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A Rabbit Model of Systemic Lupus Erythematosus, Useful for Studies of Neuropsychiatric SLE

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1. Introduction

The aim of this review is to present in one place a summary of the development of a rabbit model of SLE conducted using pedigreed rabbits bred and selected at the National Institute of Allergy and Infectious Diseases (NIAID), NIH. We provide an overview of the knowledge gained by using rabbits *(Oryctolagus cuniculus)* as models for SLE, and eliciting autoantibodies typical of those produced by patients with SLE. We present here summaries of work by ourselves and coauthors that can contribute to improved understanding of neuropsychiatric SLE (NPSLE) (Sanches-Guerro et al., 2008). Our gene expression studies (Rai, et al., 2010) and extensive evaluations of autoantibody responses coupled with observed clinical symptoms of animals immunized against peptides from the Smith antigen (Sm) or the NMDA glutamate receptor have shown promise to improve understanding NPSLE.

An overview of immune system development, genetic diversity of immunoglobulin genes and somatic diversification during B-cell development in rabbits can be found in a review by Mage et al., (2006) and reference therein. Investigations of autoantibodies found in patients with NPSLE and the problems of diagnosis and specific treatments are addressed in other chapters in this volume.

2. Our model

2.1 Earlier studies of SLE models by other laboratories using rabbit

We set out to develop a model of SLE in pedigreed rabbits because an earlier report showed that immunization of non-pedigreed rabbits with peptides such as PPPGMRPP, derived from the Sm B/B′ subunit of the spliceosomal Smith autoantigen led to epitope spreading, SLE-like autoantibody production and clinically observed seizures. This peptide sequence is one of the major regions of reactivity in SLE patients and may mimic the peptide PPPGRRP from the EBNA-1 component of Epstein-Barr virus (EBV) (James et al., 1995). Another study attempted to reproduce this report but only found some evidence for epitope spreading with no suggestion of induced autoimmunity (Mason et al., 1999). We hypothesized that the different results may have been obtained because small numbers of rabbits were studied,

and there may have been different genetic susceptibilities among outbred rabbits studied by the two groups. Since our work already depended on breeding and maintenance of immunoglobulin allotype-defined pedigreed rabbits, we were in a position to pursue this idea further. In addition to using the MAP-8-PPPGMRPP immunogen used in these previous studies (termed SM-MAP-8 in the following sections), we chose a new immunogen termed GR-MAP-8 based on a report by DeGiorgio et al (2001) that some anti-DNA antibodies cross-react with the NMDA glutamate receptor.

2.2 The model in pedigreed rabbits

We established a rabbit model of Systemic Lupus Erythematosus (SLE) in which peptide immunization led to lupus-like autoantibody production including anti-Sm, -RNP, -SS-A, -SS-B and -dsDNA. Some neurological symptoms in form of seizures and nystagmus were observed (Rai et al., 2006). The animals were selectively bred within the colony of pedigreed, immunoglobulin allotype-defined but non-inbred rabbits at the NIAID. We continued breeding responders from the first three groups studied (Rai et al., 2006, 2010, Puliyath et al., 2008, Yang et al., 2009a,b). Details about genetics, gene expression and cellular studies are in the sections below. The genetic heterogeneity of the pedigreed animals studied may correspond to that found among patients of a given ethnicity.

2.2.1 Methods

Rabbits: All rabbit experimentation and immunization protocols were reviewed and approved by the Animal Care and Use committees of the NIAID, NIH and of Spring Valley Laboratories where the animals were bred, housed and monitored. The animals' designations, sexes, and allotypes at the V_H a immunoglobulin heavy chain and Cκ b light chain loci are summarized in Tables 1A and B. Rabbits of groups 1, 3, 4, 5 and 6 received peptides (SM or GR) synthesized on MAP-8 branched lysine backbones and rabbits of group 2 received the peptides on MAP-4. BB indicates control rabbits that received backbone alone. SM animals received MAP-peptide derived from the sequence of the Smith antigen spliceosomal B/B' complex. GR animals received MAP-peptide derived from the NMDA glutamate receptor sequence.

Antigens: The peptide immunogens "GR" and "SM" used for the initial rabbit immunizations (Rai et al., 2006), were synthesized on branched lysine MAP-8 and MAP-4 backbones (BB) (AnaSpec) The SM peptide sequence PPPGMRPP corresponds to major antigenic regions at 191-198, 216-223 and 231-238 of the nuclear protein Sm B/B' (James et al, 1995). The GR peptide sequence DEWDYGLP corresponds to a known rabbit sequence of an extracellular epitope of the NR2b subunit of neuronal postsynaptic NMDA receptor. The MAP-BB without peptide was used as a control antigen. For subsequent studies, (Puliyath et al., 2008, Yang et al., 2009b, Rai et al. 2010), MAP-8 was the BB of choice because it appeared to elicit more diverse autoantibody responses.

Immunization: Rabbits each received subcutaneous (s.c.) injections of one of the MAP-peptides or control BB (0.5 mg/0.5 ml, in borate buffered saline, pH 8.0) emulsified with 0.5 ml of complete Freund's adjuvant (CFA). Boosts were given s.c. at 3-week intervals with the same antigen concentration emulsified with incomplete Freund's adjuvant (IFA). Controls that received only CFA followed by IFA were included in one study (Yang et al., 2009b). Sera collected immediately before immunization (pre-immune) and 1 week after each boost (post-boost) were stored at -20°C in multiple aliquots for assays.

