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The Mechanism of Autoantibody Production Caused by Circulating Bacteria

探討血液循環系統中的細菌感染引發自體抗體生成之機制

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本論文係葉子瑄欣君（學號R03445118）在國立臺灣大學微生物學所完成之碩（博）士學位論文，於民國105年7月28日承下列考試委員審查通過及口試及格，特此證明

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Abstract

Infections play roles in the induction of autoimmune diseases, including rheumatic fever and Guillain–Barré syndrome. Our previous data showed that autoantibodies such as anti-dsDNA antibodies can be found in patients with infective endocarditis and disseminated systemic infection. The anti-dsDNA antibodies can be induced in rat intravenously infected with *Streptococcus mutans*. Spleen plays roles in the clearance of circulating bacteria and antibody production; therefore, we hypothesized spleen may produce autoantibodies induced by circulating bacteria. We found that anti-dsDNA antibodies could be induced within 5-7 days after intravenous infection in the mouse model. The anti-dsDNA IgG levels were decreased in the mice with splenectomy, suggesting spleen can produce autoantibodies induced by circulating bacteria. The purified anti-dsDNA antibodies can cross-react with a *S. mutans* surface protein, glucosyltransferase B. On the other hand, systemic lupus erythematosus patients usually have high levels of anti-dsDNA antibodies. We found that a ribosomal protein named L7/L12 may not be an inducer of anti-dsDNA antibodies in SLE patients. Taken together, spleen play roles in autoantibody production caused by circulating infection, and the GtfB and L7/L12 may induce the cross-reactive autoantibody production.

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中文摘要

感染可能引發風溼熱、吉蘭-巴雷氏症狀群等自體免疫疾病。本實驗室先前的研究指出，感染性心內膜炎及擴散性全身性感染患者體內含有抗雙股去氧核糖核酸抗體。當大鼠經由靜脈感染轉糖鏈球菌後，體內會產生抗雙股去氧核糖核酸抗體。脾臟對於清除血液中的細菌及產生抗體扮演一定的角色；因此，我們假設血液中的細菌可能引發脾臟產生自體抗體。我們發現，當小鼠經由靜脈感染後，在 5 至 7 天內能產生抗雙股去氧核糖核酸抗體;利用脾臟被切除的小鼠進行同樣的實驗，發現小鼠體內抗雙股去氧核糖核酸抗體的量下降，顯示血液中的細菌能促使脾臟產生自體抗體。我們發現純化的抗雙股去氧核糖核酸抗體和轉糖鏈球菌的表面蛋白: 葡萄糖基轉移酶 B 有交叉反應。另外，紅斑性狼瘡患者體內通常具有大量的抗雙股去氧核糖核酸抗體，我們發現有一種核醣體蛋白: L7/L12 在紅斑性狼瘡患者體內可能無法誘發產生抗雙股去氧核糖核酸抗體。以上結果顯示血液循環系統中的細菌感染能使脾臟產生自體抗體，而葡萄糖基轉移酶 B 及 L7/L12 可能產生交叉反應促使自體抗體的產生。

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Chapter 1. Introduction

1.1 The relationship between infection and autoantibody production

Infections play roles in the induction of autoimmune diseases such as rheumatic fever, Guillain-Barré syndrome, and type 1 diabetes [1-3]. For instance, *Streptococcus pyogenes* infection can lead to rheumatic fever, which is characterized by damaged central nervous system, heart and joint. On the surface of *S. pyogenes*, there is a sugar product called N-acetyl-β-D-glucosamine carbohydrate (GlcNAc), which can cross-react with human ganglioside and cardiac myosin in the myocardium [4]. Moreover, *Campylobacter jejuni* infection can induce antibodies that crossreact with gangliosides at nerve membrane, and lead to Guillain-Barre syndrome [5]. These crossreactivities between pathogens and self-structures are characterized as "molecular mimicry". Molecular mimicry is first named by R. Damian in 1964, suggesting that this similarity can help microorganisms to escape from host's immune response. Recently, molecular mimicry is changed to another meaning that microbes may harm to host via the induction of autoimmune response [6].

1.2 Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by

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production of autoantibodies and immune complexes, leading to chronic systemic inflammation and may damage to various organs, such as kidneys and joints. This disease usually accompanied with many symptoms, including nephritis, neuropsychiatric manifestations, and cardiovascular events, in a high mortality rate [7, 8]. Anti-double stranded DNAs (anti-dsDNAs) are the hallmarks of SLE for diagnosis [9]. Although anti-dsDNA antibodies are believed to induce pathogenesis by depositing in the kidney or promoting cytokine production, the expression of anti-dsDNA antibodies is not related to the disease activity [10]. Among patients with SLE, infection is a main cause of morbidity and mortality. Clinical studies show that pathogens, including Epstein-Barr virus (EBV), cytomegalovirus (CMV) and tuberculosis (TB), can lead to the pathogenesis of SLE [11, 12]. It has been reported that a bacterial antigen, amyloid fiber (curli), have the ability to bind extracellular host DNA (eDNA). The curli/eDNA complex was used to inject lupus-prone mice, and the mice were tested positive for SLE within 2 weeks after exposure [13, 14]. It also has been found that mice treated with *Helicobacter pylori* urease can produce anti-dsDNA antibodies. In some of female African American patients, infection of *H. pylori* may protect them from developing SLE [15].

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1.3 *Streptococcus mutans*

Streptococcus mutans is first identified by J Kilian Clarke in 1924. *S. mutans* is gram-positive, facultatively anaerobic bacteria, commonly found in human oral cavity. Distinct from other coccus-shaped streptococci, *S. mutans* is in a bacillary form. This microbe is α-hemolysis on blood agar, so it belongs to viridians streptococci. *S. mutans* is a main microbe to cause dental caries through forming biofilms on the tooth and making a low pH environment [16]. In addition, during dental surgery or after trauma in the mouth, *S. mutans* can leave the oral cavity and enter into the bloodstream to cause bacteremia. Once the patients have injured cardiovascular endothelium or artificial heart valves, this microbe can easily colonize on the valves, form vegetations and lead to subcutaneous infective endocarditis (IE) [17, 18]. For causing these diseases, *S. mutans* express a lot of virulent factors, such as glucosyltransferases (Gtfs) and glucan-binding protein B (GbpB) [19-21].

1.4 Glucosyltransferases

In dental plaque, glucans can help oral microorganisims to accumulate on the tooth surface [22]. Several oral streptococci, including *S. mutans*, *S. sobrinus*, *S. salivarius* and *S. gordonii*, are able to produce different kinds or functions of

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glucosyltransferases, which can convert sucrose into glucan [23]. *S. mutans* express three Gtfs, GtfB (162 kDa), GtfC (149 kDa) and GtfD (155 kDa) [24]. Gtfs can synthesize water-soluble or –insoluble glucans, depending on their different types of branching and glycosidic linkage. GtfB can synthesize water-insoluble glucans rich in α (1-3) linkages, GtfD synthesizes water-soluble glucans rich in α (1-6) linkages, and GtfB can produce both water-soluble and –insoluble glucans with mostly α (1-6) linkages [25]. GtfB, GtfC are bacterial surface proteins, and they share about 75% amino acid sequence identity. GtfD is a secretary protein sharing about 50% identity to GtfB and GtfC [22]. Gtfs have two functional domains, a glucan-binding domain located in the C-terminal region, and a catalytic domain located in the N-terminal region. Glucan-binding domain is essential for glucan synthesis, and catalytic domain is related to hydrolyze sucrose [26]. Gtfs are highly immunogenic that in children and young adults, anti-GTF-specific immunoglobulin A (IgA) in saliva or IgG in serum may be detected [27]. It also has been reported that Gtfs can directly stimulate T-cell proliferation and induce monocytes to produce IL-6 [28, 29].

1.5 Glucan-binding protein B (GbpB)/60-kDa immunodominant glycoprotein (IdG60)

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There are four glucan-binding proteins in *S. mutans*, including GbpA, GbpB, GbpC and GbpD. Their functions are related to biofilm formation, cell wall stability, dextran-dependent aggregation, and lipase activity, respectively [25]. Among them, GbpB is first purified by Smith et al in 1994 [30]. The molecular weight of GbpB is 59kDa by SDS-PAGE. It has been found that there is a protein with a size of approximately 60kDa on Western blots can be probed with either saliva IgA or serum IgG from humans [31]. According to the molecular weight, immunodominant surface antigen and the function of posttranslational modification by glycosylation, GbpB was also named 60-kDa immunodominant glycoprotein (IdG60) [32, 33]. GbpB play important roles in cell-wall formation, cell-division and cell maintenance [21]. In addition, rats immunized with GbpB or GbpB-derived peptides can induce protective immunity against dental caries. It also has been found that GbpB is the antigen, which commonly induces antibody response in saliva of young children. Therefore, GbpB may be related to the virulence of *S. mutans* [34-37].

