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Formation of kidney specific tertiary lymphoid structures during the initiation of anti-dsDNA antibodies and development of lupus nephritis-the role of IL-7 and IL-7R

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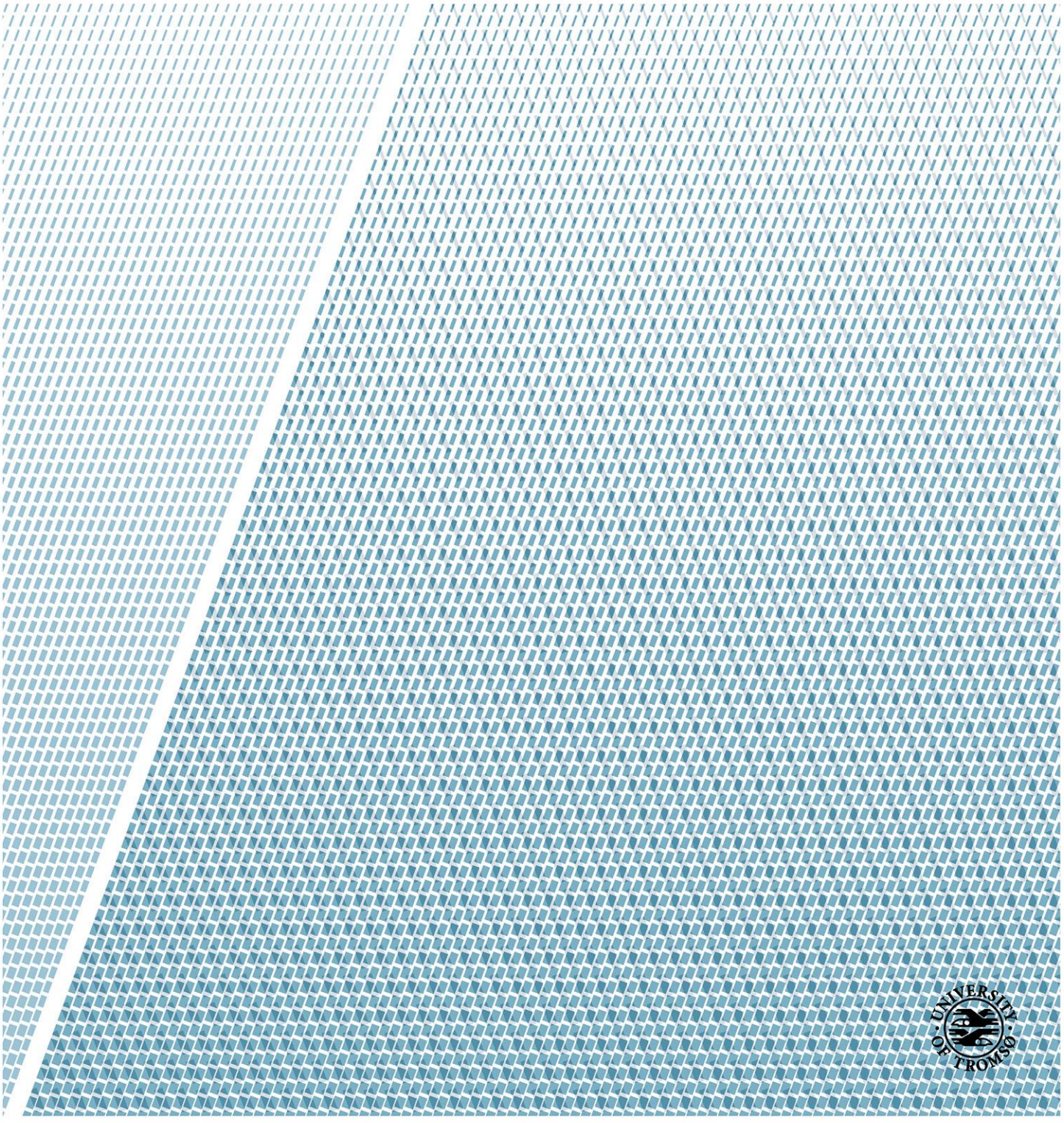

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Abbreviations

Ab	Antibody
Anti ds-DNA	Anti double stranded DNA
ct-DNA	calf thymys-DNA
EDTA	Ethylenediaminetetraacetic acid
ELISA	Ezyme linked immunosorbent assay
FAM	Fluorochrome, 6-carboxyfluorescein
IL-1β	Interleukin-1β
IL-7	Interleukin-7
IL-7R	Interleukin-7 receptor
LN	Lymph node
SLE	Systemic lupus erythematosus
TBP	TATA-binding protein
TLS	Tertiary lymphoid structures
TNFα	Tumor Necrosis Factor α
Wo	Weeks old

Abstract

Tertiary lymphoid structures are accumulation of lymphocytic cells that may occur in many organs. These structures bear a resemblance to spleen and lymph nodes which are so called secondary lymphoid organs. The formation of such structures can aggravate diseases. These structures have been shown to occur in the kidney when anti-dsDNA antibodies are produced. The development of TLS seems to occur early in the disease before the development of lupus nephritis.

In this study the expression of IL7 and IL7R in the kidney of (NZBxNZW) F1 mice and expression in spleen and LNs were examined using different methods like ELISA, immunoblotting and gene expression analysis.

The results show that there is some difference in expression between the organs and the different age groups of the mice.

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1 Introduction

1.1 Systemic lupus erythematosus

Systemic lupus erythematosus **(**SLE) is an autoimmune disease that may affect multiple organs and systems, the most serious being effects on the brain and kidneys.(1) SLE can affect both sexes in all ages but is most common in females of childbearing age. There is also a higher frequency of SLE among ethnic groups like African, Asian or Hispanic.(2) The immune system produces autoantibodies against self-antigens and that leads to attacks against the body's own tissue. In SLE the autoantibody is against DNA, RNA, nuclear proteins (histones).(3) The anti-dsDNA will form immune complexes that bind to organs. These immune complexes cause inflammation and tissue damage.(4) SLE is diagnosed according to 11 criteria's from the American College of Rheumatology, table 1.1. Four or more of the eleven criteria's has to be met to be able to set a diagnosis.(5) The criteria's have to be present serially or simultaneously.(6)

Table 1.1: American College of Rheumatology Criteria for Classification of Systemic Lupus Erythematosus(7)

1. Malar Rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician
5. Nonerosive arthritis	Involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Pleuritis or pericarditis	1. Pleuritis--convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion ***OR*** 2. Pericarditis--documented by electrocardigram or rub or evidence of pericardial effusion
7. Renal disorder	1. Persistent proteinuria > 0.5 grams per day or > than 3+ if quantitation not performed ***OR*** 2. Cellular casts--may be red cell, hemoglobin, granular, tubular, or mixed
8. Neurologic disorder	1. Seizures--in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance ***OR*** 2. Psychosis--in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance
9. Hematologic disorder	1. Hemolytic anemia--with reticulocytosis ***OR*** 2. Leukopenia--< 4,000/mm^3 on ≥ 2 occasions ***OR*** 3. Lyphopenia--< 1,500/ mm^3 on ≥ 2 occasions ***OR*** 4. Thrombocytopenia--<100,000/ mm^3 in the absence of offending drugs
10. Immunologic disorder	1. Anti-DNA: antibody to native DNA in abnormal titer ***OR*** 2. Anti-Sm: presence of antibody to Sm nuclear antigen ***OR*** 3. Positive finding of antiphospholipid antibodies on: 1. an abnormal serum level of IgG or IgM anticardiolipin antibodies, 2. a positive test result for lupus anticoagulant using a standard method, or 3. a false-positive test result for at least 6 months confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test
11. Positive antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs

1.2 Lupus nephritis

Lupus nephritis is a serious clinical manifestation of SLE. It is caused by immune complexes that are bound to the glomerular basement membranes (GBM) and in the mesangial matrix (MM).(8) This will cause inflammation in the glomeruli. The glomeruli are composed of arterial capillaries that filtrates the blood for ions and other molecules. The inflammation will cause a reduction of the filtration of ions and molecules. Damage to the glomerulus can cause leaking of albumin into the urine, this is known as proteinuria.(9) At this stage the damage to the glomerulus can be severe and really difficult to treat. Chronic inflammation in the kidneys can also lead to development of tertiary lymphoid structures.(10) The International Society of Nephrology (INS) and the Renal Pathology Society made in 2003 an improvement of the modified 1982 WHO classification of LN. It is used to aid renal biopsy interpretation and pathological studies.(11)