Rabbit ID	Rabbit no.	Allotype	Sex
Group 1			
SM1	XX129-3	a1/1, b4/5	M
SM2	2XX127-4	a1/1, b5/5	F
SM3	2XX288-2	a1/1, b9/9	M
SM4	1XX288-4	a1/1, b5/9	F
SM5	2XX127-2	a1/1, b9/9	M
SM6	2XX92-06	a1/1, b9/9	F
GR7	XX129-5	a1/1, b4/5	M
GR8	2XX127-5	a1/1, b9/9	F
GR9	1XX288-3	a1/1, b5/5	M
GR10	2XX288-6	a1/1, b5/9	F
BB11	2XX127-1	a1/1, b5/5	M
BB12	1XX78-8	a1/1, b9/9	F
Group 2			
SM13	LL191-1	a1/1, b4/5	M
SM14	LL191-2	a1/1, b5/5	F
SM15	2LL179-1	a1/1, b9/9	M
SM16	1LL163-3	a1/1, b9/9	F
SM17	LL164-4	a1/1, b9/9	F
GR18	1LL178-2	a1/1, b9/9	M
GR19	1LL178-3	a1/1, b4/4	F
GR20	1LL178-4	a1/1, b9/9	F
GR21	1LL178-5	a1/1, b4/9	M
GR22	1LL178-6	a1/1, b4/9	F
GR23	1LL178-8	a1/1, b4/4	F
BB24	LL164-1	a1/1, b9/9	M
BB25	LL164-3	a1/1, b9/9	F
BB26	2LL179-3	a1/1, b5/9	M
BB27	1LL163-4	a1/1, b5/9	F
Group 3			
GR28	LL108-1	a1/1, b5/9	M
GR29	LL108-3	a1/1, b5/5	F
GR30	LL108-4	a1/1, b9/9	F
BB31	2LL179-2	a1/1, b9/9	M
Group 4			
SM32	1QQ299-2	a1/1, b5/5	M
SM33	1QQ299-3	a1/1, b4/5	F
SM34	6QQ299-1	a1/1, b4/9	M
SM35	6QQ299-2	a1/1, b4/5	F
GR36	3QQ299-1	a1/1, b4/5	M
GR37	3QQ299-2	a1/1, b5/9	M
GR38	3QQ299-4	a1/1, b5/9	M
GR39	4QQ299-1	a1/1, b5/9	M
GR40	5QQ299-2	a1/1, b5/9	F
GR41	5QQ299-3	a1/1, b5/5	F
BB42	1QQ299-1	a1/1, b5/5	M
BB43	5QQ299-4	a1/1, b5/5	F
BB44	6QQ299-3	a1/1, b4/9	F
PB45	1QQ173-1	a1/1, b5/9	M
PB46	1QQ173-2	a1/1, b5/5	M
PB47	1QQ173-3	a1/1, b5/5	M

Table 1. A. Designations of sexes and allotypes of Groups 1-4.
PB45, 46, and 47 received injections with phosphate buffered saline only.

Group 5			
GR 48	UA345-1	a2/2, b9k/9k	M
GR49	1UA344-1	a1/2, b9k/9k	M
GR50	1UA344-5	a1/2, b9k/9k	F
GR51	1YY119-6	a1/1, b9/9	F
GR52	1YY119-8	a1/1, b9/9	F
GR53	2YY119-6	a1/1, b9/9	M
GR54	2YY299-5	a1/1, b4/9	F
GR55	2YY299-3	a1/1, b4/9	F
GR56	UA345-2	a1/2, b4/9k	M
GR57	1UA344-2	a1/2, b5/9k	M
GR58	1UA344-6	a2/2, b5/9k	F
GR59	1YY119-7	a1/1, b9/9	F
GR60	1YY327-2	a1/1, b5/9	M
GR61	2YY327-9	a1/1, b4/5	F
GR62	2YY299-4	a1/1, b4/9	F
GR63	2YY119-8	a1/1, b9/9	F
BB64	UA345-4	a2/2, b9k/9k	F
BB65	2UA344-1	a1/2, b9k/9k	F
BB66	1UA344-3	a2/2, b5/9k	M
BB67	2YY327-8	a1/1, b4/5	M
BB68	UA345-6	a1/2, b4/9k	F
BB69	1YY327-4	a1/1, b5/9	F
BB70	1YY119-5	a1/1, b9/9	M
BB71	2YY119-7	a1/1, b9/9	M
Group 6			
GR72	UA345-5	a2R3/2R3, 4/9k	F
GR73	UA269-3	a1/1, b4/9k	F
BB74	6YY328-4	a1/1, b5/9	M
BB75	2YY125-6	a1/2, b9k/9k	M
CF1	6YY328-3	a1/1, b5/9	M
CF2	1UA161-1	a1/1, b9/9	M
GR76	2YY119-9	a1/1, b9/9	F
GR77	UA269-1	a1/1, b4/5	M
BB78	YY118-6	a1/1, b9/9	M
BB79	1UA161-2	a1/1, b9/9	M
CF3	1YY125-4	a2/2, b4/9k	M
CF4	2YY125-4	a2/2, b5/9k	M
GR80	XA345-1	a1/2, b9/9k	F
GR81	2UA14-2	a1/1, b5/9	F
BB82	XA346-2	a1/1, b9/9	M
BB83	2UA14-3	a1/1, b5/9k	F
CF5	XA345-2	a1/2, b9/9k	F
GR84	XA234-2	a1/2, b5/9	F
GR85	XA346=1	a1/1, b9/9	M
BB86	XA234-2	a1/2, b5/9	M
B87	3XA203-2	a1/ali	M
CF6	2XA344-2	a1/1, b9/9	F
CF7	1XA344-1	a1/1, b9/9	M

Table 1. B. Designations of sexes and allotypes of Groups 5 and 6.

Clinical Assessments: Rabbits were housed in a separate room equipped with video surveillance so that abnormal behavior such as seizure activity and other neurological dysfunctions could be detected. They were observed daily and also received periodic complete health evaluations. Hematology using a Bayer Advida, model 120 hematology analyzer and blood chemistry assessments of each rabbit were carried out in a Veterinary diagnostic laboratory (Antech Diagnostics, Lake Success NY).

