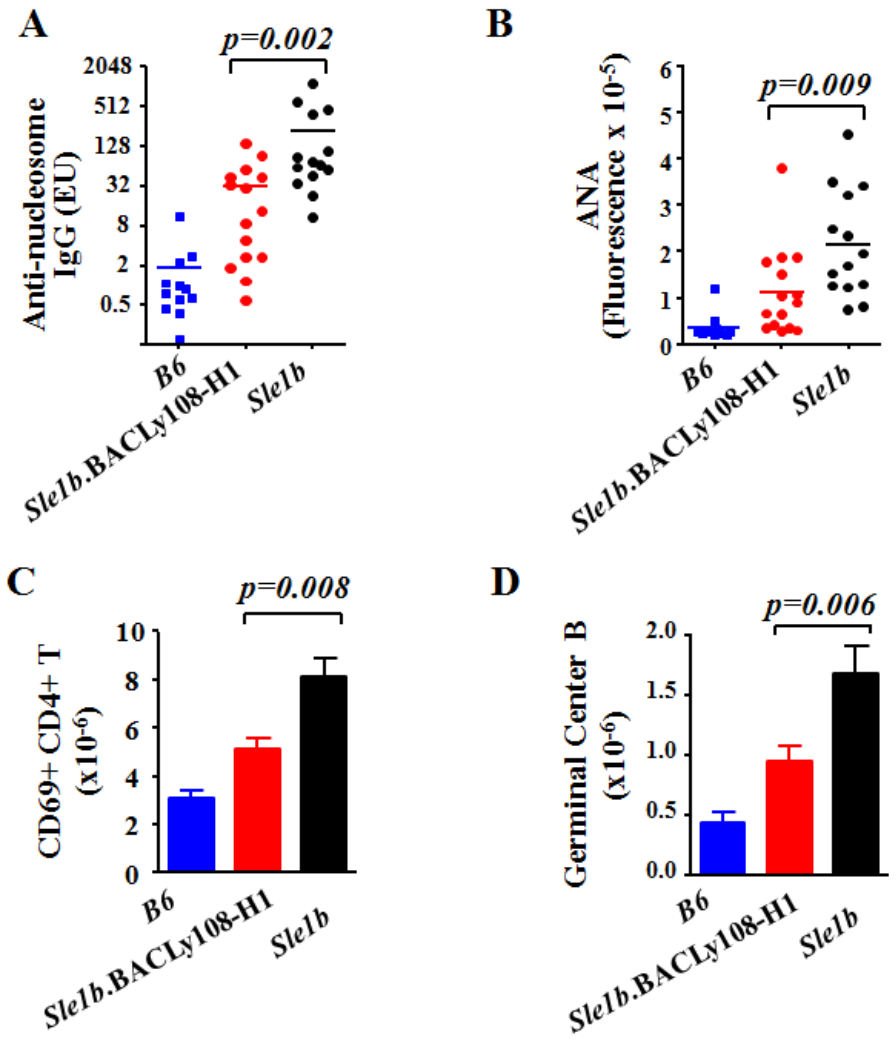

Figure 12. The BAC Ly108-H1 transgene ameliorates the spontaneous SLE phenotype in *Sle1b* mice. Autoimmunity was assessed in 6-8 months old female *B6.Sle1b*, *Sle1b*. BACLy108-H1 and *B6* mice. (A) Anti-nucleosome antibody titers in sera were measured with ELISA and with quantitative fluorescent microscopy (B) after incubating sera with monolayers of permeabilized Hep-2 cells and detecting bound IgG with anti-mouse IgG-Alexa488. (C) Flow cytometry analysis of splenocytes shows amelioration of spontaneous CD4+ T cell activation and germinal center formation (D) in *Sle1b*. BACLy108-H1 mice.

mice, we analyzed a 6-8 month old aged cohort of *Sle1b*.BACLy108-H1 hemizygous mice and *B6.Sle1b* transgene-negative littermate controls along with wild-type *B6* females. As expected, we detected high titers of anti-nucleosome antibodies (**Figure 12A, 12B**) as well as expansion of activated (CD69+) CD4+ T cells (**Figure 12C**) and germinal center B cells (GL7+FAS+B220+) (**Figure 12D**) in the aged *B6.Sle1b* cohort as compared to the wild-type *B6* mice. These autoimmune phenotypes were markedly reduced in the littermate *Sle1b*.BACLy108-H1 hemizygous transgenic animals.