Table 1.2: ISN/RPS (2003) classification of LN(11)

Class I	Minimal mesengial LN
Class II	Mesangial proliferative LN
Class III	Focal LN 1.Active lesions 2. Active and chronic lesions 3. Chronic lesions
Class IV	Diffuse LN Diffuse segmental or global LN with: 1.Active lesions 2. Active and chronic lesions 3. Chronic lesions
Class V	Membranous LN
Class VI	Advanced sclerosing LN

1.3 Tertiary lymphoid structures

Tertiary lymphoid structures or organs are accumulation of ectopic lymphoid cells by the process of lymphoid neogenesis in chronic inflamed tissue.(12)

Inflammatory cytokine expression, stromal cells producing lymphoid chemokines and development of high endothelial venues (HEVs) promote the TLS formation. The TLS are very similar to lymph nodes (LNs), which is a secondary lymphoid organ (SLO). Both the TLS and LNs contain HEVs, lymph vessels (LV), T lymphocytes, B lymphocytes, dendritic cells (DCs) and follicular dendritic cells.(FDC)(13)

SLO is developed by upregulation of CXCL13 chemokine that will attract lymphoid tissue inducer (LTi) cells. These LTi cells will express lymphoid tissue β (LTβ) cells and make the local fibroblasts to become lymphoid tissue organizer (LTo) cells. The LTo cells start producing more CXCL13 chemokines that will attract B lymphocytes. CCL19 and CCL21 chemokines, which will attract T lymphocytes and DCs. The fibroblasts will upregulate cell adhesion molecules like the intracellular adhesion molecule 1(ICAM1) and vascular cell-adhesion molecule 1(VCAM1). These adhesion molecules will make the LTis and T and B lymphocytes that were recruited by the chemokines to stick together. This results to an organized structure with separate zones for B and T lymphocytes. HEV, afferent and efferent lymph vessels are developed by local angiogenic growth factors.(14)

In TLS formation, LTαβ expression will promote CXCL13 and CCL21 expression. This will form a positive feedback loop that will increase the secretion of these chemokines. As in SLO development, the CXCL13 will attract B lymphocytes and CCL21 will attract DCs.(15) This leads to the separate zones with B and T lymphocytes, HEVs, stromal cells and the chemokine CCL21.(16) The difference between TLS and SLO is that TLS are in non-lymphoid organs such as the kidneys. TLS are unlike the LNs not encapsulated and are not connected to an afferent lymph vessel.(17, 18)

1.4 Interleukin 7 and interleukin 7 receptor

Interleukin 7 (IL-7) is a cytokine responsible for T and B lymphocyte development and maintaining the survival of T lymphocytes. IL-7 is expressed in the stromal cells of primary and secondary lymphoid organs like the thymus, bone marrow, or spleen and lymph nodes, respectively. IL-7 can also be expressed in keratinocytes, hepatocytes and intestinal epithelial cells.(19). The stromal organizing cells VCAM1 and ICAM1 produce IL-7, LTi cells survival is then promoted by the stromal cell-derived IL-7.(20) As mentioned before, LTi is very important in SLO development. The common gamma chain (γc) and the interleukin 7 receptor α (IL-7Rα) are used by the IL-7 for signaling.(21) Interleukin 7 receptor (IL-7R) is composed by the γc and IL-7Rα. The γc is expressed on hematopoietic cell types while the IL-7Rα chain is mainly expressed by developing T, B lymphocytes and NK cells and mature T lymphocytes.(22) The γc chain of the IL-7R is also used by other cytokines like IL-2, IL4, IL-9, IL-15 and IL-21. (23). In other chronic inflammatory diseases like rheumatoid arthritis (RA) and Sjögrens syndrome (SS) there is noticeable expression of IL-7. Expression of IL-7, IL-7Rα and γc where increased in ectopic lymphoid follicle samples from RA patients.(22) There was also an increase of IL-7Rα in the synovial fluid of RA patients.(24). The increased levels of IL-7α leads to activation of T, B lymphocytes and macrophages which lead to inflammation in the joints.(24). Mice with IL-7 or IL-7Rα deficiency will have incomplete LNs and reduction of lymphoid cells,(25) since the lack of IL-7 will affect the LTi cell survival. Overexpression of IL-7 may on the other hand lead to development of TLS.(26)

1.5 Tumor Necrosis Factor α and Interleukin-1β

Tumor Necrosis Factor α (TNFα) is a proinflammatory cytokine and is produced by different cells like macrophages, monocytes, T lymphocytes, mast cells and LTi.(14, 27) Through ligand binding to the tumor necrosis factor receptor type 1 and type 2 (TNFR-1 and TNFR-II) biological effects of the TNFα are mediated.(28) TNFα will through the TNFR-I receptor initiate stimulation of proinflammatory genes and cell survival or apoptosis and cell death.(29) Overexpression of TNFα can lead to chronic inflammation and autoimmune disorders like RA.(30, 31) Lymphotoxin α (LT-α) and lymphotoxin β (LT- β) are along with TNFα part of the TNF superfamily that consist of at least 19 ligands and 29 receptors.(32) Interleukin-1β (IL-1 β) is also proinflammatory cytokine produced by monocytes and macrophages but can also be produced by T lymphocytes, B lymphocytes, natural killer cells and neutrophils.(33-35) It is a part of the interleukin-1 cytokine superfamily with interleukin-1α, interleukin receptor antagonist and interleukin-18.(35) (36)

2 Aim of the study

TLS may occur in many organs and the formation of such structures can aggravate diseases. These structures have been shown to occur in the kidney when anti-dsDNA antibodies are produced. This event seems to occur early in the disease before the development of lupus nephritis.

A major problem with kidney diseases is that there are not any good diagnostic assays that can detect changes in the kidney at an early stage. Once the patient gets proteinuria, the disease has gone too far and can be difficult to treat.

The aim of this study was to follow the expression of IL7 and IL7R in the kidney of (NZBxNZW) F1 mice and compare the expression in spleen and LN from the same mice

3 Material and methods

3.1 Animals and tissue samples

In this study kidneys, spleen, lymph nodes and sera from (NZBxNZW)F1 mice was used. The mice were obtained from Harlan (Oxon, England) and Jackson Laboratory (Bar Harbor, Maine, USA). Tissue samples used was preserved by snap freezing, in RNAlater solution and by formalin or zinc fixation in paraffin blocks. Sera samples used in ELISA were stored at -20°C

3.2 Enzyme-linked immunosorbent assay (ELISA)

Background

ELISA is a way to detect interaction between an antigen and the equivalent antibody. Antigen is attached to the wall of a multiwell plate and exposed to liquid with the suspected antibody, in this case sera from mice. The antibody will bind to the antigen and the leftover antibody will be washed away. After the addition of a secondary antibody and a substrate solution there will be a colour change. This change in colour can be measured at 492nm with an ELISA plate reader. The intensity of the colour reflects on how much antibody is bound to antigen.(37) ELISA is used to test urine or blood for viruses, microorganisms, infectious diseases or other autoimmune diseases. ELISA is mostly used as a screening tool as it can detect biomolecules at very low concentrations.(37)

Method

A 96 well plate (Nunc) was coated with calf thymus dsDNA (ctDNA)(D8661, Sigma-Aldrich, Saint Louis, USA) by adding 50µl of a 10mg/ml solution in PBS per well. The plate was sealed with a plastic cover and incubated overnight at 37ºC.
A new plate with two fold dilutions of the primary antibody (sera) was made the day after. See Table 3.1 for details on the dilution

The plate from the day before with the calf-thymus dsDNA was washed 3 times with Wellwash 4 (Labsystems) with PBST 0.05%

The primary antibody dilutions were added to the newly washed plate and incubated at 37°C for 1 hour and washed 3 times with Wellwash 4.

Secondary antibody (Anti-mouse IgM in a 1:2000 dilution in PBST 0.02%) was added to the washed plate from the step before and incubated at 37°C for 1 hour and washed 3 times with Wellwash 4

A 0.2M NaPhosphate buffer and a 0.1M citric acid buffer were made.

The two buffers were mixed with each other and dH_2O to make the phosphate-citric-buffer. The pH was adjusted to 5.0.

The substrate solution was made by mixing 6ml phosphate-citric-buffer pH 5.0 with a 4mg tablet of O-phenylenediamine dihydrochloride and 5µl H_2O_2 under the hood

50µL substrate solution was added to the wells and incubated for 6 minutes.