ELISA for anti-peptide antibodies, anti-dsDNA and autoantibodies to nuclear antigens: Serum antibody responses to the MAP-peptides and control immunogens were measured by solid phase ELISA as previously described (Rai et al 2006). "Polystyrene 96-well plates (Corning Inc, Corning, NY, Cat # 3590) were coated with 50 µl/well of either SM-, GR- or BB- (MAP-8 or MAP–4) at 10 µg/ml in bicarbonate buffer, (pH 9.6) and incubated overnight at 4^0C. Plates were washed three times with PBS (pH 7.2) containing 0.1% Tween 20 and blocked with 100 µl blocking solution for 1 hr at 37^0C (Quality Biological Inc, Gaithersburg, MD). Wells were then incubated 1 hr, at 37^0C with 50 µl/well of sera titrated by four-fold dilutions in blocking solution, washed 5 times, incubated for 1 hr at 37^0C with 50 µl of a 1:2000 dilution (0.4 ng/µl) of affinity-purified horseradish peroxidase conjugated (HRP) goat anti-rabbit IgG (H+L) secondary antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA), developed with 3, 3', 5, 5'- tetramethylbenzidine (TMB) (Inova Diagnostics, Inc., San Diego, CA) and the resulting OD read at 450 nm."

Commercially available human diagnostic kits (INOVA Diagnostics) were adapted and used to assay serum autoantibodies to total extractable nuclear Ags (ENA) and to component Ags Sm, Rnp, SS-A, SS-B. Assays for autoantibodies to calf thymus dsDNA were adapted similarly using two different commercially available kits (Vidia, Vestec (Kit A); Zeus scientific, NJ (Kit B). Briefly, 100 µl rabbit sera diluted 1:100 in the proprietary sample diluents were added to antigen-coated wells and incubated for 60 min. at 37°C (Kit A) or 30 min. at RT (Kit B). Wells were then washed, incubated for 60 min. at 37°C (Kit A) or 30 min at RT (Kit B) with secondary antibody HRP-goat anti-rabbit IgG Fc (Jackson Immunoresearch Laboratories, Inc.) and developed with TMB for reading OD at 450 nm. For groups 5 and 6, anti-dsDNA, -ANA, -RNP and -Sm were assayed with the Quantalite kits (Inova Diagnostics) substituting affinity purified HRP-goat anti-rabbit IgG Fc for the anti-human secondary reagent (Puliyath et al., 2008, Yang et al., 2009b).

Detection of anti-nuclear antibodies (ANA) by indirect immunofluorescence: Commercially available slides coated with fixed Hep-2 cells (Antibodies Inc., Davis, CA) were incubated with rabbit antisera diluted 1:20 in 5% goat serum (Jackson Immunoresearch Laboratories Inc.) for 30 min. at RT. ANA binding was detected by fluorescence microscopy following 30 min incubation at RT with 12.5 ng/µl of FITC-goat anti-rabbit IgG Fc (Southern Biotech Inc., Birmingham, AL). Fluorescent binding patterns were compared with reference pictures provided by Antibodies, Inc.

Flow cytometry: Anti-human antibodies that cross reacted with rabbit B-cell activation factor (BAFF) (biotin conjugated goat anti-human BAFF polyclonal antibody), transmembrane activator and CAML interactor (TACI) (biotin conjugated goat anti-human TACI polyclonal antibody)(Antigenix, America, Inc.), BAFF receptor (BR3) (purified goat anti-human BR3 antibody) (R&D systems) were used for staining. Briefly, purified PBMCs were incubated on ice for 40 min with primary antibody before washing twice with cold PBS containing 1% FCS, then subsequent incubation with various secondary reagents or secondary antibodies. For BR3 detection, a biotinylated donkey anti-goat IgG was used as

secondary antibody. Biotinylated antibodies were visualized by PE-conjugated streptavidin (Jackson ImmunoResearch laboratories, Inc.). After washing, cells were analyzed using a FACS-Calibur flow cytometer (BD Pharmingen) and FlowJo analytical software (Tree Star). Cells were gated on the side scatter x forward scatter (SSCxFSC) profiles to include both small and large lymphocytes, as well as monocytes but exclude red blood cells and granulocytes; dead cells were excluded by propidium iodide staining. Rabbit IgM^+ B cells were detected by FITC-conjugated goat anti-rabbit IgM (Southern Biotechnology Associates).

Gene Expression studies: *RNA extraction and synthesis of cDNA and cRNA*

Peripheral white blood cells (PWBCs) were lysed with TRIzol (Invitrogen, CA) and total RNA was extracted using RNAeasy Mini columns following the manufacturer's instructions (Qiagen, CA). The cRNA probes were prepared from mRNA using the Affymetrix gene chip eukaryotic small sample target labeling protocol assay version II (Affymetrix, Santa Clara, CA) using 2 cycles of cDNA synthesis and *in vitro* transcription (IVT) reactions. The cRNA thus obtained was used in the final IVT cycle for obtaining biotinylated cRNA using CTP and UTP (EnzoBioarray, Enzo Life Sciences, Farmingdale, NY) (Rai et al 2010).

Microarray analysis Affymetrix U95A human microarray chips were used and hybridization of the labeled cRNA was carried out according to the manufacturer's recommended protocol. Non-normalized MAS5 signals were used to compare raw probeset intensity values between human and rabbit samples. Final rabbit study analyses were conducted with expression values summarized using dChip, log2 transformed and Loess normalized using an R package (http://www.elwood9.net/spike). Analyses of the gene sets were done using Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/knowledgebase/) and Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Mountain View, CA; www.ingenuity.com).

Quantitative real time PCR: Quantitative real time PCR analysis of mRNAs was performed on a 7900HT Sequence Detection System (Applied Biosystems). The cDNA synthesized from isolated PWBCs was directly used as template for real-time PCR by using TaqMan 2x PCR Master Mix Reagents Kit (Applied Biosystems). Each sample from three independent experiments was run in duplicate. The unit number showing relative mRNA levels in each sample was determined as a value of mRNA normalized against Peptidylprolyl isomerase A (PPIA). RT-PCR data were analyzed by using the $2^{-\Delta\Delta C_T}$ method. Based on its uniform expression among rabbit groups in the microarray analysis, rabbit peptidylprolyl isomerase A (*PPIA*; cyclophilin A) was selected as the housekeeping gene control and used for the calculation of ΔC_T. Where rabbit sequences were unavailable, primers were designed after searching for rabbit sequences with corresponding human gene sequences in the database containing the trace archives of the whole genome shotgun sequence of the rabbit (*Oryctolagus cuniculus*) generated by the Broad Institute of MIT and Harvard University (NCBI trace archive: cross- species Megablast at http://www.ncbi.nlm.nih.gov/blast/tracemb.shtml) and in assemblies of rabbit scaffolds at Ensembl and UCSC (see NCBI Rabbit Genome Resources site) at: http://www.ncbi.nlm.nih.gov/projects/genome/guide/rabbit/