1.6 Spleen

Spleen is one of the secondary lymphoid organs, in which immune responses are induced. The structure of spleen can be divided into two types of tissue, namely red

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pulp and white pulp, each has different functions. The red pulp can eliminate older or abnormal red blood cells. It also contains some phagocytes that engulf microorganisms from circulation. The white pulp can be further separated into periarteriolar lymphoid sheaths (PALS), lymph follicles and marginal zone. Inside white pulp, there are many lymphocytes, which in turn produce antibodies. Therefore, spleen play roles in both innate and adaptive immunity, especially in host defence against blood-borne pathogens [38]. People who receive splenctomy, a surgery to remove spleen, are at a high risk of infection. It has been reported that splenectomized patients are easily infected with encapsulated bacteria and may lead to death in 40% to 54% of mortality rate [39]. Because of the high mortality rate, patients are received vaccines againt *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitides* before splenectomy [40].

1.7 Marginal zone B cells

Marginal zone is an area of spleen between the white pulp and red pulp. B cells residing in this area, called marginal zone B (MZ B) cells, can quickly respond to blood-borne pathogens or antigens [41]. It has been reported that MZ B cells can produce low-affinity immunoglobulin M (IgM), IgG and some IgA as early as 1-3

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days after exposure to blood-borne microbes through T cell-independent (TI) pathway [42]. In addition, MZ B cells also participate in T cell-dependent (TD) immune response through the capture of circulating antigens and generate long-lived plasma cells that secret high-affinity antibodies [43].

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Chapter 2.Purpose and Specific Aim

Infection can induce autoimmune disease such as rheumatic fever and Guillain–Barré syndrome. Clinical data shows various autoantibodies, including anti-dsDNA antibodies and anti-phospholipid antibodies, can be found in patients with infective endocarditis (IE) and disseminated systemic infection (Table 1). Our previous data showed that anti-dsDNA antibodies can be induced in rats intravenously infected with *S. mutans*. However the mechanism of anti-dsDNA production after intravenous infection remains unknown. Spleen can clear bacteria in the bloodstream and produce antibodies; therefore, we hypothesized spleen may play roles in autoantibody production induced by circulating bacteria. Besides, the cross-reactivity between bacterial proteins and anti-dsDNA antibodies is another question we are interested in. Because the titers of anti-dsDNA antibodies in SLE patients are high, we can use these patients' serum to investigate the relationship in the cross-reaction.

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Chapter 3. Materials and methods

3.1 Animals

Six to eight weeks-old BALB/*c* mice were purchased from National Laboratory Animal Center (NLAC), Taipei, Taiwan. All animal experiments in this study were approved by National Taiwan University Institutional Animal Care and Use Committee. Mice were intravenously (i.v.) injected with bacteria or bacterial proteins. Blood were collected from the facial vein of the mice by using lancets. The sera were prepared after centrifugation at 8000 rpm for 5 minutes at room temperature.

3.2 Bacterial strains and growth conditions

The isogenic mutants GS5DD, NHR1DD, LN62DD, NHS1 and NHS1DD, which are provided by H. K. Kuramitsu (State University of New York, Buffalo), are derived from *Streptococcus mutans* GS-5. These mutant strains expressed with GtfB/C, GtfB, GtfC , GtfD or without Gtf protein, respectively. All of the Streptococcal strains were grown in Brain Heart Infusion (BHI) broth (Difco Laboratories Inc.) or on agar plate (Difco Laboratories Inc.) in anaerobic condition (5% CO_2 and 95% N_2) at 37℃ overnight. *S. mutans* NHR1DD, LN62DD and NHS1DD were grown in BHI contained erythromycin (5μg/ml) and tetracycline

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(10μg/ml). *S. mutans* NHS1 and GS5DD were grown in the broth supplemented with erythromycin (5μg/ml) or tetracycline (15μg/ml), respectively.

3.3 Streptococcal cell-wall-associated (CA) proteins extraction

S. mutans GS-5, GS5DD, NHR1DD, LN62DD, NHS1, NHS1DD and IdG60 K.O. mutants were grown in 50ml BHI broth contained antibiotics, as required, for 14 to 16 hours at 37℃ in anaerobic condition. Then, the bacteria were sub-cultured to 1L TTY broth (2.25% tryptone, 0.6% tryptose, 0.6% yeast extract, 0.2% K_2PHO_4, 0.2% Na_2CO_3, 0.2% NaCl, 0.5% KH_2PO_4, 1% glucose) for 14 to 16 hours at 37℃. Bacteria were collected after centrifugation and washing three times with 10 mM sodium phosphate (NaPB) buffer in pH 6.0 at 6000 rpm (JA-14), 4℃ for 20 minutes. The bacteria was suspended with 45ml 8M urea and stirred at room temperature for 2 hours to extract the cell-wall associated proteins. After centrifugation at 10000 rpm, 4℃ for 30 minutes, the supernatant was collected. The supernatant was dialyzed for 4 times in cold 10 mM NaPB buffer (pH=6.0) at 4℃ for 1 hour, and it was dialyzed overnight at the last time. The extract was concentrated by 60% (saturation) ammonium sulfate precipitation and stirred at 4℃ overnight. The precipitate was centrifuged at 10000 rpm, 4℃ for 30 minutes and the pellet was dissolved with 1 or 2

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ml 10 mM pH 6.0 NaPB buffer (dependent on the pellet size) and dialyzed for 6 times in the same dialysis buffer at 4℃ for 0.5 hour, and subsequently dialyzed overnight at the last time. The concentration of CA proteins were measured by bicinchoninic acid (BCA) protein assay (Pierce®).

3.4 Transform and purification of rIdG-60

IdG60 K.O. mutant was grown in 10ml BHI broth contained erythromycin (5μg/ml) for 14 to 16 hours at 37℃ in anaerobic condition. Then, the bacteria were sub-cultured to 5% horse serum in BHI broth at 1:20 dilution. Bacteria was grown to an OD550 of 0.18 to 0.28. The bacteria were transferred to a new tube, and the his-tagged recombinant IdG-60 (rIdG-60) plasmid (1λ) was added. After 30 minutes incubation, 700 λ BHI broth was added and continuously incubated for 1.5 hours. The 100λ bacterial solution was cultured onto BHI agar plate contained erythromycin (5μg/ml) and spectinomycin (500μg/ml). His-tagged rIdG60 expressed in IdG60 K.O. mutant was purified by chromatography on an Ni^{2+} affinity resin. Homogeneity of the purified proteins was confirmed by SDS-PAGE, followed by coomassie blue staining. Identity of the protein was analyzed by immunoblots with anti-His-tag monoclonal antibody. Protein concentrations were measured with BCA assay (Pierce®).

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3.5 Enzyme-linked immunosorbent assay (ELISA)

For the anti-dsDNA antibody assay, the calf-thymic DNA (Sigma) was dissolved in ddH_2O and DNA-coating buffer (Pierce®) for 10 minutes. One μg dsDNA in 50 μL per well of calf-thymic dsDNA was coated to ELISA plates overnight at room temperature. The plates were washed with 0.05% Phosphate Buffered Saline Tween 20 (PBST) for 3 times. After blocking with 1% bovine serum albumin (BSA) in PBS at 37℃ for 2 hours, mice sera at 1:500 dilutions in PBS contained 1% BSA were added and incubated at 4℃ overnight. The plates were washed with 0.05% PBST for 3 times and bound antibodies were detected by horseradish peroxidase (HRP) conjugated secondary antibodies at dilutions of 1:5000 (for anti-dsDNA IgG), and incubated at 37℃ for 2 hours. The plates were washed with 0.05% PBST for 3 times and the 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was added. The reaction was stopped by 2N H_2SO_4 and the absorbance was measured at 450 nm.

For the anti-CA or purified bacterial proteins assay, the CA proteins and bacterial purified proteins were dissolved in ELISA coating buffer (pH=9.0) at the concentration for 20μg/ml and 10μg/ml, respectively. The ELISA plate wells were coated with 1μg /50 μl CA proteins or 0.5μg /50 μl in coating buffer at 4℃ overnight. The plates were washed with 0.05% PBST for 3 times. After blocking with 1% BSA

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in PBS at 37℃ for 2 hours, mice sera at 1:1000 dilutions in PBS contained 1% BSA were added and incubated at 4℃ overnight. The plates were washed with 0.05% PBST for 3 times and bound antibodies were detected by HRP conjugated secondary antibodies at dilutions of 1:5000 (for anti-dsDNA IgG), and incubated at 37℃ for 2 hours. The plates were washed with 0.05% PBST for 3 times and the TMB substrate was added. The reaction was stopped by 2N H_2SO_4 and the absorbance was measured at 450 nm.

For the anti-bacterial antibody assay, the bacteria were grown in the broth for 14-16 hours and centrifuged at 3000rpm, room temperature for 15 minutes. After washing with PBS, the pellet was dissolved in ELISA coating buffer (pH=9.0) at an OD550 of 0.8 to 1.2. Each well was coated with 50μl bacterial solution at 4℃ overnight. The following steps are as same as anti-CA proteins assay.

3.6 Antibody elution from ELISA plate wells

As described in ELISA, after blocking with 1% BSA in PBS, sera samples were added to Calf-thymic dsDNA-coated ELISA plates at 4℃ overnight. Supernatants were discarded and plates were washed 3 times with 0.05% PBST. Bound antibodies were eluted from the plates by adding 50 μl elution buffer (0.2 M Glycine/HCl

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pH=2.5) for 10 minutes at room temperature, and neutralization buffer (1 M Tris/HCl pH=9.0) were added. The antibodies were dialyzed in 10 mM NaPB buffer (pH=6.0) for 6 times at 4℃ for 0.5 hour. After condensing, the antibodies were stored at -20℃.