T-cell dependent autoimmunity is ameliorated by Ly108-H1 in the bm12 Chronic Graft-vs-Host Disease transfer model of murine SLE.

To evaluate whether Ly108-H1 expression affects CD4+ T cell function, we purified naïve CD4+ T cells from *B6*, *B6.Sle1b* and *Sle1b*.BACLy108-H1 mice and injected them into bm12 hosts. After 4 weeks, we analyzed sera for autoantibodies and found that the BACLy108-H1 transgene ameliorated dsDNA and ssDNA responses in *B6.Sle1b* mice. This suggests that the reduced overall spontaneous lupus phenotype in *Sle1b*.BACLy108-H1 is at least partially a consequence of a repaired T cell function.

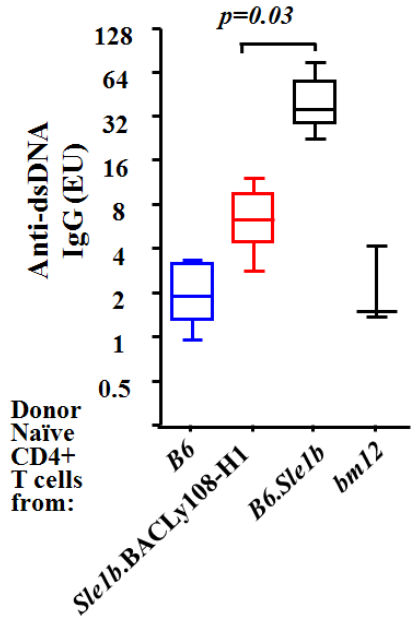

Figure 13. *B6.Sle1b* naïve CD4+ T cells induce severe cGVH disease, which is markedly reduced in *Sle1b.BACLy108-H1*. cGVH disease was induced by i.p. injection of $2.5x10^6$ purified naïve (CD62L+) CD4+ T cells. Anti-dsDNA autoantibodies were measured 4 wks after disease induction by ELISA.

CHAPTER 5: DISCUSSION

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

Genetic association studies of candidate genes are an important tool for exploring genetic susceptibility factors of multifactorial diseases. Here we examined genetic associations between polymorphisms of four immunoregulatory candidate genes (MCP-1, RANTES, TNFα and mannose binding lectin) with childhood asthma. All of the examined polymorphisms in these genes or their promoters had been described to significantly impact expression/function of the protein.

We 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).

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 control children. There was no association between these parameters, indicating that serum levels were independently regulated from this polymorphism.

Glucocorticoids have been shown to inhibit MCP-1 expression in vitro (Nickel et al. 1999). We therefore examined whether the observed decrease in MCP-1 serum levels in

asthmatic children was a consequence of treatment with steroids. There was no significant difference in MCP-1 serum levels between inhaled corticosteroid users and non-users (**Table 3**). We hypothesize that this reduction in MCP-1 levels in asthmatic patients is a result of a systemic anti-inflammatory feedback response, most likely mediated by endogenous corticosteroids. Although the data regarding serum levels of endogenous corticosteroids in asthma are controversial, some groups have reported higher cortisol levels in asthmatic patients as compared to nonasthmatic controls (Haen et al. 1991). This hypothesis is supported by our observation that MCP-1 serum levels are lower in high IgE responders, atopic patients and children with the highest asthma severity score; patients with the most severe forms of the disease would also have the strongest anti-inflammatory feedback response.

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 that 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.

In summary, we have found a negative correlation between the serum levels of MCP-1 and the presence of childhood asthma. Our results indicate MCP-1 as a possible diagnostic tool and therapeutic target in asthma.