2.2.2 Genetics and autoantibody responses

Figure 1 shows the pedigree and an overview of antibody responses of the rabbits immunized and selectively bred during the project to develop a rabbit model of SLE. There were 31 1- to 2-year-old rabbits in the initial studies (Rai et al., 2006). Rabbits of groups 2 or

3 were descendants of rabbits of groups 1 or 2 and/or their siblings. Rabbits that did not respond with autoantibody production after immunization during this initial study by Rai et al., (2006) are not shown. The fourth group was described in Rai et al., (2010) and their mRNA included along with mRNA from the first three groups for gene expression profiling of a total of 46 pedigreed control- or immunized-rabbits as detailed below (section 2.2.3). Controls that received only phosphate buffered saline are designated PB. Because the GR peptide generally elicited better autoantibody responses than the SM peptide, the two subsequent groups [5 (Puliyath et al., 2008) and 6 (Yang et al., 2009b)] were immunized with GR-MAP-8 or control BB-MAP-8. The final 6th group also included controls that received complete followed by incomplete Freund's adjuvant but no MAP-BB or MAP-peptide, to investigate whether adjuvants alone led to any autoantibody production (designated CF).

An overview of autoantibody responses, and the relationships of males (squares) and females (circles) in six immunization groups is shown in Figure 1. The four quadrants indicate post-immunization elevations of levels of anti-dsDNA (upper left), anti-Sm and/or anti-RNP (lower left), ANA by IFA (upper right) and ANA by ELISA (lower right). For the 5th (Puliyath et al. 2008) and 6th groups (Yang et al., 2009b), darker shades indicate high autoantibody responses. The large circles and squares represent the 6th group developed from selective breeding using responders from earlier groups. Figure originally published by Yang et al., (2009b) Investigations of a rabbit (*Oryctolagus cuniculus*) model of systemic lupus erythematosus (SLE), BAFF and its receptors. *PLoS ONE* Vol. 4, 2009.

The selective breeding led to subsequent progeny (groups 5 and 6) exhibiting more consistent autoantibody production. In the pedigree, we can trace the ancestry of some responder rabbits back to the first high responders (SM1 and GR9) that also exhibited seizures. For example, GR54 and GR55 from litter 2YY299 had high-responder grandsires SM1 and SM15 (Puliyath et al. 2008). The model developed using selectively bred pedigreed rabbits remains a promising one for further genetic investigations.

As is found in human sera (Li et al., 2011), some rabbits had detectable pre-immune anti-nuclear antibodies (ANA) by ELISA. ANA of sixteen of twenty-four rabbits in group 5, including four immunized with only MAP-8 backbone had an increased ELISA value (delta OD) above pre-immune of 1.0 or more optical density units after the third boost. Anti-dsDNA increased in 12/24 rabbits after the fifth or seventh boost (Puliyath et al., 2008). Figure 2 shows examples of indirect immunofluorescence (ANA-IFA) studies of some sera from group 6 (Yang et al. 2009b). As in human SLE sera, the ANA-IFA patterns reflect responses to one or more autoantigens in different individuals. Littermates that received GR peptide such as UA269-3 and -1 (GR73 and GR77) developed similar patterns after the 3rd boost. ANA staining with sera of littermates XA346-1 and -2 (GR85 and BB82) resulted in different patterns. GR85 serum exhibited some cytoplasmic and peripheral nuclear staining not seen with the serum of BB82. Puliyath et al., (2008) also noticed that GR-immunized littermates had similar ANA-IFA staining patterns but that BB immunized animals' patterns generally differed.

2.2.3 Gene expression studies

We extended the information about the rabbit model of SLE by microarray-based expression profiling of mRNA from peripheral blood leukocytes following peptide immunization (Rai et al., 2010). Data obtained in studies of gene expression in the first four groups of immunized rabbits were deposited in the Gene Expression Omnibus and became