3.7 Western blotting (WB)

Samples containing 20 μg of CA proteins or 2 μg of purified proteins were electrophoresed on 10% SDS-PAGE gels, and then transferred to 0.22 μm polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with blocking buffer (4% non-fat milk in ddH_2O containing 10 mM Tris and 150 mM NaCl). Separated proteins were probed with mouse, rat, human, rabbit sera, or eluted antibodies at dilution of 1:2000, 1:5000, 1:1000, 1:5000, or 1:100, respectively, overnight at 4℃.The membranes were then incubated with mouse, rat, human, rabbit corresponding HRP conjugated secondary antibody at dilution of 1:10000, 1:5000, 1:10000, 1:10000, respectively, for 2 hours at room. The specific protein bands were visualized using an Enhanced luminal reagent (Western Lightning^{TM}).

3.8 The preparation of splenocytes

Mice spleen were collected and homogenized with 40μm sieve and plunger into

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a 50ml tube. The sieve was washed with cold HBSS and the cells were spun down at 1500 rpm for 10 minutes at 4℃ for 2 times. The supernatant was discarded and added 10 ml cold RBC lysis buffer. After centrifugation for 10 minutes at 1500 rpm at 4℃, the pellet was suspended in RPMI medium, and the splenocytes were collected.

3.9 Cell sorting and *in vitro* stimulation

Splenocytes were stained by anti-B220-APC-Cy7, anti-CD23-PE and anti-CD21-PE-Cy7 (Biolegend) for 30 minutes. The cells were sorted on a BD FACSAria IIIu sorter. Sorted MZ B and follicular (FO) B cells were labeled by CFSE. And these cells (1×10^6 cells/ml) were cultured in 96 well plates (1×10^5 cells/well) for 3 days with the stimulation of *S. mutans* CA proteins. After 3 days, the proliferation of MZ B and FO B cells was analyzed by flow cytometry.

3.10 Statistical analysis

Data were presented as mean (± SEM) and analyzed with the Student's *t*-test to compare the mean level of anti-dsDNA antibodies. Differences with the p value < 0.05 were considered statistically significant.

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Chapter 4. Results

4.1 Antibody production in mice intravenously or intraperitioneally infected with *S. mutans*

To investigate whether bacterial infection can induce anti-dsDNA antibodies, the BALB/*c* mice were received intravenous (i.v.) or intraperitioneally (i.p.) infection with *S. mutans*. Anti- *S. mutans* antibodies in BALB/*c* mice could be produced within one week through intravenous infection (Figure 1A). Anti-dsDNA IgG in intravenously injected mice were also rapidly generated (Figure 1B). In intraperitioneally infected mice, anti-*S. mutans* antibodies and anti-dsDNA antibodies could still be induced but with a delayed and decreased level. These data showed mice were intravenously infected with *S. mutans* could rapidly induce anti-dsDNA IgG and anti-*S. mutans* IgG within 5-7 days, but the antibody level in intraperitioneally infected mice was decreased. Therefore, bacterial infection through bloodstream can promote anti-dsDNA antibody production.

4.2 The reactivity of eluted anti-dsDNA antibodies to *S. mutans* proteins

To identify which proteins induce anti-dsDNA antibody production, anti-dsDNA antibodies of *S. mutans* infected mice was eluted to probe *S. mutans* proteins. We

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found that eluted anti-dsDNA antibodies could recognize around 130-180 kDa proteins, which may be Gtfs (Figure 2A). We further used eluted anti-dsDNA antibodies to blot against various Gtfs mutant strains of *S. mutains*, including GS5DD, NHR1DD, LN62DD, NHS1, NHS1DD, which expressed GtfB/C, GtfB, GtfC, GtfD or none of Gtfs, respectively. Interestingly, the eluted anti-dsDNA antibodies could cross-react with GtfB rather than GtfC or GtfD (Figure 2B). These results indicated that anti-dsDNA antibodies could cross-react with GtfB. Due to the cross-reactivity of anti-dsDNA antibodies, GtfB may act as a source of anti-dsDNA antibody generation.

4.3 The anti-dsDNA antibody production in the mice infected with *S. aureus*

Our previous data showed that the autoantibody production can be induced by the intravenous infection of *S. mutans*. To investigate if the phenomenon was strict in *S. mutans* infection, we used the other vascular infection pathogen, *S. aureus*, as a target. After intravenous or subcutaneous infection with *S. aureus*, the anti-dsDNA IgG production was measured by ELISA (Figure 3A and 3B). In the intravenously infected mice, the anti-dsDNA antibodies could be rapidly generated, but not in subcutaneously immunized mice. We also eluted anti-dsDNA IgG from intravenously infected mice to probe with proteins of *S. mutans*. Consistently, the eluted

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anti-dsDNA IgG from the mice infected with *S. aureus* could recognize GtfB. These results suggested that intravascular bacterial infection could produce anti-dsDNA antibodies, and these antibodies may be induced by GtfB.

4.4 The anti-dsDNA antibody production in the mice infected with *S. mutans*, NHR1DD or NHS1DD

To investigate whether GtfB could induce anti-dsDNA antibodies *in vivo*, we intravenously infected mice with *S. mutans*, NHR1DD or NHS1DD (Figure 4). In *S. mutans* and NHR1DD infected mice, the anti-dsDNA antibodies could be induced, but the antibody level in NHS1DD infected mice was decreased (Figure 4A and 4B). These data implied that anti-dsDNA antibodies could be induced by GtfB *in vivo*.

4.5 Spleen may play roles in anti-bacterial and anti-dsDNA antibody production in mouse model

The spleen plays roles in antibody production and circulating bacterial clearance, so we wanted to know whether spleen is involved in anti-bacterial and anti-dsDNA antibody production. One week before intravenously infected with *S. mutans*, mice received a surgery to remove the spleen. Compared with the normal mice, the anti- *S.*

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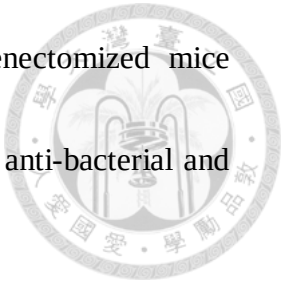
mutans and anti-dsDNA IgG levels were decreased in the splenectomized mice (Figure 5A and 5B). These data suggested that spleen play roles in anti-bacterial and anti-dsDNA antibody production induced by circulating bacteria.



4.6 The antibody production in the normal and splenectomized mice

To identify which *S. mutans* protein induces anti-bacterial and anti-dsDNA antibody production, we performed the Western blotting assay by using the murine sera isolated from the mice with or without splenectomy, which were intravenously infected with *S. mutans* (Figure 6A and 6B). We found that two of the band, with the molecular weight of approximately 130 kDa and 60 kDa, were decreased in splenectomized mice. The identities were confirmed by using anti-Gtfs and anti-IdG60 antibodies (Figure 6C and 6D). These results showed spleen plays roles in anti-Gtfs and anti-IdG60 antibody production.

4.7 The stimulation of MZ B and FO B cells by using streptococcal proteins

Because anti-dsDNA antibodies were rapidly produced after intravenous infection and GtfB may be a source of anti-dsDNA antibody production, we investigated whether MZ B cells play roles in anti-GtfB antibody production. We

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purified MZ B and FO B cells from the mouse by sorting, and stimulated MZ B cells or FO B cells by using CA proteins of *S. mutans*, NHS1DD and NHR1DD, the isogenic mutant strains of *S. mutans* and deficient in GtfB/C/D and GtfC/D, respectively. Three days after stimulation, the proliferation of MZ B and FO B cells was analyzed by flow cytometry (Figure 7). The result showed NHR1DD CA protein can stimulated the proliferation of MZ B cells, compared to *S. mutans* and NHS1DD CA proteins. In contrast, all the CA proteins could not significantly stimulate the proliferation of FO B cells. Therefore, GtfB may trigger MZ B cells to proliferate and produce antibodies.

4.8 The role of the bacterial ribosomal protein, L7/L12, in SLE

Because the sera of SLE patients contained high levels of auto-reactive antibodies, we used the sera from SLE patients and non-SLE people to probe *S. mutans* CA proteins. Interestingly, most of sera from SLE patients could recognize 10 to 17 kDa proteins, but not the sera from the non-SLE people. (Figure 8A). The identity of the bacterial protein, which could be recognized by the SLE sera, was further confirmed by using the specific antibody against the bacterial ribosomal protein, L7/L12 (Figure 8B). More interestingly, the anti- *S. aureus* sera from mice

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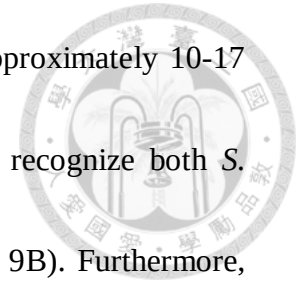

could also recognize the protein with the molecular weight of approximately 10-17 kDa (Figure 9A). The specific antibodies against L7/L12 could recognize both *S. mutans* and *S. aureus* CA proteins at the similar position (Figure 9B). Furthermore, we used the sera from mice infected with *S. aureus* to probe L7/L12 purified protein, *S. mutans* and *S. aureus* CA proteins (Figure 9C), and the anti-*S. aureus* sera could recognized L7/L12 purified protein. These data implied that the role of L7/L12 in the autoantibody production in SLE patients.