Reaction was stopped with 25µL stop solution and the plate was measured with an ELISA plate reader.

Table 3.1: 1:100 dilution followed by two fold dilutions of the primary antibody (sera)

Sample	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200	1:102400
Week 20	120µl= 119µl PBST 0.02% + 1µl serum	60µl PBST 0.02% +60µl of 1:100	60µl PBST 0.02% +60µl of 1:200	60µl PBST 0.02% +60µl of 1:400	60µl PBST 0.02% +60µl of 1:800	60µl PBST 0.02% +60µl of 1:1600	60µl PBST 0.02% +60µl of 1:3200	60µl PBST 0.02% +60µl of 1:6400	60µl PBST 0.02% +60µl of 1:12800	60µl PBST 0.02% +60µl of 1:25600	60µl PBST 0.02% +60µl of 1:51200
Week 21	~	~	~	~	~	~	~	~	~	~	~
Week 22	~	~	~	~	~	~	~	~	~	~	~
Week 23	~	~	~	~	~	~	~	~	~	~	~
Week 25	~	~	~	~	~	~	~	~	~	~	~
Week 24	~	~	~	~	~	~	~	~	~	~	~
Positive control	120µl= 119µl PBST 0.02% + 1µl positive control	60µl PBST 0.02% +60µl of 1:100	60µl PBST 0.02% +60µl of 1:200	60µl PBST 0.02% +60µl of 1:400	60µl PBST 0.02% +60µl of 1:800	60µl PBST 0.02% +60µl of 1:1600	60µl PBST 0.02% +60µl of 1:3200	60µl PBST 0.02% +60µl of 1:6400	60µl PBST 0.02% +60µl of 1:12800	60µl PBST 0.02% +60µl of 1:25600	60µl PBST 0.02% +60µl of 1:51200
Negative control 0.02 % PBST	120µl PBST 0.02%	60µl PBST 0.02%	60µl PBST 0.02%	60µl PBST 0.02%	60µl PBST 0.02%	60µl PBST 0.02%	60µl PBST 0.02%	60µl PBST 0.02%	60µl PBST 0.02%	60µl PBST 0.02%	120µl PBST 0.02%

Buffers and solutions

0.2M NaPhosphate buffer:
3.56g of Na_2HPO_4-dihydrate in 100ml dH_2O

0.1M Citric acid buffer:
2.1g of citric acid monohydrate in 100ml dH_2O

100 ml Phosphate-citric-buffer:
25.7ml of 0,2M NaPhosphate buffer
24.3ml of 0,1M Citric acid buffer
50ml dH_2O
pH 5.0

Substrate solution:

6mL Phosphate-citric-buffer

4mg O-phenylenediamine dihydrochloride

5µl H_2O_2

Stop solution 3M HCl

3.3 Protein isolation, SDS-PAGE and Immunoblotting:

Background

To be able to extract proteins from tissue, the cell membranes have to be physically disrupted by force with the help of beads in a tube and by using detergents (NP-40 and Triton X-100) The lipid barrier surrounding the cell membrane will be broken by the detergents. This is called cell lysis. A buffer called RIPA buffer, contains the detergents and a chelating agent. The chelating agent (EDTA) will bind the metal ions in the lysate while the detergents will solubilize the proteins.(38) By binding metal ions, the metal-dependent protease will be inhibited. Protease inhibitor cocktail has to be used to inhibit other protease reactions in the lysate to prevent denaturation.(39)
The protein concentration is measured by using the Direct Detect® Spectrometer from Merck Millipore. The instrument uses IR spectroscopy to measure amide bonds in protein chains. The amide bonds have a really strong bond at the 1600-1690cm^{-1} area of the IR spectrum which absorbs electromagnetic radiation. The instrument will measure the peak height of the Amine I bond in that area.

A small chip with four spots will be used. One spot will be a blank with only the buffer and the three other spots will have the lysate samples.
By measuring the intensity of Amide I peak and subtracting the signal from the buffer (from the blank spot in the chip) in that same area of the spectrum, an accurate measurement of the protein concentration is calculated. (40)
SDS-PAGE is a very common technique used to separate proteins according to their molecular weight. The samples are prepared by mixing them with LDS sample buffer and reducing agent. The LDS sample buffer contains glycerol which will make the samples sink faster in the wells of the gel. The reducing agent will keep the samples in a reduced state after heating.
LDS or lithium dodecyl sulfate is a detergent that will remove the tertiary and secondary protein structures. LDS will also give the proteins a negative electrical charge. The negatively charged proteins will migrate through an electrical field to the positively charged anode.(41)
The method from the manufacturer's manual (NuPAGE Technical Guide, Invitrogen) was used.
During the western blot the proteins from the gel will be transferred onto a membrane with the help of an electrical field. The proteins still have a negative charge from the LDS and will move towards the positively charged electrode. The membrane that is between the gel and the positively charged anode will stop the proteins from moving any further by binding them.
After that the transfer is done, the membrane is blocked by using a buffer with non-fat milk. The blocking will prevent that the primary and secondary antibodies will bind to non-specific locations of the membrane since the milk proteins will bind to those.
Primary antibody will bind to the specific proteins and the secondary antibodies will bind to the primary antibody. The secondary antibody should be HRP-conjugated.(41) A special detection reagent will react with the HRP and the reaction will generate light emission. With the use of ImageQuant LAS 3000 the light emission can be detected and photographed.

Method:

A slice of snap frozen spleen or kidney from mice was put in tubes filled with ceramic beads (MagNa Lyser Green Beads) and 300µl of the RIPA buffer and protease inhibitor solution. The slices were about 2 mm thin.
The samples were homogenized at 6000rpm for 30 seconds in 2 turns. The homogenate was added to Lo Bind Protein tube and centrifuged at 12000 rpm for 20minutes at 4°C. The

supernatant which contains the proteins was saved in Lo Protein tubes. Concentration of the proteins was measured with Direct Detect Spectrometer

The samples were mixed with 5µl NuPAGE LDS Sample Buffer (4x), 2µl NuPAGE Reducing Agent (10x) from the manufacturer and H_2O and denatured at 70ºC for 10 minutes. The samples were then loaded on a NuPAGE Novex Bis-Tris Mini gel with 15 wells. 10µl See Blue Pre-stained standard (LC5925) and 5µl MagicMark XP was also loaded into the first well to be used as ladders. The gel was run at 200 V for 35min in an Xcell SureLock Mini-cell. Inner chamber was filled with 200ml running buffer and 500µl NuPAGE Antioxidant. Outer chamber was filled with 600ml running buffer.

The gel was transferred to a membrane using the manual from XCell II Blot Module, Invitrogen. The membrane was first submerged in methanol for 3 seconds, 10 seconds in Milli-Q water and at least 5 minutes in transfer buffer. The gel was put between a filter paper and the membrane and put into the blot module. The membrane and gel should be really close to each other for best result and to avoid bubbles.

After the western blotting the membrane got incubated one hour with 10ml blocking buffer. The membrane was then incubated overnight with primary antibody in a 1:500, 1:1000 or 1:2000 dilutions depending on the antibody.
The membrane was washed three times with 5 ml blocking buffer for 5 minutes.
After washing the membrane was incubated with secondary antibody for one hour in room temperature.
After one hour the membrane was washed with blocking buffer (5mlx3, 5 minutes) and PBST 0.05% (2x5 minutes) and incubated with SuperSignal West Pico Chemilumiescent Substrate for 5 minutes. ImageQuant LAS 4000 was used to take pictures of the membrane.

The primary antibody could also only be incubated for one hour in room temperature while the secondary antibody was incubated overnight.
It was also possible to incubate only in room temperature and skip the overnight step.

Buffers:

RIPA buffer (Radio Immunoprecipitation Assay buffer)
-150 mM sodium chloride
-1.0% NP-40
-0.25% Triton X-100
-1 mM EDTA (*Ethylene Diamine-Tetra-acetic Acid)*
-50 mM Tris, pH 7.4
Running buffer:

50mL 20X NuPAGE MES SDS Running Buffer
950mL Milli-Q water
1000mL

Transfer buffer:

NuPAGE Transfer Buffer (20x)	50mL
NuPAGE Antioxidant	1mL (if samples are reduced)
Methanol	100mL
Deionized water	849mL (850mL if NuPAGE Antioxidant is not used)
Total Volume	1000mL

Blocking buffer:
7.5g milk powder
150µL Tween-20
150mL PBS buffer

3.4 Immunohistochemistry

Background

Immunohistochemistry (IHC) is a method of staining tissue with antigens. It is based on the same principle as the ELISA that a specific antibody will recognize a specific antigen, and by binding to it form a complex that can be detected. In this case detection is by light microscopy. Sections of kidneys will be exposed to antibodies against specific antigens to see if there is any expression of these particular antigens in different groups of mice. Too see if there is more or less expression in sick or in young and healthy mice.