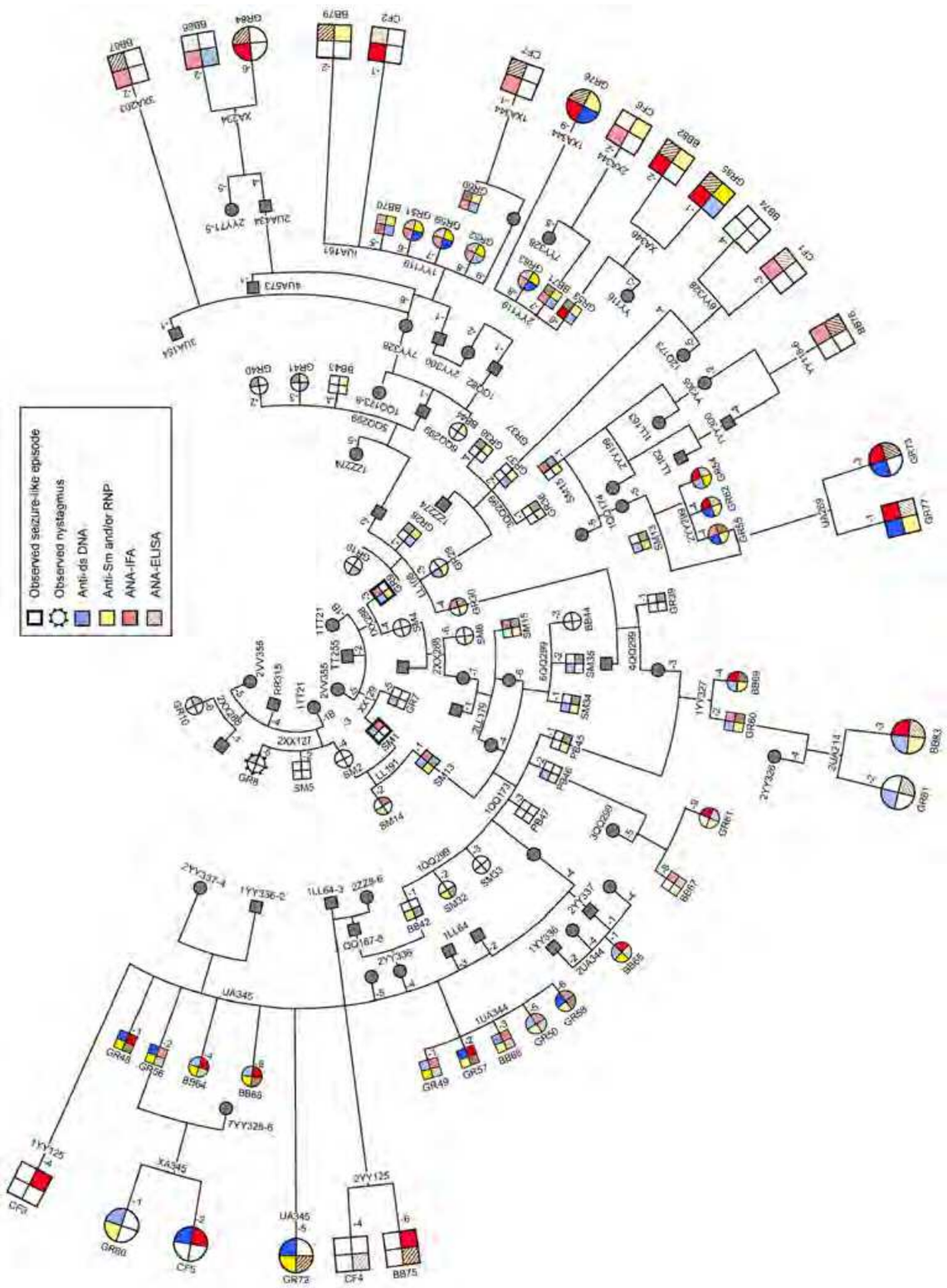

Fig. 1. Pedigree and summary of autoantibody responses.

public on Jul 23, 2010 at the NCBI website: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23076
Experiment type: Expression profiling by array. GEO accession: Series GSE23076 Query DataSets for GSE23076

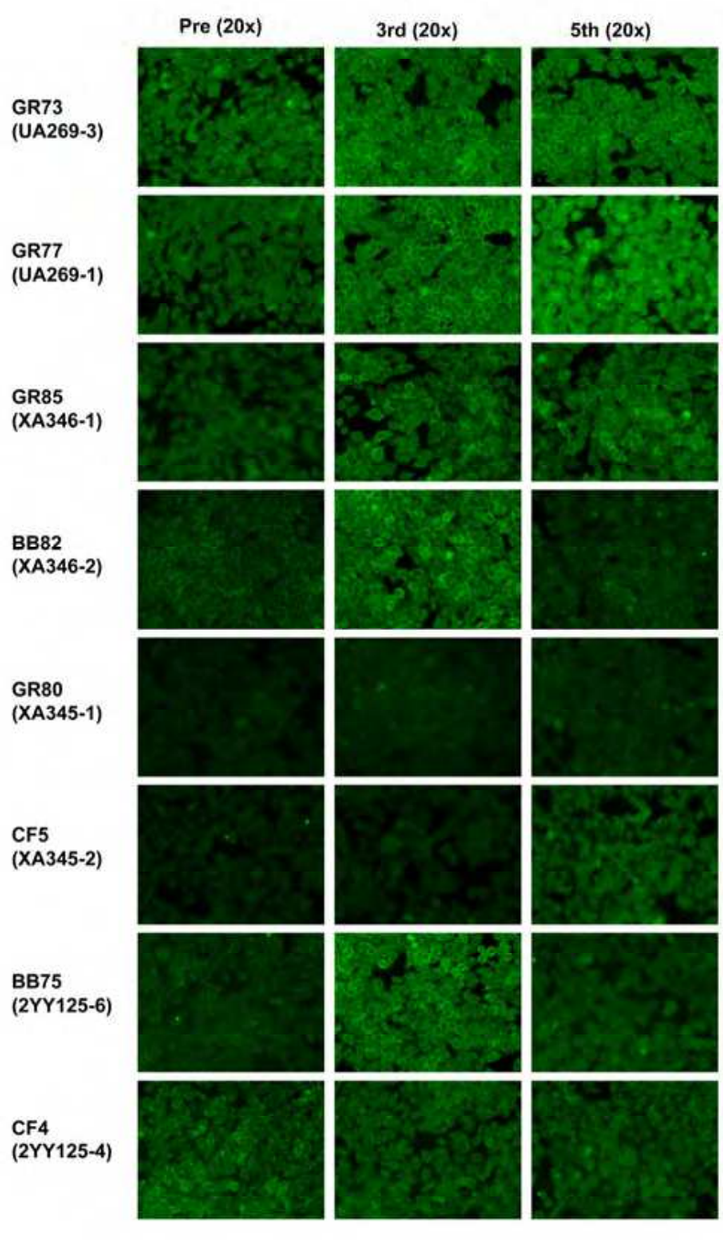

Fig. 2. Examples of indirect ANA-IFA assays of pre- and post-immune sera. Figure originally published by Yang et al., (2009b) Investigations of a rabbit (*Oryctolagus cuniculus*) model of systemic lupus erythematosus (SLE), BAFF and its receptors. *PLoS ONE* Vol.4, 2009.

At the time of the gene expression studies, microarrays specific for study of gene expression profiles were not available for rabbits. We therefore first conducted comparisons of identically prepared rabbit and human cRNA binding to the Affymetrix U95 microarray available for human gene expression analyses. We showed that the human microarray could be used with rabbit cRNA to yield information on genetic pathways activated and/or suppressed in autoantibody-producing immunized rabbits. After demonstrating that human expression arrays could be used with rabbit RNA to yield information on molecular pathways, we designed a study evaluating gene expression profiles in a total of 46 rabbits from 4 groups of the pedigreed control and immunized rabbits. We discovered unique gene expression changes associated with lupus-like serological patterns in immunized rabbits. Our results also demonstrated that caution must be applied when choosing the structure of the carrier Multiple Antigen Peptide (MAP-peptide) for immunization. We discovered that using MAP-4 rather than MAP-8 significantly altered patterns of immune response and gene expression.