4.9 The correlation of anti-dsDNA antibodies and anti-bacterial antibodies in SLE patients

To further investigate the correlation of anti-dsDNA antibodies and anti-bacterial antibodies, we analyzed the data of the sera from SLE patients to interact with *S. mutans* CA proteins by western blotting (Figure 10A and 10B). The result showed that L7/L12 did not correlate with anti-dsDNA antibodies in SLE patients, suggesting that L7/L12 may be an autoantigen but not an inducer of anti-dsDNA antibodies in SLE patients. On the other hand, my previous data showed GtfB may induce antibody production (Figure 2), the sera from SLE patients were used to interact with NHR1DD CA proteins, and analyzed by western blotting (Figure 10C and 10D).

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Interestingly, the mount of anti-GtfB antibodies was correlated to anti-dsDNA antibodies in SLE patients, confirming that role of GtfB in the anti-dsDNA antibody production.

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Chapter 5. Discussion

5.1 Intravascular bacterial infection may be important for the autoantibody production in autoimmune disease

Our data showed that intravascular bacterial infection could induce anti-dsDNA antibody production in mouse models, and spleen was involved in autoantibody production. We also found GtfB, one of the *S. mutans* surface proteins, may trigger MZ B cells to proliferate. In addition, anti-GtfB antibodies can correlate with anti-dsDNA antibodies in SLE patients. These data imply GtfB may be responsible for the anti-dsDNA antibody production induced by *S. mutans*. However, another ribosomal bacterial protein, L7/L12, did not correlate with anti-dsDNA antibodies in SLE patients. Taken together, spleen plays roles in autoantibody production caused by intravascular bacterial infection, and the GtfB and L7/L12 may induce the cross-reactive autoantibody production in SLE patients. In order to confirm the correlation of GtfB and anti-dsDNA antibodies, we could collect more clinical data. Although the data of L7/L12 indicates L7/L12 is not the protein responsible for anti-dsDNA antibody production, the antibodies of L7/L12 may be used as a marker for the diagnosis of SLE. Furthermore, the mechanism of anti-dsDNA antibody production by intravenously bacterial infection needs further investigation. The

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recombinant GtfB protein could be constructed to investigate whether GtfB could induce anti-dsDNA antibody production in murine models.

5.2 The role of neutrophil extracellular traps (NETs) in autoantibody production

It has been reported that during sepsis, neutrophils can trap the microorganisms by releasing neutrophil extracellular traps (NETs) [44]. NETs are extracellular DNA fibers that capture and eliminate microbes. These extracellular DNA fibers, composed of dsDNAs and neutrophil granulate proteins can serve as autoantigens for autoantibody production [45-47]. NETs were released into circulation when mice were intravenously infected with bacteria, and may play some roles in anti-dsDNA antibody production. Compared to *S. mutans*, one of the pathogens, *S. aureus*, can easily induce NETs formation during sepsis [48, 49]. In our experiments, anti-dsDNA antibody production after *S. mutans* or *S. aureus* infection might have the effectiveness of NETs (Figure 1 and 3). To exclude the NETs effect, we can use DNase I to digest free DNAs in bloodstream [50]. However, the outcome of DNase I might be limited because DNase I just digests DNAs but can't digest NETs components. Peptidylarginine deiminase 4 (PAD4) is an enzyme, which plays important roles in NETs formation [51]. PAD4-deficiency mice may be a better

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choice for us to prevent the effectiveness from NETs [48, 52].

5.3 The cross-reactivity of GtfB and anti-dsDNA antibodies

In our data, anti-dsDNA antibodies could cross-react with GtfB but not other Gtfs (Figure 2 and 3). We also found in the NHR1DD infected mice, the anti-dsDNA antibody production was higher than NHS1DD infected mice (Figure 4). In addition, anti-GtfB antibodies can correlate with anti-dsDNA antibodies in SLE patients (Figure 10C and 10D). These data imply GtfB may be responsible for anti-dsDNA antibody production, but the binding sites or the epitopes of cross-reaction should be further investigated. Although GtfB and GtfC shares 75% amino acid sequence identity [22], the anti-dsDNA antibodies only cross-reacted with GtfB. Therefore, we compared the amino sequence identity between GtfB and GtfC by BLAST. The result showed GtfB and GtfC are mainly different in the N-teriminal and C-terminal regions, which contained catalytic domain and glucan-binding domain, respectively [25, 53]. It has been found that there is a ca. 360-amino-acid variable region (VR) in the N-terminal region of GtfB, which is specifically existed in *S. mutans* and/or GtfB, is an epitope that shows promise for the development of a caries vaccine [54]. The 3D structure of the catalytic domain of Gtfs has been reported [55], we can compare the

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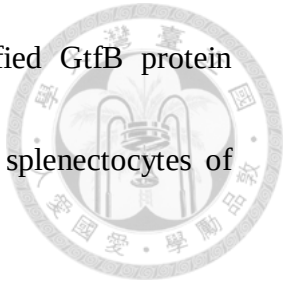
structural identities between dsDNA and GtfB. Moreover, purified GtfB protein should be used to intravenously infect mice *in vivo* or stimulate splenectocytes of mice *ex-vivo.*



5.4 The origin of anti-dsDNA antibodies

We found that anti-dsDNA antibodies were produced in 5-7 days after intravenous infection in mouse models (Figure 1 and 3). This phenomenon is unusual, because antigen-specific IgGs are generally produced in bacterial infected animal models after 2 weeks. This rapid antibody production manner may be induced through T-cell independent response [56-58]. In T-cell independent pathway, marginal zone B cells and B1 cells play important roles in antibody production [42]. Our experiments showed MZ B cells proliferated with the stimulation of NHR1DD, which is deficient in GtfC/D (Figure 7). It has been reported that MZ B cells can produce IgG as early as 1-3 days after exposure to blood-borne microbes through T cell-independent pathway [42]. This may be linked to MZ B cells with the stimulation of GtfB can proliferate and generate anti-dsDNA antibodies. The evidence of this mechanism has to be further investigated. In addition to MZ B cells, B1 cells also have been reported to produce polyreactive IgM and IgA to defense the blood-borne pathogens in a fast

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manner [59, 60]. B1 cells are major reside in peritioneal and pleural cavities, they are also found in spleen. Moreover, B1 cells can become to phagocytes that produce antibodies [60, 61]. Therefore, B1 cells may play some roles in anti-dsDNA antibody production.

5.5 The role of L7/L12 in SLE patients

Anti-ribosomal antibodies can be detected in some SLE patients, and these patients have high titers of anti-dsDNA antibodies [62]. It has been reported that in naïve mice, anti-ribosomal P antibodies can induce nephritis, which occurs in 40-60% of SLE patients [63]. In addition, these antibodies were also found to accelerate the development of glomerulonephritis in lupus prone mice [64]. L7/L12 is a ribosomal 50S protein and present in two forms: L7 has aminoacylated N-terminal serine, while L12 has a free N-terminus [65, 66]. It is similar to our findings that anti-L7/L12 antibodies were detected in SLE patients (Figure 8). However, although some SLE patients have anti-ribosomal antibodies contained high titers of anti-dsDNA antibodies [62], our data showed anti-L7/L12 antibodies were not related to anti-dsDNA antibodies in SLE patients, suggesting that L7/L12 may be a inducer of cross-reactive antibodies in SLE patients (Figure 10). Although anti-L7/L12

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antibodies did not correlate with anti-dsDNA antibodies, it could be a marker for the diagnosis of SLE.

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Chapter 7. Table

Table. Autoantibody production in patients with infective endocarditis and other disseminated systemic infections

	Male-to-female ratio	Age	Anti-dsDNA no.(%)	APL (Antiphospholipid antibodies)			APL no.(%)	APL+ anti-dsDNA no.(%)
				AntiCardiolipin IgG-no.(%)	AntiCardiolipin IgM-no.(%)	Antib2-Glycoprotein I IgG-no.(%)		
All disseminated systemic infection (n=50)	1.63	53.4 ± 20.7	13 (26)	22 (44)	23 (46)	10 (20)	27 (54)	30 (60)
IE (n=21)	2	52.4 ± 22.1	5 (23.8)	9 (42.1)	9 (42.8)	5 (23.8)	11 (52.3)	12 (57.1)
other disseminated systemic infection (n=29)	1.23	54.2 ± 19.9	8 (27.5)	13 (44.8)	14 (48.2)	5 (17.2)	16 (55.1)	18 (62)