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

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. Our work aimed to elucidate the role of *Slamf* genes in the pathogenesis of SLE using congenic, knockout and transgenic mouse strains. Although murine SLE does not fully recapitulate the human syndrome (Daniel Jeffrey Wallace, Bevra Hahn, & Edmund L.Dubois 2011), lupus-prone mice are important tools in the dissection of multiple factors that result in this autoimmune disease (Kanta & Mohan 2009). A growing body of evidence indicates that SLAM genes are key players in the fine-tuning of humoral immune responses (Calpe et al. 2008a;Cannons et al. 2010) and are closely linked to the processes which are responsible for breaching tolerance against nuclear antigens (Detre et al., 2010).

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.

By comparing spontaneous SLE development in *B6.129* congenic knockout strains, we found that the control *B6.129chr1b* mice, the *Slamf1*$^{-/-}$ and *Slamf2*$^{-/-}$ *[B6.129]* strains developed autoantibodies and glomerulonephritis at a comparable age. However, *Slamf2*$^{-/-}$ *[B6.129]* mice developed symptoms of autoimmunity at much earlier age. On the *B6* background, a null mutation of the *Slamf4* gene, which is the ligand for *Slamf2*, caused

mild autoimmunity, indicating that interaction *of Slamf2-Slamf4* plays a role in maintaining self tolerance.

In contrast to *Slamf1*$^{-/-}$ and *Slamf2*$^{-/-}$, the *Slamf6 (Ly108)-/- [B6.129]* 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 GC formation) *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.

CHAPTER 6: CONCLUSIONS

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

6.2 Elucidation of the interplay of the SLAM-family (SLAMF) of 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* embryonic stem 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.
- Knocking out the SLAM-family adaptor proteins does not induce autoimmunity.
- *B6.Sle1b* lupus prone congenic mice have an intrinsic peripheral CD4+ T cell phenotype.
- Ly108 isoforms are differentially expressed in *B6* and *B6.Sle1b* CD4+ T cells.
- 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-vs-Host Disease transfer model of murine SLE.

SUMMARY

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

My studies focused on two multifactorial immunopathologies: 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 internal (nuclear) epitopes. Despite seeming like two entirely disparate conditions, both diseases involve exaggerated humoral immune responses in the host. Association studies of candidate genes, linkage analysis and genome-wide association studies indicate that both asthma and SLE pathogenesis are primarily dependent on alterations in cytokines, cell-surface receptors and the connected intracellular signaling molecules.

In our asthma studies, we were examining genetic association between polymorphisms of several candidate genes (MCP-1, RANTES, TNFα and mannose binding lectin) with childhood asthma. We found that among these polymorphisms, only the MCP-1 -2518A/G biallelic variant had a significant genetic association with asthma and demonstrated that serum MCP-1 levels were significantly lower in asthmatic children. Atopy or elevated serum IgE was associated with a further reduction in MCP-1 levels compared to the non-atopic patients. However, there was no significant association between the MCP-1 -2518 genotype and MCP-1 serum concentration in asthmatic children.

My subsequent work involved elucidating the contribution of the SLAM-family (*SLAMF*) of co-stimulatory receptors in the pathogenesis of SLE using gene-targeted knockout and transgenic mouse models**.** Genome-wide linkage scans of SLE patients and studies of murine congenic mouse strains consistently demonstrated the presence of a lupus susceptibility locus on chromosome 1 (1q23) and on the syntenic mouse region. Interestingly, these regions include seven SLAMF (Slamf in mice) genes.

The systematic analysis of lupus prone congenic mouse strains suggests a role for two isoforms of the Slamf6 (Ly108) receptor in the pathogenesis of the disease. We demonstrated that Ly108 is involved in the pathogenesis of a lupus-like autoimmunity in mice by comparing the SLE phenotype in *Slamf1*, *Slamf2*, *Slamf3* and *Ly10*8 congenic knock-out strains. In addition, using transgenesis, we determined that Ly108-1, a known lupus candidate gene, is able to drive spontaneous lupus. More importantly, we identified a third protein isoform, Ly108-H1, which is absent in two lupus prone congenic animals. Introduction of a Ly108-H1 expressing transgene markedly diminishes T cell dependent autoimmunity in congenic *B6.Sle1b* mice. 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.