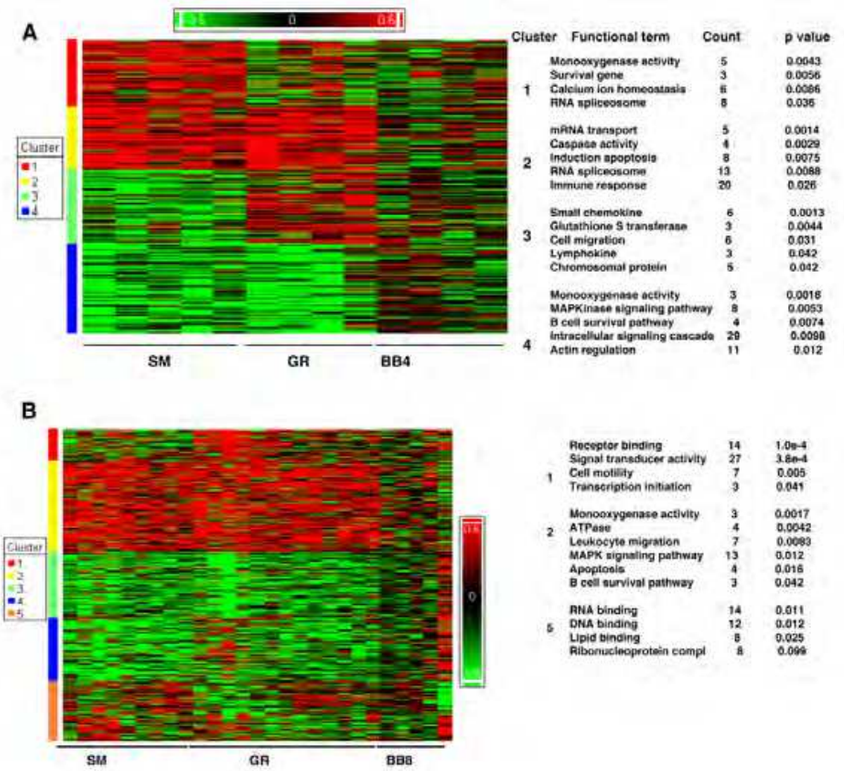

Fig. 3. Gene expression patterns differ when the backbone of immunogen is MAP-4 (A) or MAP-8 (B). In panel A, Cluster 1 genes were overrepresented in the SM group, Cluster 2 were common to both, Cluster 3 genes were overexpressed in the GR group and Cluster 4 genes were decreased in expression in both groups. Count indicates the number of different genes associated with each functional term. Figure modified from one originally published in The Journal of Immunology. Rai, G., Ray S., Milton, J., Yang, J., Ren, P., Lempicki, R., and Mage, R.G. 2010. Gene expression profiles in a rabbit model of systemic lupus erythematosus autoantibody production. J. Immunol. 185:4446-4456. Copyright © [2010] The American Association of Immunologists, Inc.

Figure 3 shows that distinct patterns and clusters of functionally related genes were found to be upregulated when peptides SM or GR on MAP-4 backbone (A) were used as immunogens compared to when MAP-8 backbone was used (B) (Rai et al., 2010). Validation of gene expression data by quantitative real-time PCR was conducted for two genes for which primer sequences were available beta2-microglobulin (B2M) and p-21-protein (Cdc42/Rac)-activated kinase 1 (PAK1) (Figure 6 in Rai et al., 2010). These genes appear in the interactive pathway shown in Figure 4 below. Among the genes significantly upregulated in SLE rabbits were those associated with NK cytotoxicity, antigen presentation, leukocyte migration, cytokine activity, protein kinases, RNA spliceosomal ribonucleoproteins, intracellular signaling cascades, and glutamate receptor activity (Rai et al., 2010).

Functional Annotation	**p-Value**	**Number of molecules**
Inflammatory Disorder	1.6E-11	25
Immunological Disorder	3.1E-11	23
Rheumatic Disease	5.4E-11	19
Autoimmune Disease	2.7 E-08	18
Rheumatoid Arthritis	1.7E-06	13
Glomerulonephritis	2.6E-06	5
Inflammation	2.5E-05	7
Lupus Nephritis of Mice	3.2E-04	3

Table 2. The top functional annotations found using Ingenuity Pathways Analysis (IPA) in comparisons of upregulated genes of rabbits making anti-dsDNA to those only making other anti-nuclear antibodies.

Figure 4 and Table 2 summarize the patterns of upregulated gene expression found in the rabbits from the three groups immunized with MAP-8-peptides that made anti-dsDNA compared to those that only made other anti-nuclear antibodies. Twenty-five genes associated with inflammatory disorders were significantly upregulated in expression. Subsets of these were associated with various immunological disorders in the IPA databases including Autoimmune, Rheumatic, and inflammatory diseases. The results linked increased immune activation with up-regulation of components associated with neurological and anti-RNP responses, demonstrating the utility of the rabbit SLE model to uncover biological pathways related to SLE-induced clinical symptoms, including NPSLE. We suggested that our finding of distinct gene expression patterns in rabbits that made anti-dsDNA should be further investigated in subsets of SLE patients with different autoantibody profiles (Rai et al., 2010). In Figure 4, the connecting lines indicate direct interactions among the products of these genes. The shapes classify the proteins found as transmembrane receptors e.g. CD 40, cytokines/growth factors, e.g. CCL2, kinases, e.g. TYK2, peptidases, e.g. MMP9, other enzymes, e.g. ARF1 and transcriptional regulators, e.g. STAT5B. Genes shown were common to the pathways listed in Table 2 that were upregulated in the anti-dsDNA positive rabbits. Figure 4 was modified from one originally published in the Journal of Immunology. Rai, G., Ray S., Milton, J., Yang, J., Ren, P., Lempicki, R., and Mage, R.G. 2010. Gene expression profiles in a rabbit model of systemic lupus erythematosus autoantibody production. J. Immunol. 185:4446-4456. Copyright © [2010] The American Association of Immunologists, Inc.