For antigen retrieval, the tissue has to be rehydrated, deparaffinized and by heating the samples up to 97°C in a PT-Link machine with a 10x citrate buffer. The heating will break the cross-linking between formalin and the proteins.(42, 43) If the cross-linkage is not broken the

recognition of antigens will be difficult. The cross-linkage may mask epitopes and thereby hinder the binding of antibody to the antigen.

Method

Samples fixated with formalin in paraffin blocks were cut using the microtome. Slices were 4µm thick and were collected from the warm water bath using an object glass. The sections were put in a rack and into a heating cabinet overnight at 60°C.

A 10 times diluted citrate buffer was prepared and poured into the well of the PT-Link machine. The machine was preheated to 65°C. (The heating step can be skipped if the tissue is fixated in Zink)

The rack with the sections was put in a container with xylene for 10 minutes, after 10 minutes the rack was put in another container with xylene for 10 minutes. Container with absolute alcohol for 2 minutes, container with absolute alcohol for 2 minutes, container with 96% ethanol for 2 minutes and container with 96% ethanol for 2 minutes.

The rack with the sections was then rinsed with running ion-exchanged water for 2 minutes.

The sections were put in the PT-Link machine and heated up to 97°C. When the machine cooled down to 65°C again the sections were taken out and put in a container with PBS buffer. The sections were blocked with 100µl 3% H_2O_2 for 10 minutes. After 10 minutes, the sections were rinsed with PBS buffer and put in a rack in a container with PBS buffer. There were three containers with PBS and the rack was in each container for 5 minutes. The sections were then blocked with 100µl goat serum (1:10 dilution with PBS) for 30 minutes.

After 30 minutes, the drop of goat serum was removed and the sections were incubated with primary antibody for 30 minutes. The primary antibody was diluted with goat serum. Dilution concentration depended on the producer's recommendation and on trituration experiment done beforehand. The sections were then rinsed with PBS buffer as before.

After rinsing, the sections were incubated with 100µl Labelled polymer Anti-Rabbit for 30 minutes and rinsed with PBS buffer afterwards.

The sections were taken into the closed hood and incubated with 50µl chromogen for 10 minutes. Sections were then rinsed with running ion-exchanged water for 30 minutes. To counterstain the sections, the rack was put in a container with haematoxylin for 30 seconds and rinsed afterwards in running ion-exchanged water until the red colour disappeared.

The rack was then put in a container with Scott's solution for 15 seconds and rinsed in running ion-exchanged water for 2 minutes. For the dehydration process the rack was dipped 10 times in containers with the following liquids: 96% ethanol, 96% ethanol, absolute alcohol, absolute alcohol, xylene and xylene. A drop of histokit was dripped on the section and covered with cover glass.

3.5 RNA Isolation, Quantification and Quality

Background:

In order to isolate total RNA the tissue has to be homogenized and lysed. A special lysis buffer containing phenol and guanidine-thiocyanate is used to prevent RNase. After centrifuging with chloroform, the proteins and DNA will be in the organic phase while the RNA is in the aqueous phase. Mixing ethanol with the aqueous phase will make the RNA bind easily to the silica-based membrane in the mini-column. Contaminants are washed away with different buffers while the RNA is bound to the silica-membrane. Low molecular weight RNA like 5.8S rRNA, 5S rRNA and tRNA are washed away while RNA molecules that are 200 nucleotides or longer are purified. The RNA is then eluted with RNAse-free water and stored.(44, 45)

Concentration is measured by using nanodrop. The concentration is calculated by using Beer-Lambert equation which is modified to use extinction coefficient with units of ng-cm/ml. Equation with the extinction coefficient: **c= (A*e)/b**. **A** is absorbance, **e** is the extinction coefficient and **b** is the path of length in cm and **c** is the concentration of the RNA in ng/ml. For RNA the extinction coefficient is 40ng-cm/µl.

The quality is measured by using the RNA Integrity Number (RIN) software algorithm from Agilent Technologies.

Method:

RNeasy Mini Kit from QIAGEN was used for the extraction of RNA with the protocol from MiRNeasy Mini Handbook (Purification of Total RNA, Including Small RNAs, from Animal Tissues)

700µl QIAzol Lysis Reagent from QIAGEN was added to MagNa Lyser Green Beads 2ml tubes. Samples stored in RNAlater were cut and weighed (≤25µg) and put it into the tubes. The tubes were put in a Precellys 24 homogenisator and homogenized at 6000 RPM for 30 seconds. Afterwards the tubes were put on ice while the homogenisator cooled down and homogenized once more at 6000 RPM for 30 seconds.

The homogenates were incubated at room temperature for 5 minutes and pipetted (600µl) into a new collection tube. Chloroform (120µl) was added to the tubes and shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes. The tubes were centrifuged at 12000g for 15 minutes at 4°C

The upper aqueous phases was transferred to new collection tubes and mixed with 365µl absolute alcohol. 580µl of the mixture was added into a RNeasy Mini column in a 2 ml collection tube. The tubes were centrifuged at 8000g for 30 seconds. The flow trough was discarded. 700µl Buffer RWT was added into each RNeasy Mini Column and centrifuged at 8000g for 30 seconds. The flow trough was discarded and 500µl Buffer RWT was added to the column tubes and centrifuged at 8000g for 30 seconds. The flow trough was once again discarded and the column tubes were filled with 500µl Buffer RPE and centrifuged for 2 minutes at 8000g. The columns were put into new 2 ml collection tubes and centrifuged at max speed (20238g) for 1 minute. The columns were transferred onto new 1.5 ml tubes. 50 µl RNAse-free water was pipetted directly onto the membrane inside the column and centrifuged at 8000g for 1 minute. The RNA samples were stored in a -80°C freezer for future use.

The RNA samples concentration were measured with NanoDrop ND-1000 (Thermo Scientific). The sampling arm of the instrument was in the open positon and a drop of RNA-free water was used as a standard. The drop of water was pipetted on the lower measurement pedestal. The sampling arm was closed and measurement was started using the software. The pedestal was wiped and a drop of RNA sample was pipetted onto the pedestal and spectral measured.

The quality of the RNA was measured with the Agilent 2100 Bioanalyzer system (Agilent Technologies) by using an Agilent RNA 6000 Nano chip(Agilent Technologies). The RNA dye concentrate was had to be in room temperature for 30 minutes before use. The RNA dye concentrate was vortexed for 10 seconds. 1 µl of the RNA dye concentrate was added into a 65µl aliquot of filtered gel. The solution was vortexed and the tube was spun down at 13000 g for 10 minutes at room temperature. The nano chip was put on a priming station and 9µl of

the gel-dye mix was pipetted into the well marked with G symbol. The plunger in the priming station has to be at 1ml position. The chip priming station is closed and the plunger was pressed until it was held by the clip. After 30 seconds the clip was released. After 5 seconds the plunger was slowly pulled back to the 1ml position. 9µl gel-dye mix as pipetted into a well marked with a different G symbol. 5µl of RNA marker is pipetted into all 12 sample wells and in the well with a ladder symbol. 1 µl of prepared ladder is pipetted into the well marked with the ladder symbol. 1µl sample is pipetted into all of the 12 sample wells. If some of the sample wells are not used, 1µl RNA marker is pipetted into those wells. The chip was put horizontally in the IKA vortexer and vortexed for 1 minute at 2400 rpm. The chip was then analyzed with the Agilent 2100 Bioanalyzer system.

3.6 cDNA synthesis

Background:

In order to synthesize a complementary DNA strand from RNA, a kit with reverse transcriptase, deoxynucleotide triphosphates (dTNPs), Reverse Transcription buffer and random primers will be used. The random primers will bind all along the RNA transcript, usually a primer would bind at the 3' end of the RNA. The reverse transcriptase will then with the dTNPs synthesize the cDNA strand, this will happen at 37°C for two hours. The reverse transcriptase is deactivated at 85°C.(46, 47)

Method:

RNA for the reverse transcription should be in the concentration range of 0.002-0.2µg/µl. High capacity cDNA Reverse Transcription kit (Applied Biosystems) was used. Components from the kit needed for the reverse transcription PCR master mix was calculated.