ÖSSZEFOGLALÁS (Summary in Hungarian)

A komplex vagy multifaktoriális betegségek, amelyek kialakulását számos genetikai és környezeti faktor is befolyásolja, vezető halálozási okként szerepelnek világszerte.

Ebben a tézisben két multifaktoriális immunopatológiás kórképet vizsgáltunk: az allergiás asztmát és az autoimmun szisztémás lupusz eritematózuszt (SLE). Míg az allergiás asztmát külső antigének, az SLE-t saját (sejtmagi) epitópok elleni antitestek jellemzik. Annak ellenére, hogy ez a két betegség lényegesen eltér egymástól, mindkét patomechanizmusra jellemző a szervezet túlzott humorális immureakciója. Kandidáns géneken végzett genetikai asszociációs vizsgálatok, genetikai kapcsoltság analízis és teljes genom-asszociációs vizsgálatok eredményei szerint, mind az asztma, mind a lupusz patogenezis fő meghatározói a citokinek, sejtfelszíni receptorok és a hozzájuk kapcsolódó jelátviteli hálózatok fehérjéi.

Asztmához kapcsolódó kutatásaink során számos kandidáns gén polimorfizmusait vizsgáltuk (MCP-1, RANTES, TNFa és MBL). A vizsgált génváltozatok között csak az MCP-1 -2518A/G polimorfizmus hajlamosított gyermekkori asztma kialakulására. Bemutattuk, hogy a szérumban mérhető MCP-1szint szignifikánsan alacsonyabb volt az

asztmás gyermekekben, mint a kontroll csoportban. Atópia vagy megnövekedett szérum IgE az asztmás betegekben alacsonyabb MCP-1 koncentrációval járt a nem-atópiásokhoz viszonyítva. Várakozásainkkal ellentétben, nem találtunk korrelációt az MCP-1 -2518 génpolimorfizmus és az MCP-1 szérum koncentráció között asztmás gyermekekben.

További munkám a SLAM kostimulátor család szerepének felderítését célozta a szisztémás lupusz eritematózusz patomechanizmusában, gén-kiütött és transzgenikus egérmodellek segítségével. Teljes genom asszociációs vizsgálatok SLE betegeken, valamint kongenikus egértörzseken végzett kutatások egyöntetűen bizonyítják egy lupusz szuszceptibilitás lókusz jelenlétét az 1-es kromoszóma telomerikus 1q23 régiójában, ahol az általunk vizsgált SLAM gének találhatóak.

Lupuszra genetikailag fogékony egértörzsek vizsgálata az *Ly108* (*Slamf6*) gén két izoformájának szerepére utal. *Slamf1*, *Slamf2*, *Slamf3* és Ly108 gén-kiütött kongenikus egerekkel igazoltuk, hogy az Ly108 gén kifejeződése valóban szükséges a lupusz fenotípus kialakulásához modellünkben, továbbá azt is, hogy az Ly108-1 lupusz kandidáns izoforma transzgenikus kifejeződése spontán SLE kialakulásához vezet. Munkánk során kiderült, hogy az Ly108 génnek létezik egy harmadik eddig nem ismert, általunk Ly108-H1-nek elnevezett izoformája, amely megtalálható a vadtípusú *B6* egerekben, de nem fejeződik ki a lupuszra genetikailag fogékony kongenikus *B6.Sle1b* és *B6.129chr1b* egértörzsekben. Az Ly108-H1 izoforma transzgenikus kifejezése *B6.Sle1b* egérben számottevően csökkentette a T sejt függő autoimmunitás kialakulását.

Összefoglalva, kutatásaink elsőként bizonyítják , hogy az Ly108-1 és Ly108-H1 izoformák egymással ellentétes módon szabályozzák a T sejt függő lupusz fenotípus kialakulását egérmodellekben.

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