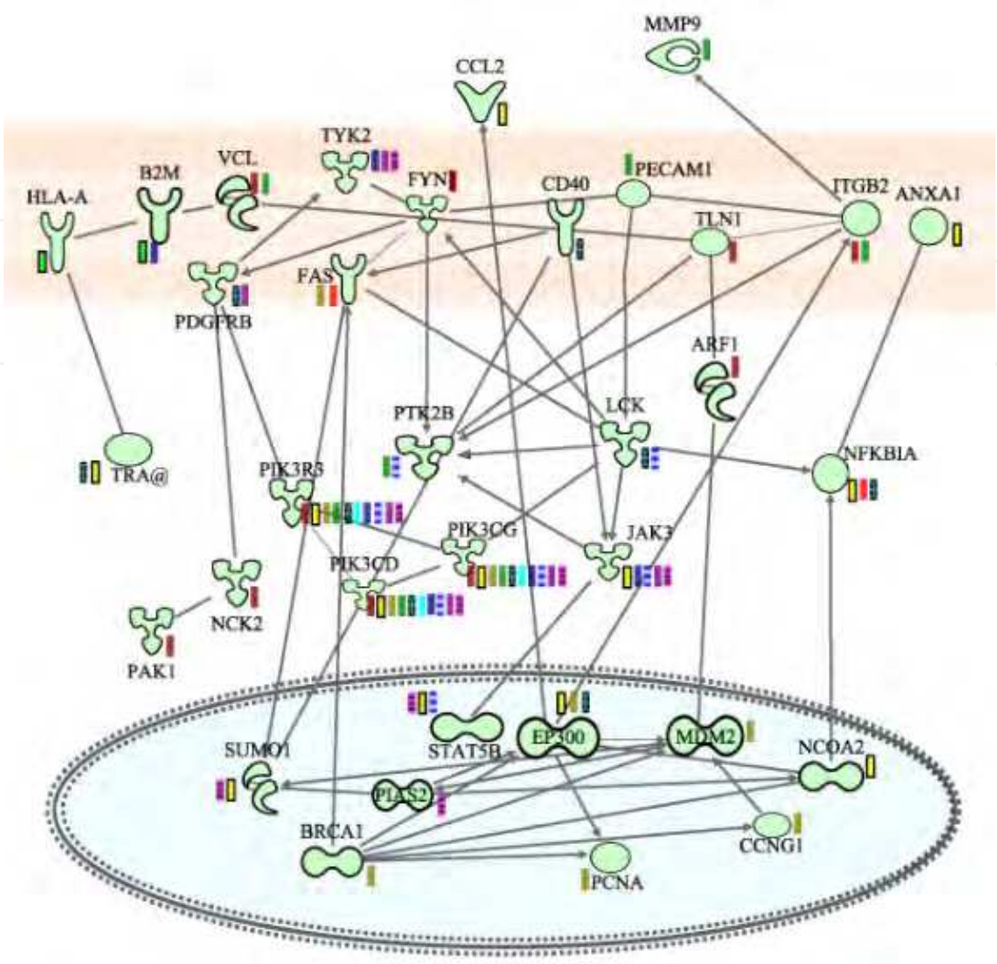

Fig. 4. Interactive pathway network of upregulated genes in anti-dsDNA positive rabbits.

2.2.4 Rabbit BAFF

Our laboratory described the expression and localization of rabbit B-cell activating factor (BAFF also termed BLys, TNFSF13b TALL1, zTNF4) and its receptor BR3 in cells and tissues of the rabbit (Yang et al., 2009a). In addition to its important role in B-cell development and survival, disease activity in human lupus patients has been reported to correlate with serum BAFF levels (reviewed in Groom et al., 2007) and with elevated expression of mRNA for BAFF and two BAFF receptors, BR3 and transmembrane activator and CAML interactor (TACI) in PBMC of lupus patients (Petri et al, 2008). We therefore also investigated BAFF and its receptors in our rabbit model of SLE (Yang et al., 2009b). We previously concluded that BAFF detected on B cells by flow cytometry represented BAFF bound to its receptors on the cells (Yang et al, 2009a). An independent study (Yeramilli, & Knight, 2010) also reported that BAFF-binding receptors on rabbit B-cells are occupied by endogenous soluble BAFF. These authors' studies also suggested that B cells in rabbit could produce BAFF. With the small number of total animals available in group 6 (Table 1B), and no reagents available to detect levels of serum BAFF, we could only measure BAFF on cell surfaces by flow cytometry. These studies found decreased surface expression of BAFF, BR3 and TACI after immunization and boosting

in most animals. However, two rabbits that produced high anti-dsDNA responses (GR76 and GR77) developed higher percentages of BAFF/CD14 and BR3/CD14 positive cells. We did observe consistently lower mean fluorescence intensities of staining of TACI on PBMC and lower percentages of TACI positive cells. We suggested that since TACI is a negative regulator of B cells in mouse and man, perhaps the decrease in TACI in the rabbits producing autoantibodies had allowed autoreactive B cells to escape regulation.

At the time these studies were conducted, clinical trials targeting BAFF/BLys and its receptors were in progress. With the FDA approval of Benlysta® (belimumab) in March, 2011, this monoclonal antibody, that inhibits binding of BLys/BAFF to receptors on B cells, became the first United States FDA approved treatment for SLE in over fifty years. Unfortunately, the clinical trials did not include SLE patients with severe active central nervous system lupus or nephritis. Post-approval trials will be required before this treatment can be recommended for these cohorts of patients.