10x Reverse Transcription Buffer, 25x dNTPs, 10x random primers and Nuclease-free H_20 were mixed in a tube. MultiScribe Reverse Transcriptase was added last.

A master mix for the control was made without the reverse transcriptase (RT). The RT was replaced with RNase free water.

50µl of master mix was pipetted into a 0.5ml Eppendorf PCR tube. 50µl RNA sample was pipetted into the same tube and mixed by pipetting up and down twice.

Controls were made by pipetting 50µl master mix without RT into a PCR tube with 50µl RNA sample.

Another set of control was made without RNA sample/template. The template was replaced with RNase free water.

The tubes were centrifuged and stored on ice until loaded into the thermal cycler. The thermal cycler was programmed to: Step 1:25°C for 10 minutes. Step 2:37°C for 120 minutes Step 3:85°C for 5 seconds and forever on 4°C .The program name was: Archive. Reaction volume was set on 100µl. After the run of the program the cDNA was stored in -20°C

3.7 Gene expression analysis

Background:

Quantitative real-time Poleymerase Chain Reaction (qPCR) is a method for accurately quantifying targeted DNA molecules. The traditional PCR measures PCR product at the endpoint of amplification while qPCR measures PCR product after each round of amplification.

The PCR is done with the Taq polymerase, which is isolated from the thermophile bacteria species *Thermus aquaticus*. The temperature is then alternated to denature and renature DNA strands. The Taq polymerase will add complementary DNA strands with a primer.

The detection of fluorescent signal during amplification of DNA is done by the use of fluorescent labeling with a probe. At the 5’ end the probe is labeled with a reporter fluorochrome, 6-carboxyfluorescein (FAM). At the 3’ end there is a quencher. When the reporter and quencher dyes are in electronic exited state there will be a transfer of energy called fluorescent resonance energy transfer. This energy transfer is distance dependent where excitation is transferred without a discharge of photons. The quencher will absorb the reporter dyes fluorescent emission and prevent detection of fluorescence signals.

The 5’-3’ nuclease activity of the Taq polymerase will cleave the probe during the primer extension step and release fluorescence signal during the amplification process. The cleaving of the probe leads to less efficient transfer of the reporter dye fluorescent emission to the quenching dye due to the increased distance between the reporter and the quencher. This leads

to an increased emission of the reporter fluorescent dye. The amount fluorescence released is proportional to the generated PCR product in each cycle. (48, 49)

Method

The cDNA samples and primer/probes were kept on ice at all times. Primer/probes are sensitive to light and were covered with aluminium foil at all times. The Taqman Fast Universal PCR Master Mix was spun down before use. A master mix was made with primer/probe, RNase free water and Taqman. A 96 well plate was placed on a plate-rack on ice. 7.5µl master mix was pipetted into each well together with 2.5µl cDNA. Controls were also pipetted in a few wells. The plate was covered with optical adhesive cover and aluminium foil and stored on ice or refrigerator until use. The plate was centrifuged to spin down content and avoid bubbles, 1080g for 60 seconds. The plate was placed into the LightCycler 96 machine for analysis.

Table 3.2: Program for LightCycler 96

		Temperature	Seconds	Ramp
Preincubation		95°C	600	4.4
2-step amp 40 cycles	Step 1	95°C	15	
	Step 2	60°C	60	
Cooling		37°C	30	

3.7.1 Statistics

Kruskal-Wallis test was performed to compare three or more groups. Only one p-value is given for the total comparison of all the groups. If there was a p-value which was statistically significant, a post-test was performed. This post-test was Dunn's Multiple Comparison Test which compared all the groups. The post-test does not provide any p-values.

Fold change was calculated by subtracting the mean threshold cycle number (Cq mean) of the housekeeping gene (TBP) from the gene we are interested in.

Expample: Cq mean (IL-7) – Cq mean (TBP) =ΔCq.

The avarage ΔCq value of the young mice is used. (ΔCq young mice)

ΔCq-(ΔCq young mice)= ΔΔCq

Fold change is then calculated by: $2^{-(\Delta\Delta Cq)}$.

4 Results

4.1 Detection of mouse anti-dsDNA IgM molecules in sera of lupus prone B/W mice

To investigate if anti-dsDNA IgM antibodies preceded the anti-dsDNA IgG antibody production in NZB/W mice, sera taken from B/W mice at different time points were analyzed for the presence of mouse IgM and IgG molecules specific for dsDNA using an anti-dsDNA ELISA. **Error! Reference source not found.** A and B demonstrates the binding to ctDNA of ouse IgM molecules in a dilution series starting at 1:100 dilution, from sera collected at different time points. The positive control was as expected the one that had the most antibodies bound to antigens while the negative control had none. The results from the 1:100 dilutions for IgG and IgM were plotted against age (weeks old) to try to pinpoint the start of antibody production (Figure 4.1 C and D, respectively). The anti-dsDNA IgG ab production was increased as the mouse got older, the production also started at a later age. It started to increase at 18 w.o with a small drop at week 23 but increased again after that. The anti-dsDNA IgM production seemed to be increased at the start 13-14 w.o and then started to decrease as the mouse got older. This indicates that the start point at 13 w.o. is to late to get the starting point of the anti-dsDNA IgM ab production in NZB/W mice.

Sera from week 14 and 15 had more antibodies bound to antigens than sera from week 12. Sera from week 16 had less antibodies bound than sera from week 17 and 18. The 1:200 dilution showed that sera from week 16 had more antibodies bound than week 17 and 18 which differs from the 1:100 dilution.

Figure 4.1, B shows that there was less antibodies bound to antigens compared to the results observed in **Error! Reference source not found.**, A. Sera from 13 w.o had more antibodies ound compared to sera taken later. There were some abnormalities like week 22 and 23 had the most antibodies bound to antigens while week 20, 21, 25 and 26 were closer to each other. The anti-dsDNA IgM ELISA need to be optimized more to give any conclusive results, but the results here indicate that anti-dsDNA IgM abs may precede the anti-dsDNA IgG abs with more than 10 weeks.

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The anti-dsDNA IgM ELISA need to be optimized more to give any conclusive results, but the results here indicate that anti-dsDNA IgM abs may precede the anti-dsDNA IgG abs with more than 10 weeks.