From National Taiwan University Hospital

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Chapter 8. Figures

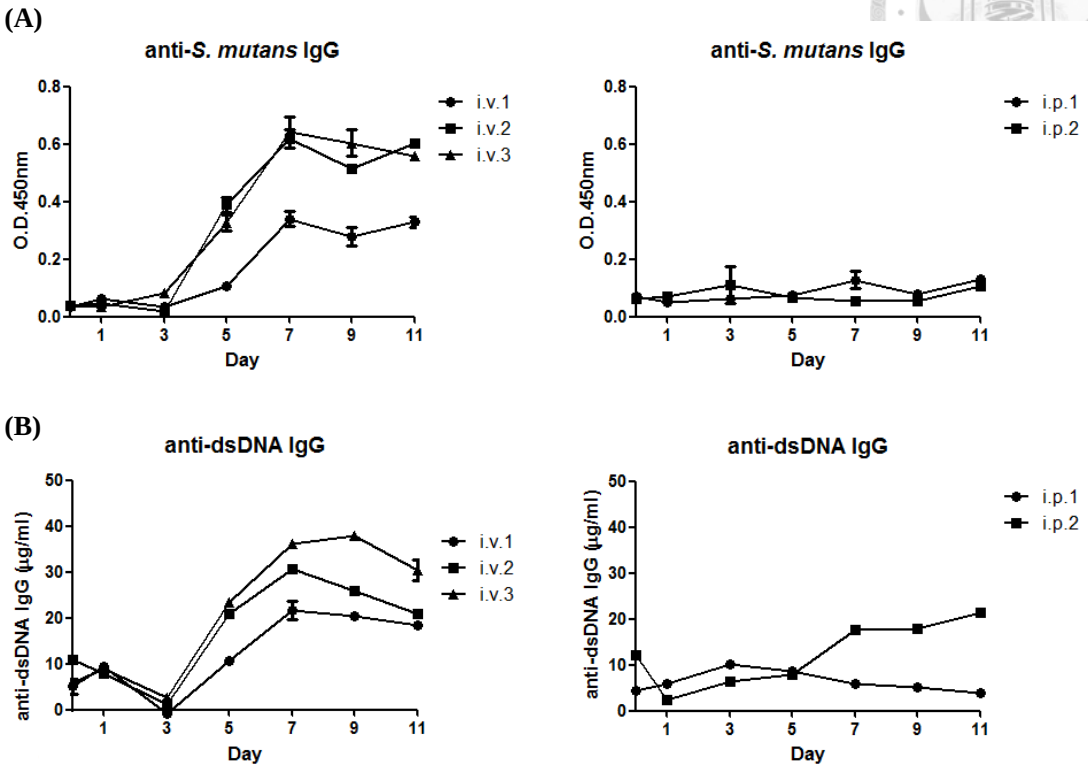

Figure 1. Antibody production in mice intravenously or intraperitoneally infected with *S. mutans*

BALB/c mice were received intravenous (i.v.) or intraperitoneal (i.p.) infection with 1×10^9 C.F.U./100μl *S. mutans*. The sera were collected and the production of anti-*S. mutans* (A) and anti-dsDNAs IgG (B) was measured by ELISA.

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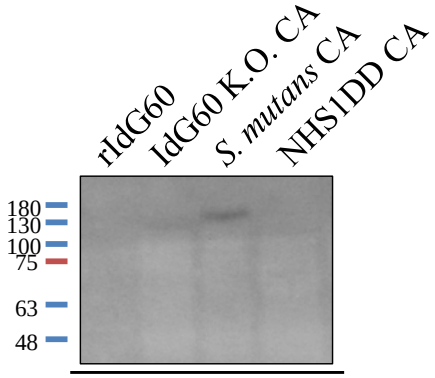
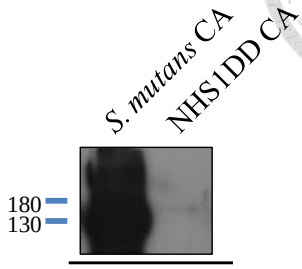
(A)

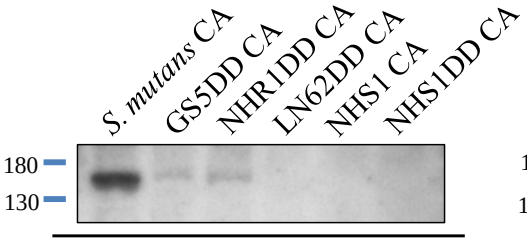


Elute anti-dsDNA Ab
(*S. mutans*)

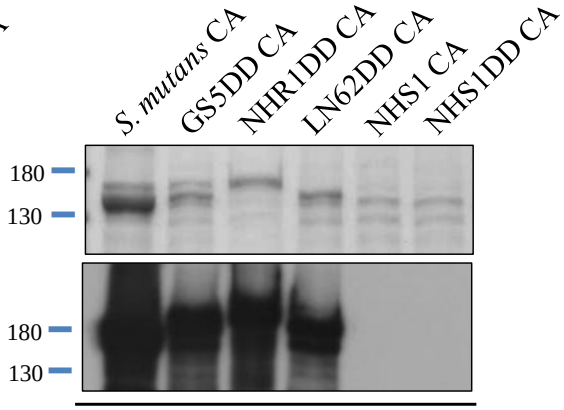


Rabbit anti-GtfB/C serum

(B)



Elute anti-dsDNA Ab
(*S. mutans*)



anti-GtfB/C serum

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Figure 2. The reactivity of eluted anti-dsDNA antibodies to *S. mutans* proteins

(A)Left panel: Eluted anti-dsDNA antibodies from *S. mutans* infected mice were probed with purified IdG60 protein, the CA proteins of IdG60 knockout (K.O.), *S. mutans* or NHS1DD (left to right). Right panel: Anti-GtfB/C serum was used as control. (B) Left panel: To identify the anti-180 kDa protein, the eluted anti-dsDNA antibodies probed with isogenic mutant strains, including GS5DD, NHR1DD, LN62DD, NHS1, NHS1DD, which is deficient in GtfD, GtfC/D, GtfB/D, GtfB/C or GtfB/C/D, respectively, were used for western blotting analysis. Right panel: Anti-GtfB/C serum was used as control.

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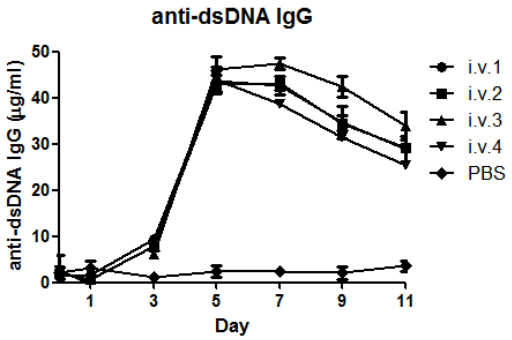
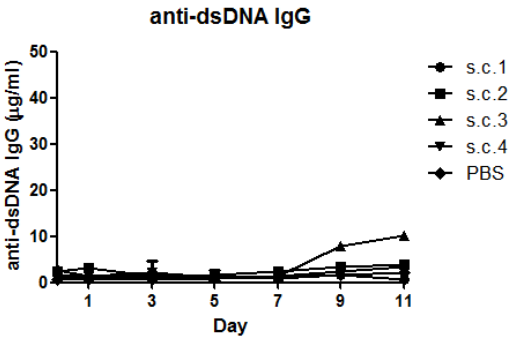
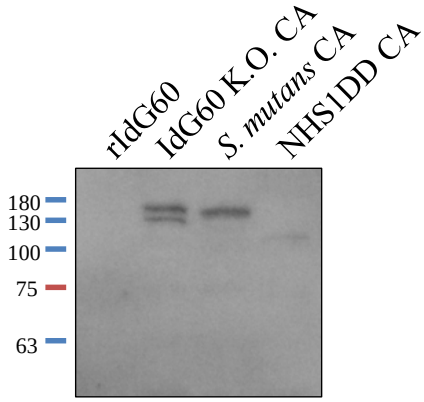
(A)



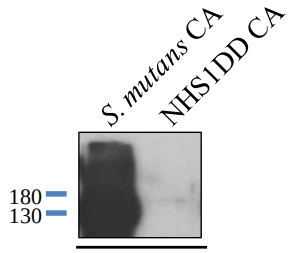
(B)



(C)

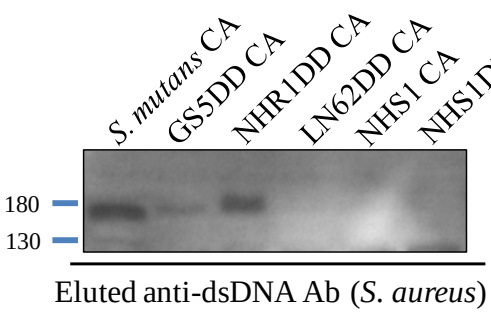


Eluted anti-dsDNA Ab (*S. aureus*)

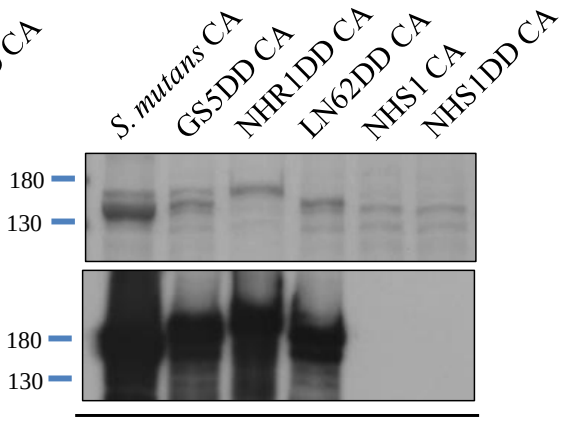


anti-GtfB/C serum

(D)



Eluted anti-dsDNA Ab (*S. aureus*)



anti-GtfB/C serum

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Figure 3. The anti-dsDNA antibody production in the mice infected with *S. aureus*

Anti-dsDNA IgG in sera from (A) intravenously or (B) subcutaneously infected mice were measured by ELISA. (C) Left panel: The eluted anti-dsDNA antibodies (Abs) from *S. aureus* infected mouse were probed with purified IdG60 protein and the CA proteins of IdG60 K.O. mutant, *S. mutans*, NHS1DD (Left to right). Right panel: anti-GtfB/C serum was used as control. (D) Left panel: The eluted anti-dsDNA antibodies from *S. aureus* infected mouse were probed with the CA proteins of *S. mutans*, GS5DD, NHR1DD, LN62DD, NHS1, NHS1DD (Left to right). Right panel: coomassie blue staining (upper panel), anti-GtfB/C serum as control (lower panel).