2.3 Future prospects

2.3.1 Detection of autoantibodies to other antigens including neuroantigens in the rabbit model

In our rabbits, the development of severe symptoms may not yet have occurred because many were euthanized to make room for immunization and testing of their progeny and for tissue collection. For example, although nephritis was not observed, our gene expression studies identified upregulation of genes associated with Glomerulonephritis and also found in mice with Lupus Nephritis (Table 2 and Figure 4). Protein arrays containing microbial and autoantigens have been used to extend information on patients' serum profiles beyond the standard tests used in diagnosis (see for example, Robinson et al., 2002; Quintana et al., 2004; Li et al., 2005; Fattal et al. 2010). Recently, Li et al, (2011) used protein microarrays to determine risk factors for ANA positivity in healthy persons and concluded that serum profiles of autoantibodies can potentially identify healthy individuals with potential to develop lupus and other autoimmune diseases. Their observations extended the widely quoted earlier observations by Arbuckle et al, (2003) that autoantibodies develop as much as ten years before the clinical onset of SLE. In a NOD mouse model of cyclophosphamide-accelerated diabetes, Quintana et al (2004) used a protein microarray to predict from autoantibody repertoires, resistance or susceptibility to the development of diabetes before the induction with cyclophosphamide. Recently Fattal et al, (2010) applied the same technology to studies of SLE patients and controls. They reported highly specific SLE profiles that typically show increases in IgG binding to dsDNA, single-stranded DNA, Epstein–Barr virus, and hyaluronic acid. Interestingly, a healthy control subject who had the SLE antibody profile was later found to develop clinical SLE. Decreases in some specific IgM reactivities to autoantigens observed in this and earlier studies (Li et al., 2005) suggest that some natural IgM autoantibodies may play a protective role. A project to determine the antibody profiles of the rabbits' serum IgG and IgM, purified anti-dsDNA, and anti-peptide on protein microarrays carrying microbial and self antigens including those from the central and peripheral nervous system is in progress.

2.3.2 NPSLE and anti-NMDA glutamate receptors

The suggestion from extensive studies in the laboratory of Betty Diamond that some anti-dsDNA antibodies may react with the NMDA receptor and contribute to neurological manifestations in some lupus patients (DeGiorgio et al. 2001; Kowal et al, 2004), has led to

numerous follow-up studies by the Diamond group, (Diamond & Volpe, 2004) and others. A recent editorial (Appenzeller, 2011) provides an updated overview of controversies in the field and discusses the accompanying paper by Gono et al., (2011) who report new analyses of 107 patients' sera for cross-reactivities of anti-dsDNA with a peptide derived from the sequence of the human NMDA receptor 2A (NR2A) compared with the similar peptide from human NR2B. They suggest that the sensitivity for detection of autoantibodies is greater with the NR2A peptide although their ELISA results directly comparing serum reactivities with each peptide were correlated with high significance ($r = 0.94$; $P<0.0001$). They conclude that assays of sera for anti-NR2A antibodies may be a better predictor of NPSLE than assays for NR2B and suggest that mixed results from other similar studies may be explained by small numbers of patients (Husebye et al., 2005) or less sensitive assays. We chose the GR peptide used in our immunization protocol based on the human sequence of NR2B because the rabbit sequence was not yet known. However, we knew that this sequence was highly conserved in several species including mouse, rat, dog, cow and chicken.

2.3.3 Rabbit genomics

Future studies of rabbit autoimmune and infectious diseases will benefit from the availability of a high quality draft rabbit genome sequence and assembly at ~7 x coverage recently completed at the Broad Institute, Boston (OryCun2.0). The donor was from a partially inbred strain. NCBI maintains a Rabbit Genome Resources website: http://www.ncbi.nlm.nih.gov/projects/genome/guide/rabbit/

Rabbit genomic sequences and assemblies from the ENCODE Project, with ~ 1% of rabbit genomic sequence from a different, outbred NZW animal are also available in GenBank. The selection of peptides for future immunization studies in rabbits can benefit from searching these resources.

3. Conclusion

The work described in this review documents that rabbits have a strong genetic component that leads to predisposition to production of autoantibodies similar to those found in SLE patients including those with NPSLE. Breeding and selection for consistent autoantibody production in the rabbit model can be accomplished over a few generations. When one of us (RGM) retired to Emeritus status at NIAID, the pedigreed colony was no longer maintained. Some animals related to those studied were distributed to others. In addition, although the pedigreed colony was dispersed, there is sperm available from two male breeders rabbits LL191-1 (SM13) and 1UA344-1 (GR49). In particular male SM13 and his progeny in the breeding scheme shown in Figure 2 generated numerous responders that made autoantibodies similar to those found in human Lupus patients. Cryovials of sperm from these animals are currently stored at the Twinbrook 3 facility of the Comparative Medicine Branch (CMB) of NIAID in liquid nitrogen storage tanks, and monitored weekly by their personnel. Further contact information can be obtained at the website of the CMB, of the NIAID, NIH at: http://www.niaid.nih.gov/LabsAndResources/labs/aboutlabs/cmb/Pages/default.aspx.

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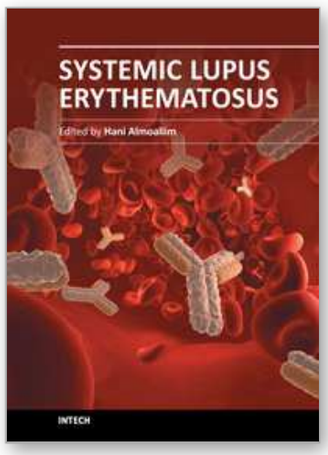

Systemic Lupus Erythematosus

Edited by Dr Hani Almoallim

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This book provides a comprehensive overview of the basic and clinical sciences of Systemic Lupus Erythematosus. It is suitable for basic scientists looking for detailed coverage of their areas of interest. It describes how advances in molecular biology have increased our understanding of this disease. It is a valuable clinical resource for practicing clinicians from different disciplines including rheumatologists, rheumatology fellows and residents. This book provides convenient access to information you need about cytokines, genetics, Fas pathway, toll like receptors and atherogenesis in SLE. Animal models have been reviewed as well. How to avoid delay in SLE diagnosis and management, in addition to various clinical manifestations including pregnancy and SLE have all been explained thoroughly in this book.

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