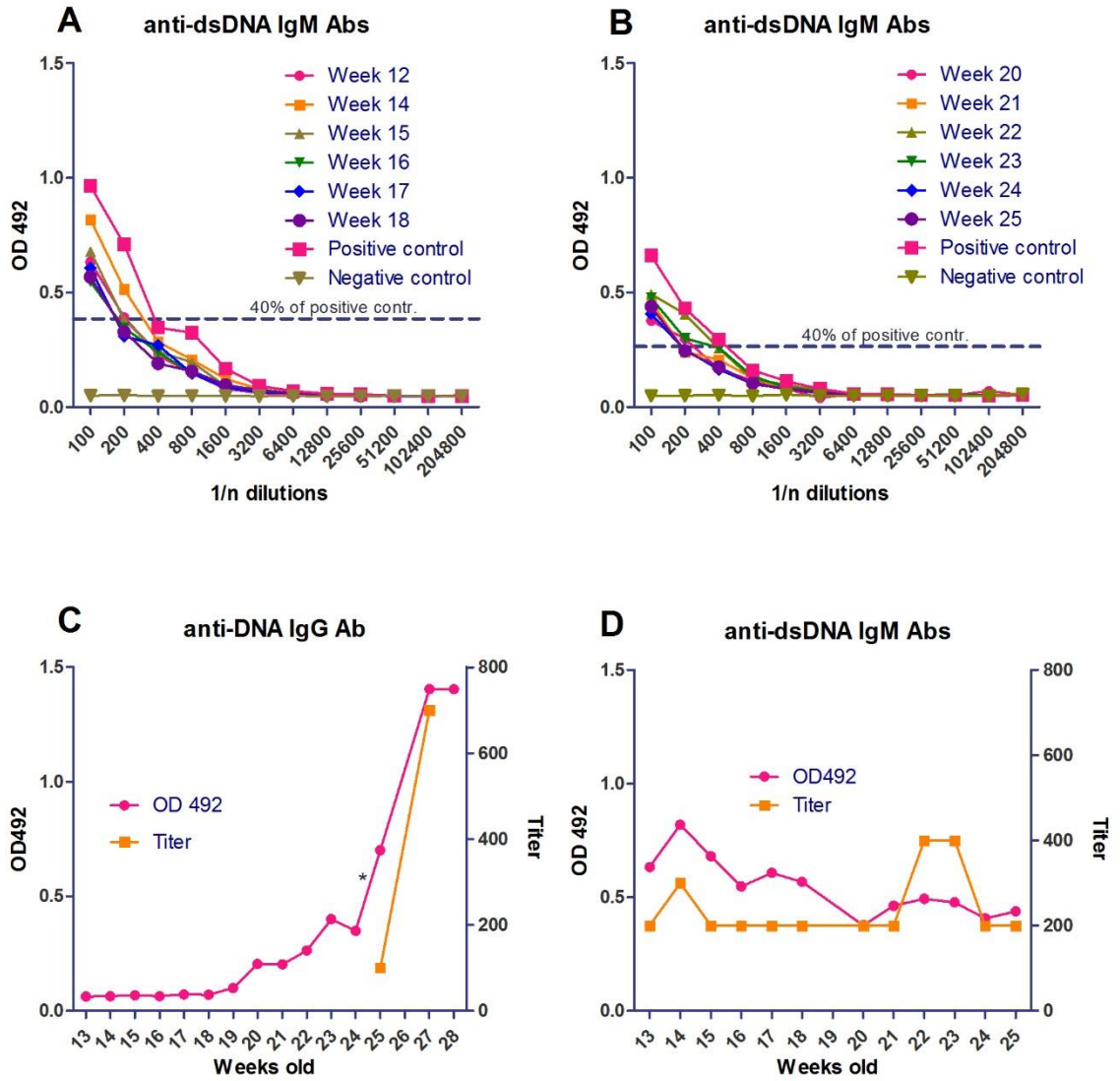

Figure 4.1: ELISA detecting anti-dsDNA IgM antibodies in sera from a 28 w.o. mouse, (A and B).
C and D compares the 1:100 of anti-DNA IgG Ab and anti-dsDNA IgM Ab dilutions with age. Anti-dsDNA IgM ab production starts early and decreases with age. Anti-dsDNA IgG ab production starts when the mouse is older and increases with age.

4.2 Detection of IL-7 and IL-7R in kidneys and spleen of NZB/W mice at different ages

Proteins isolated from kidney and spleen of B/W mice sacrificed at different ages were analyzed using the SDS-PAGE method to first separate the proteins according to their molecular weight and then using western blotting to see if different anti-antibodies would bind to the proteins on the membrane.

Figure 4.2 shows the titer experiment used to find out which protein concentration was the best to use in future experiments. Two visible bands at approximately 50 and 90-100 kDa were observed for 4-10 µg of proteins. The 2µg protein per sample did not show a band at 50kDa for the L8 mouse but had one at 100kDa. The 2µg protein per sample of the L49 mice gave the opposite result of the L8 with a band at 50kDa but nothing at 100kDa. After the titer experiment, the protein concentration per sample was set to 8-10µg for future experiments. Predicted molecular weight of IL-7R is 52kDa according to abcam.

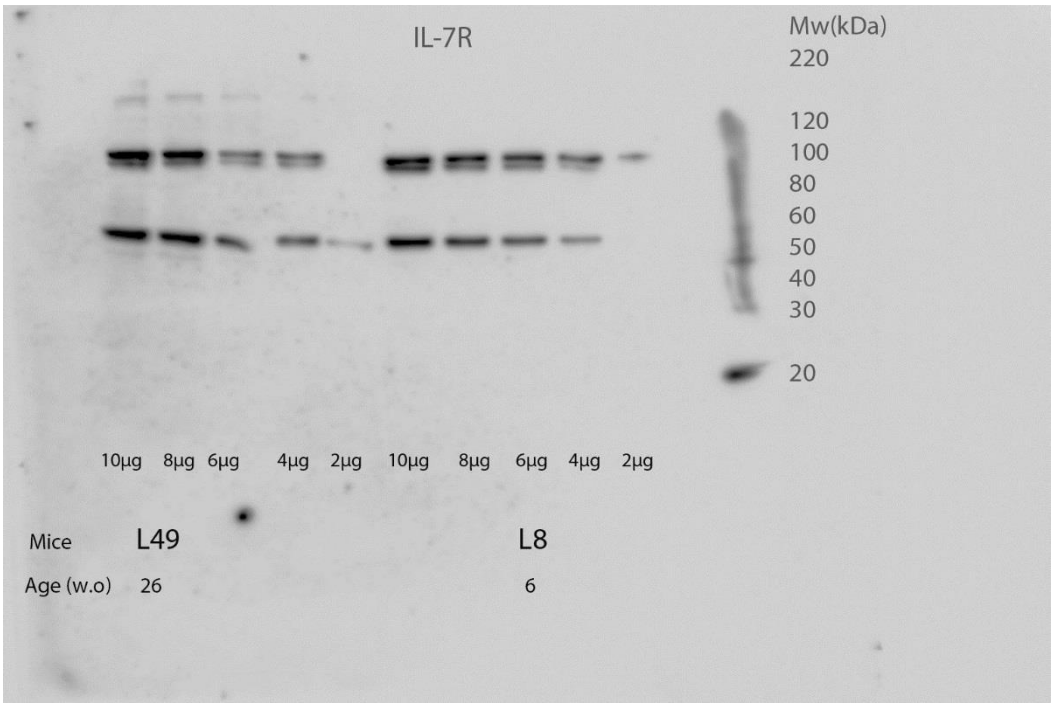

Figure 4.2: Western blot detecting IL7R on different protein concentration of kidney extracts to find the ideal concentration of protein per sample for western blotting. The blot shows two bands at 100 and 50 kDa.

Predicted molecular weight for anti-IL7 antibody is 20kDa according to abcam. Western blot detecting IL-7 performed on kidney extracts demonstrated double bonds slightly below 30kDa for all the mice except for the L8 mouse. The L8 mouse had a barely visible band. There were also bands above 40kDa for all the mice but nothing at the 20kDa range (Figure 4.3).

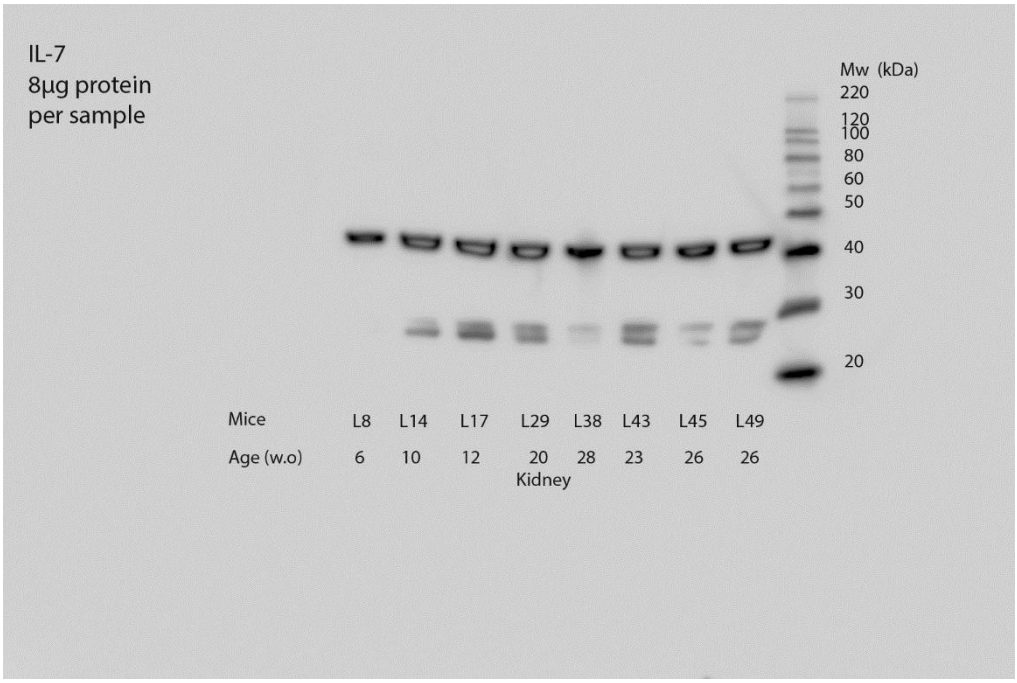

Figure 4.3: Western blot detecting IL7 on different protein extracts of kidney. The blot shows two bands, below 30 and above 40 kDa.