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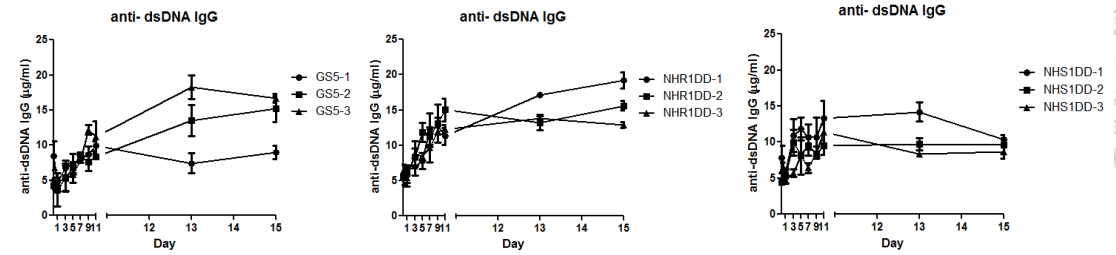
(A)



(B)

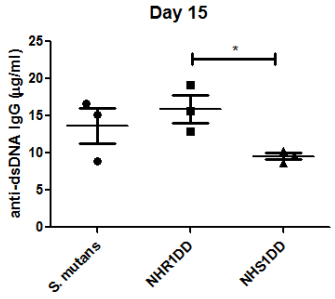


Figure 4. The anti-dsDNA antibody production in the mice infected with *S. mutans*, NHR1DD or NHS1DD

Mice were intravenously infected with 1×10^9 C.F.U./100μl *S. mutans*, NHR1DD or NHS1DD. The sera were collected and the production of anti-dsDNAs IgG (A) was measured by ELISA. (B) The anti-dsDNA IgG production after 15 days infection was presented as mean ± SEM.

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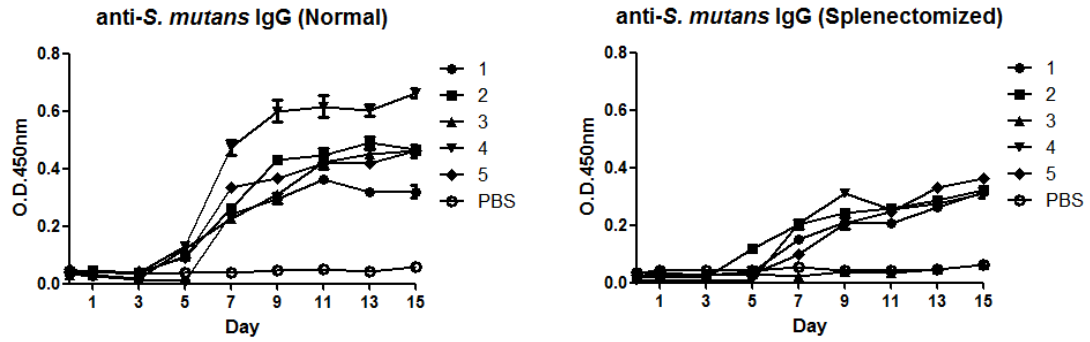
(A)



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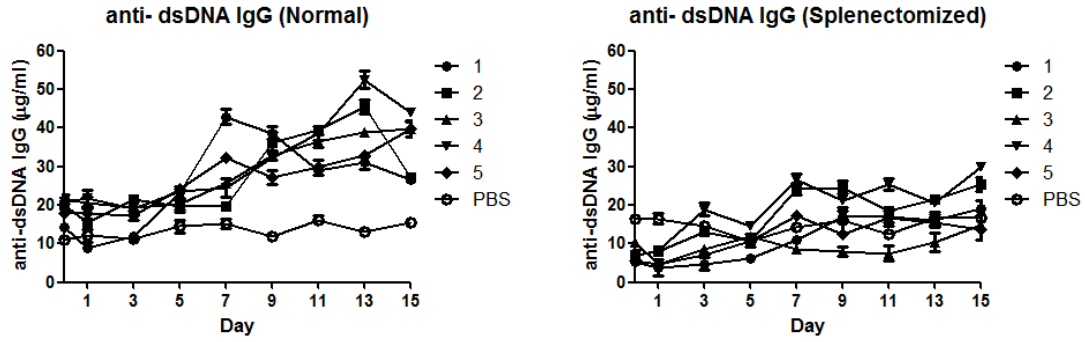

Figure 5. Spleen may play roles in anti-bacterial and anti-dsDNA antibody production in mouse model

(A) Anti- *S. mutans* IgG and (B) Anti- dsDNA IgG in sera from normal or splenectomized mice intravenously injected with *S. mutans* was measured by ELISA.

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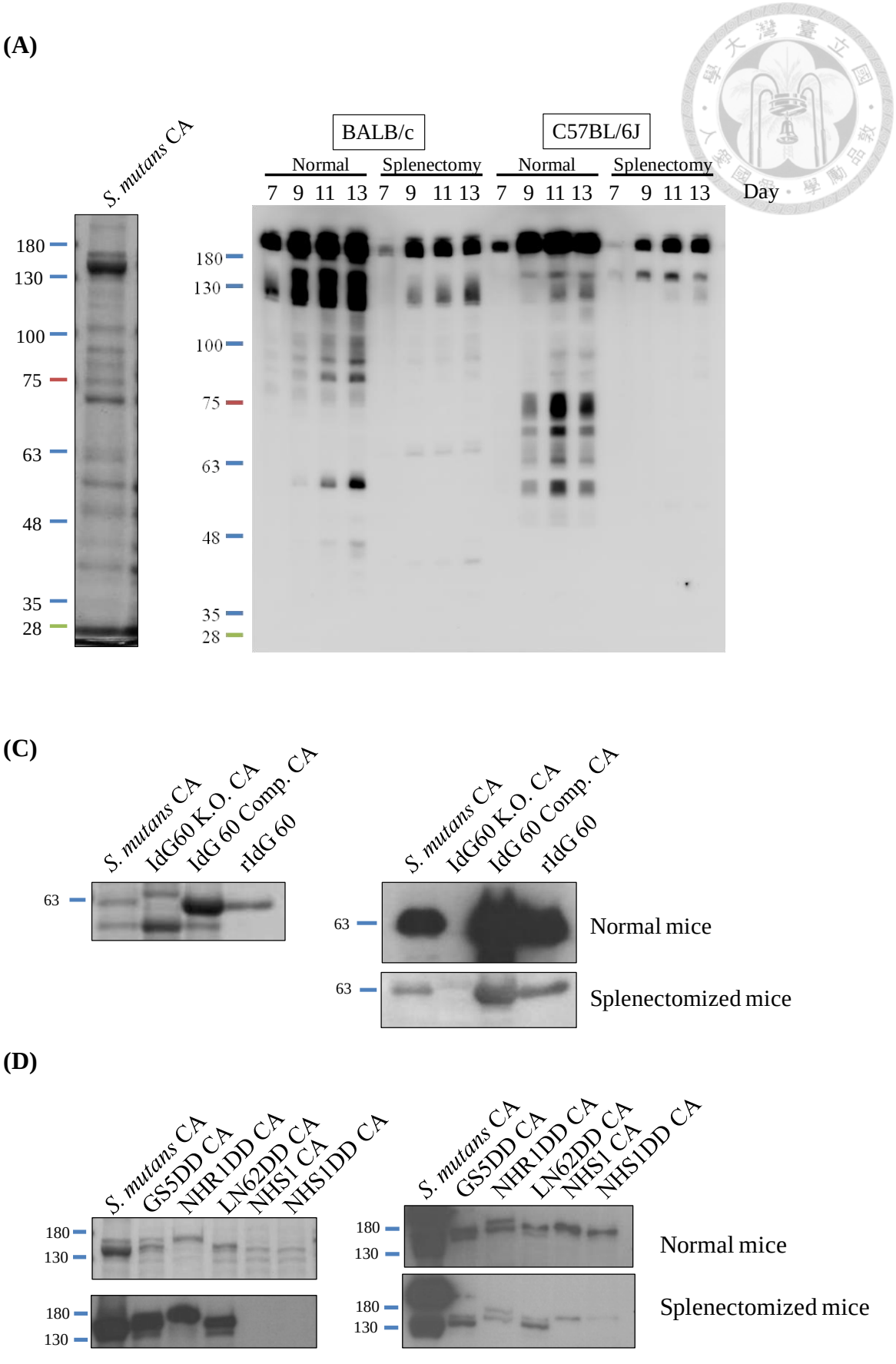

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Figure 6. The antibody production in the normal and splenectomized mice

(A) The pattern of *S. mutans* CA protein was stained by coomassie blue. (B) The sera from either normal or splenectomized mice intravenously injected with *S. mutans* in different days after infection were probed with *S. mutans* CA proteins (C) The sera from either normal or splenectomized mice intravenously injected with *S. mutans* probed with various isogenic mutants of *S.mutans*, including GS5DD, NHR1DD, LN62DD, NHS1, and NHS1DD, which are deficient in GtfD, GtfC/D, GtfB/D, GtfB/C, and GtfB/C/D, respectively. Left panel: Coomassie blue staining (upper panel), anti-GtfB/C serum as control (lower panel). Right panel: western blots analysis. (D) Left panel: coomassie blue staining as control. Right panel: western blots analysis. The CA proteins of *S. mutans*, IdG60 knockout (K.O.) strain, IdG60 complement (Comp.) strain, and the IdG60 purified protein were probed with the sera of normal (upper panel) or sp patientslenectomized (lower panel) mice.