Western blot detecting IL7-R performed on protein extract isolated from spleen samples showed bands around 50kDa which is close to the molecular weight of IL 7R(Figure 4.4). There were some bands visible below 20kDa and double bands around 100kDa. The bands around 50 and 100 kDa were the same as observed in the titer experiment, Figure 4.2.

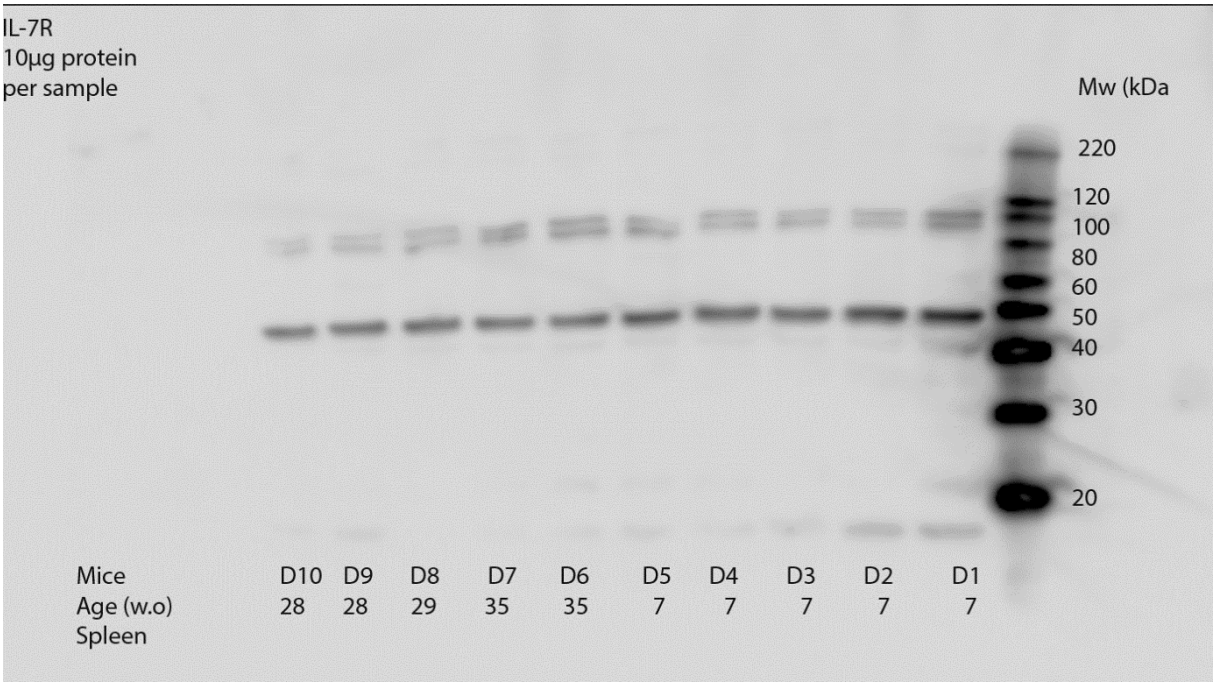

Figure 4.4: Western blot detecting IL7R on different protein extracts of spleen. The blot shows two bands at 100 and 50 kDa.

Figure 4.5 showed the same as Figure 4.4, bands around 50 and 100 kDa were detected using anti-IL7 ab. Weak bands around 20kDa were also observed for samples D1-5. The bands around 50kDa were thicker for the kidney protein extract then for the spleen extracts. There were also no visible bands around 100kDa for D4 and D5. However, this seemed to be due to insufficient transfer of proteins in the area caused by presumable an air bubble (Figure 4.5, left side).

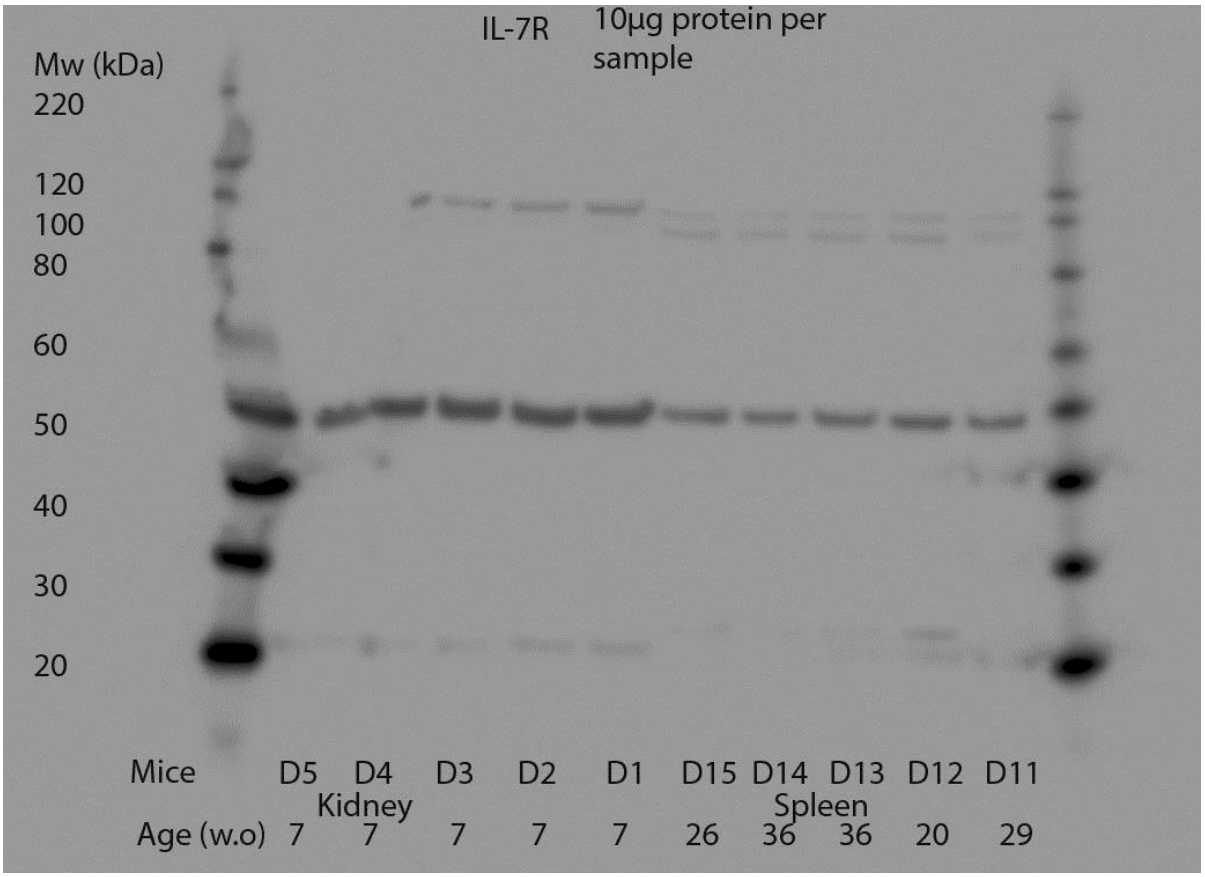

Figure 4.5: Western blot detecting IL7R on different protein extracts from kidney and spleen. The blot shows two bands at 100 kDa for the spleen extracts. Single bands at 50 kDa.

Western blot detecting IL7R performed on the rest of the kidney extract demonstrated the same as observed in Figure 4.5Figure 4.5 with thick bands around 50kDa (Figure 4.6). The bands were also thicker for the samples from older mice compared to samples from young mice. This may indicate that more IL7R were produced in older mice compared to the young mice. The bands around 20kDa were more visible compared to what was observed in Figure 4.5. There were only single bands around 100kDa for D1-D7 mice, while sample D8-D15 mice had double bands around 100kDa (Figure 4.6).