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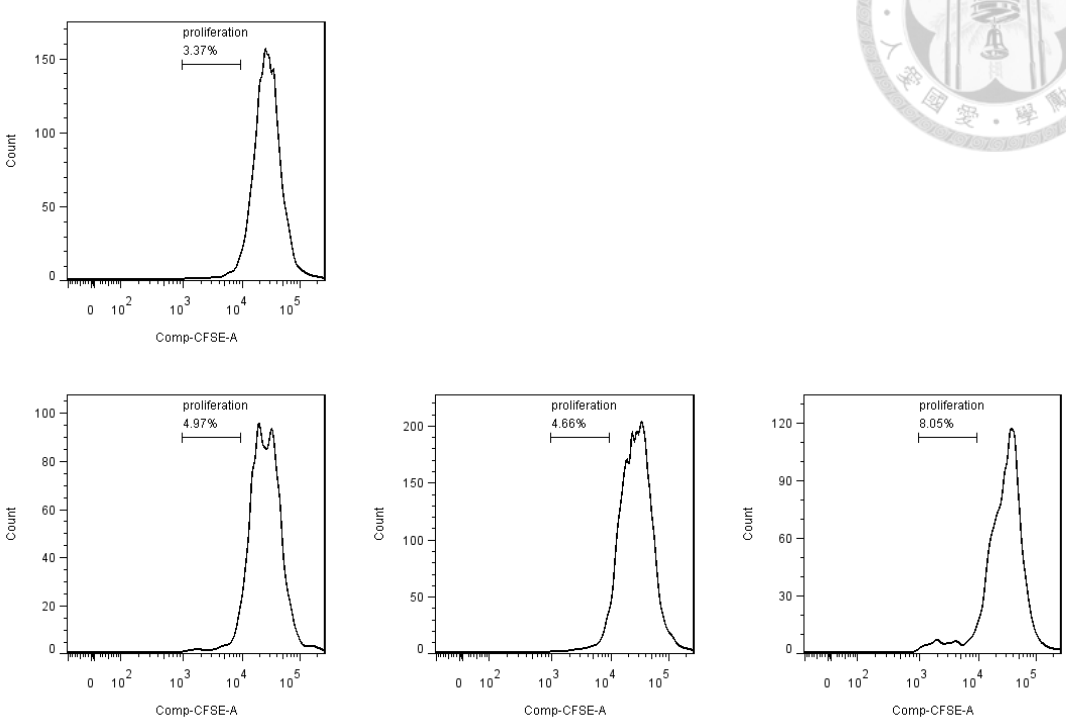
(A)



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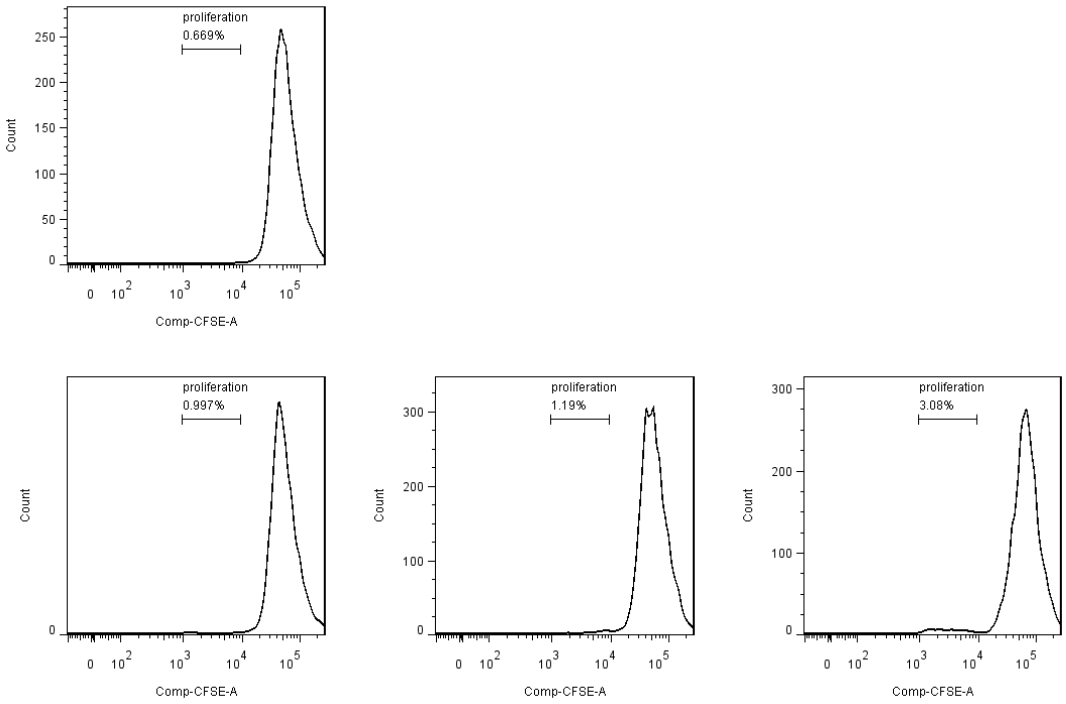

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Figure 7. The stimulation of MZ B and FO B cells by using streptococcal proteins

The MZ B and FO B cells were purified from BALB/*c* mouse by sorting, these cells were stained with CFSE to detect cell proliferation. MZ B and FO B cells were cultured in 96 wells for 3 days (1×10^5 cells/200μl). And 100 μg/ml *S. mutans*, NHS1DD and NHR1DD CA proteins were used to stimulate either MZ B or FO B cells for 3 days. NHS1DD and NHR1DD are isogenic mutant strains of *S. mutans*, which is deficient in GtfB/C/D and GtfC/D, respectively. Three days later, the proliferation of (A) MZ B and (B) FO B cells was analyzed by flow cytometry.

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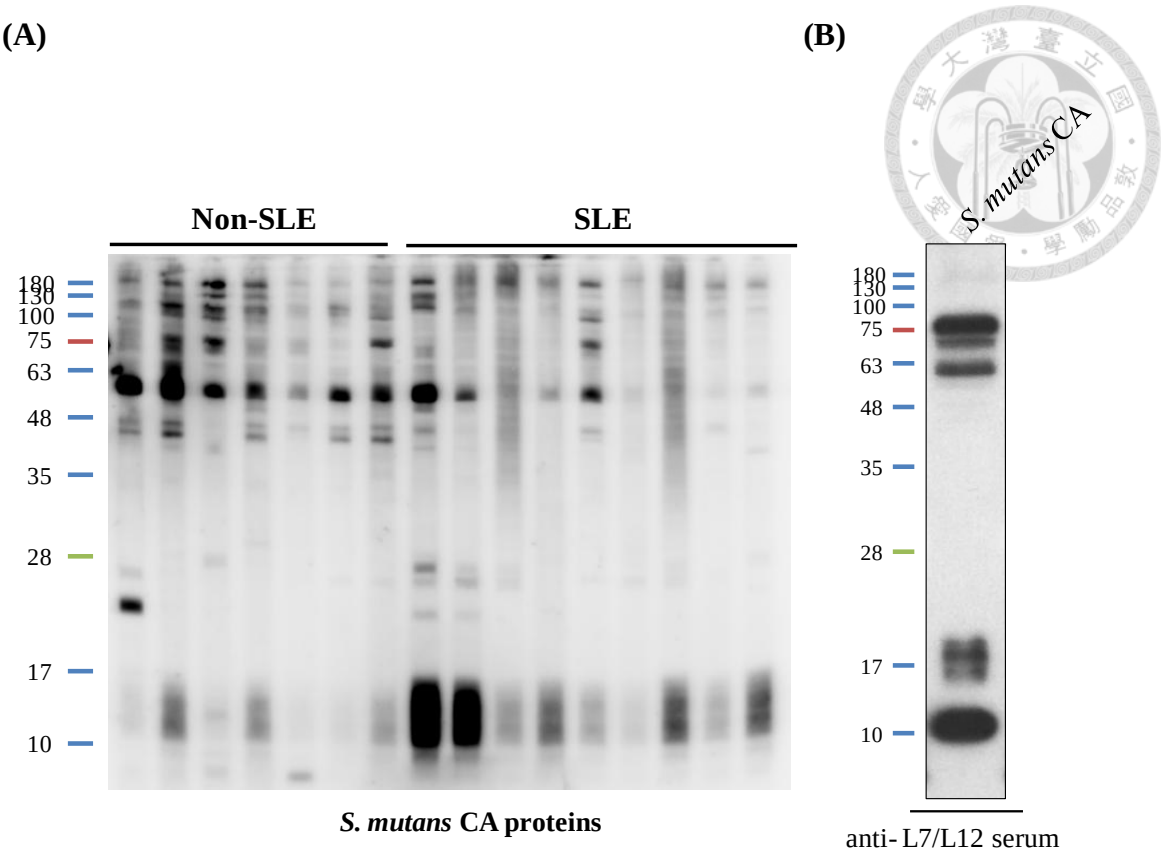

Figure 8. The role of the bacterial ribosomal protein, L7/L12, in SLE

(A) The pattern of sera from non-SLE people (n=7) and SLE patients (n=9) against *S. mutans* CA proteins was analyzed by western blots. (B) The *S. mutans* CA protein was probed with serum from L7/L12 immunized rat.

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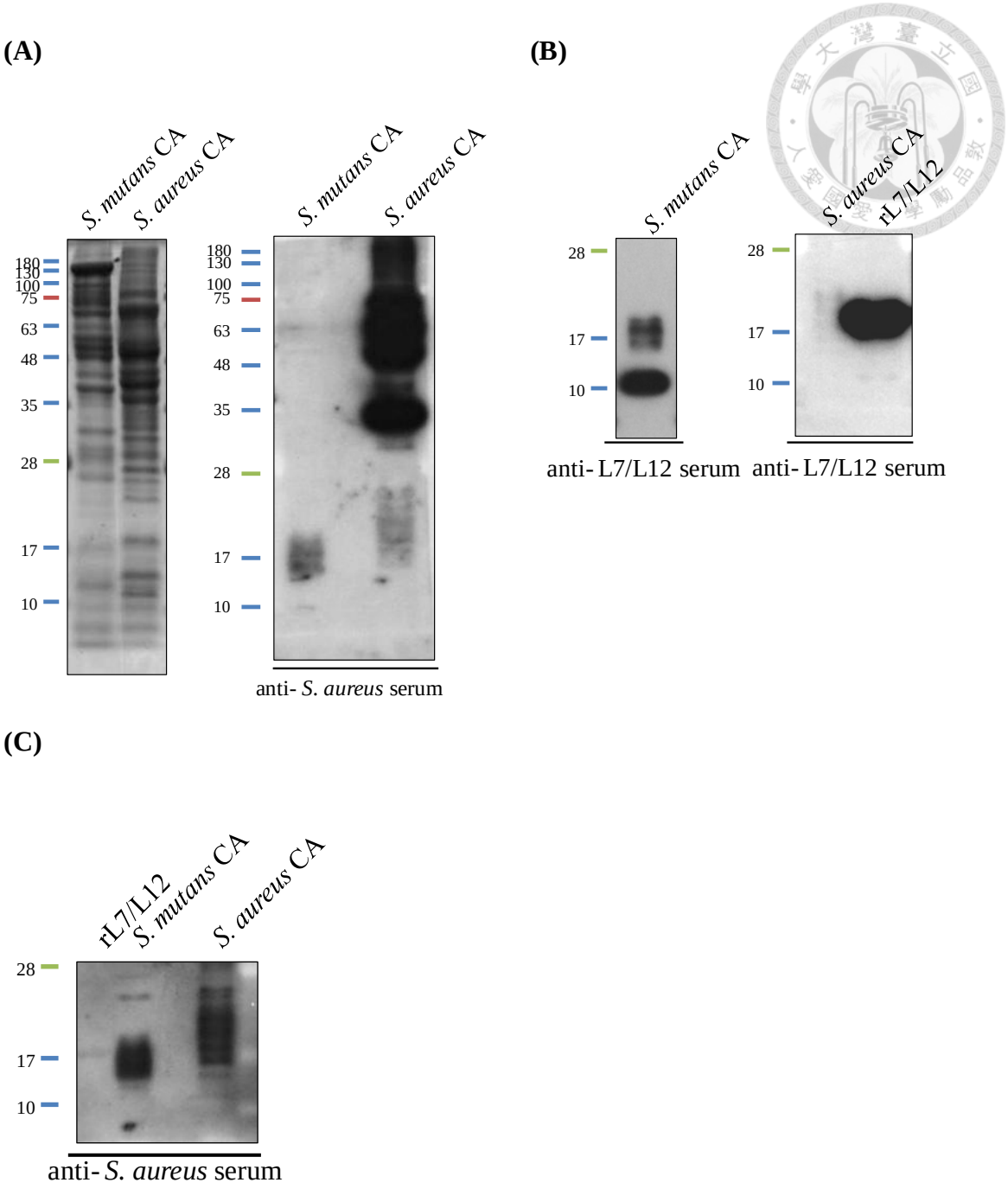

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Figure 9. The role of the bacterial ribosomal protein, L7/L12, in SLE

(A) Left panel: The CA proteins of *S. mutans* and *S. aureus* were stained by coomassie blue. Right panel: The serum of *S. aureus* infected mouse was probed with *S. mutans* or *S. aureus* CA proteins. (B) Left panel: The CA proteins of *S. mutans* were probed with anti-L7/L12 serum. Right panel: The CA proteins of *S. aureus* and purified L7/L12 proteins (rL7/L12) were probed with anti-L7/L12 serum. (C) The serum of *S. aureus* infected mouse was probed with L7/L12 purified protein (rL7/L12), *S. mutans* or *S. aureus* CA proteins.

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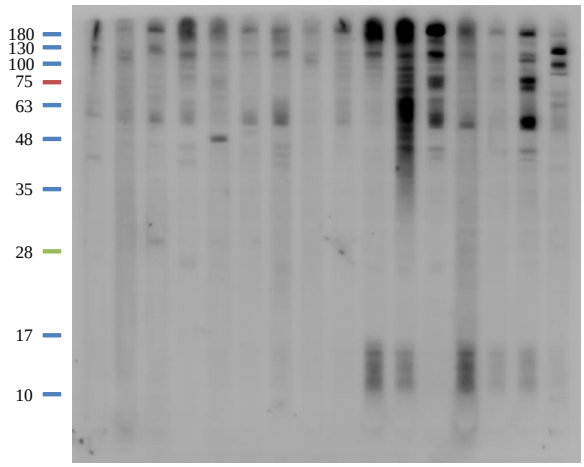
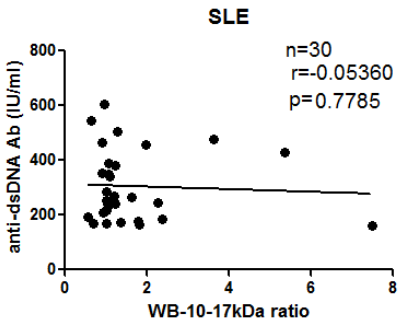
(A)



S. mutans CA proteins

(B)



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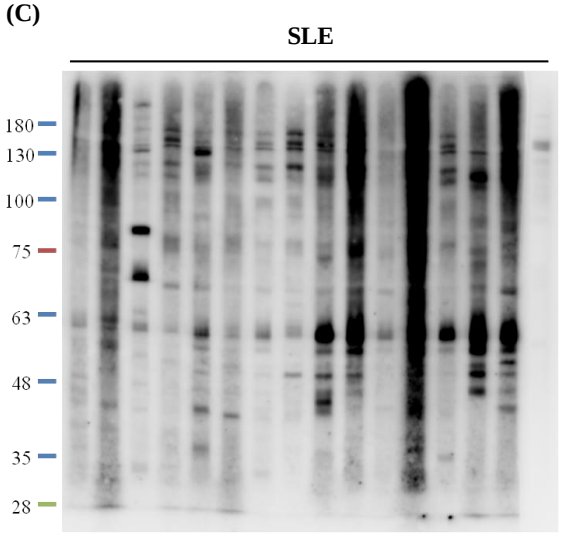

NHR1DD CA proteins

(D)

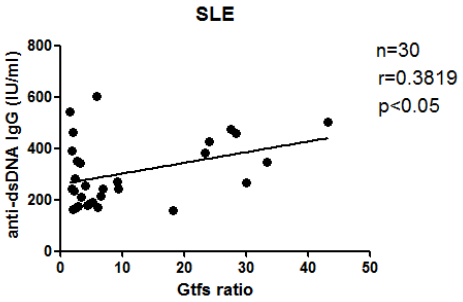

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Figure 10. The correlation of anti-dsDNA antibodies and anti-bacterial antibodies in SLE patients

(A) Western blotting analysis on sera from SLE patients (n=16) against *S. mutans* CA proteins. (B) The correlation between anti-dsDNA antibodies (IU/ml) and 10-17 kDa ratio in SLE patients (n=30). (C) Western blotting analysis on sera from SLE patients (n=16) against NHR1DD CA proteins. (D) The correlation between anti-dsDNA antibodies (IU/ml) and Gtfs ratio in SLE patients (n=30).

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