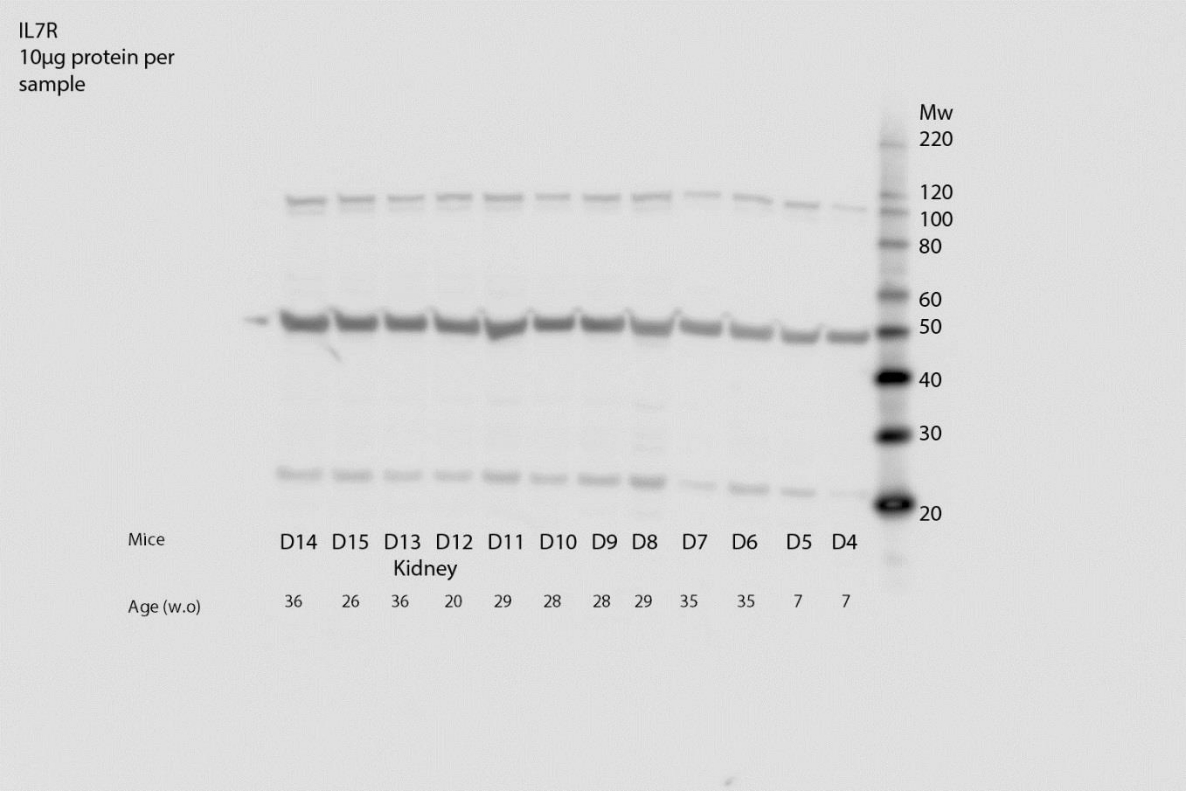

Figure 4.6: Western blot detecting IL7R on different protein extracts from kidney. The blot shows three bands at 100, 50 and 20 kDa.

4.3 Immunohistochemistry

Tissue fixated in formalin or zinc was prepared and stained with different antibodies. Different mice were compared to see if there was any difference of IL7 and IL7R expression in young mice compared to older mice, and if the expression of these two proteins could be linked to the formation of TLS. IL7 and IL-7R was detected in all mice tested (Figure 4.7 and Figure 4.9). In the cortex the IL7R was detected in proximal, distal and collecting tubules with different amount of staining intensities (Figure 4.9, A). No staining of IL7R was observed in glomeruli (Figure 4.9, A) but it looks like there was some staining of IL-7 in the glomerulus in (Figure 4.7, A). IL7 was observed in cells located in the pelvic wall were TLS are observed to form in diseased animals (Figure 4.7, B). In kidney section from mice with visible TLS the IL7 were detected around the edge of the TLS (Figure 4.7, C). Looking at a higher magnification there were some staining observed inside the TLS (Figure 4.7, D). (Figure 4.7 C, D) shows the TLS through two different objectives. The IL7 is mostly around the edge of the TLS but with a closer view there was some staining inside the TLS also. As seen in (Figure 4.8, A, B) there was a week expression of IL-7R in the tubular cells in the cortex. There was some expression of IL-7R near the pelvic wall in (Figure 4.8, F). Stromal looking cells in the TLS were

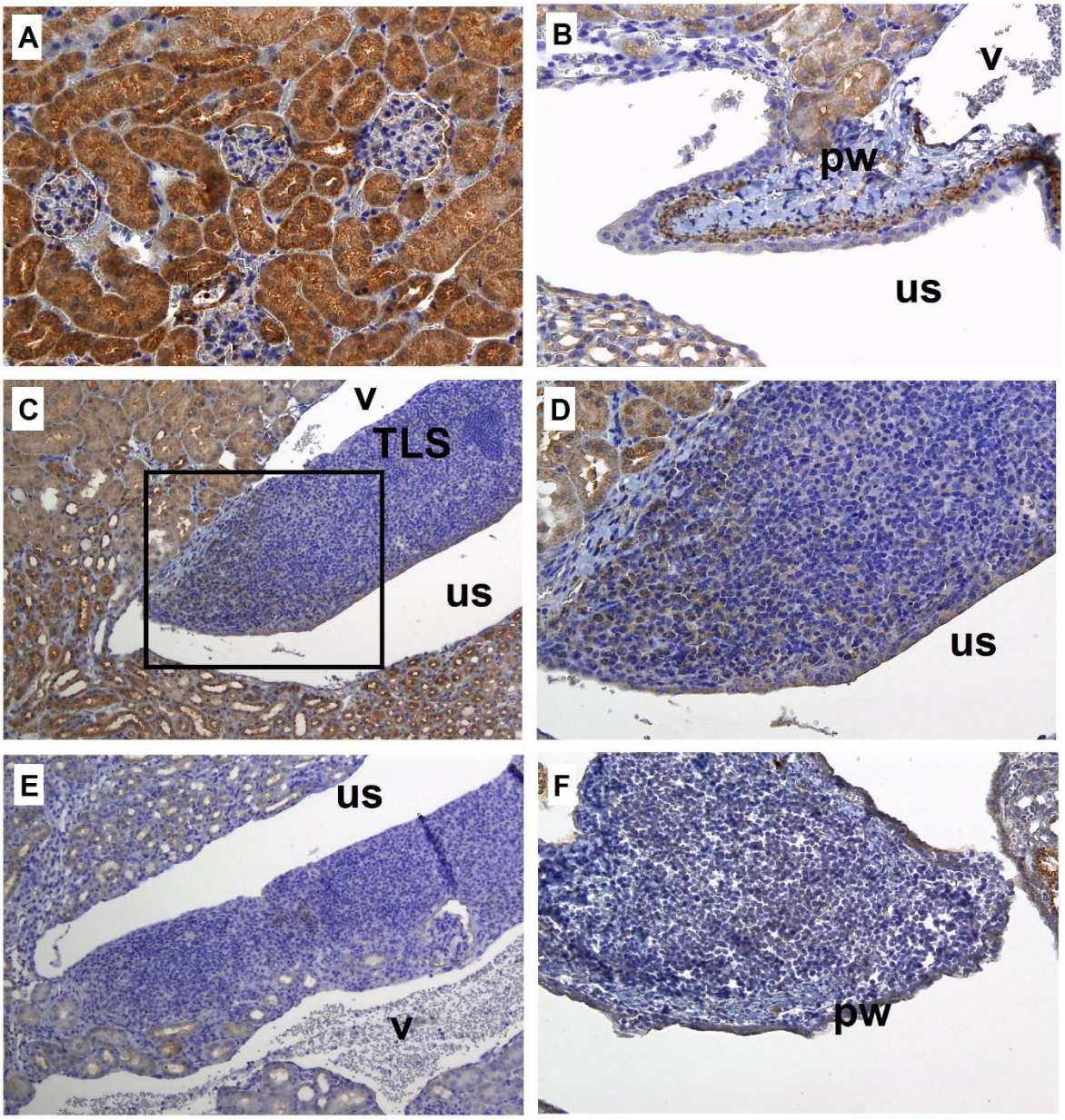

Figure 4.7: IHC detecting IL7 in formalin fixed kidney sections from a 16 w.o. mouse without anti-dsDNA abs(A and B), a 32 w.o. mouse with anti-dsDNA abs and proteinuria (D and C), and a 29 w.o. mouse with anti-dsDNA abs and proteinuria. The IHC with the anti-IL7 ab did not work on kidney samples fixed with Zn2+ as the staining pattern gave a lot of background staining (F). pw, pelvic wall; TLS, tertiary lymphoid structures; v, vein; us, urinary space. A, D and E are taken at 10 x magnification while B, D and F is taken at 20 x magnification.

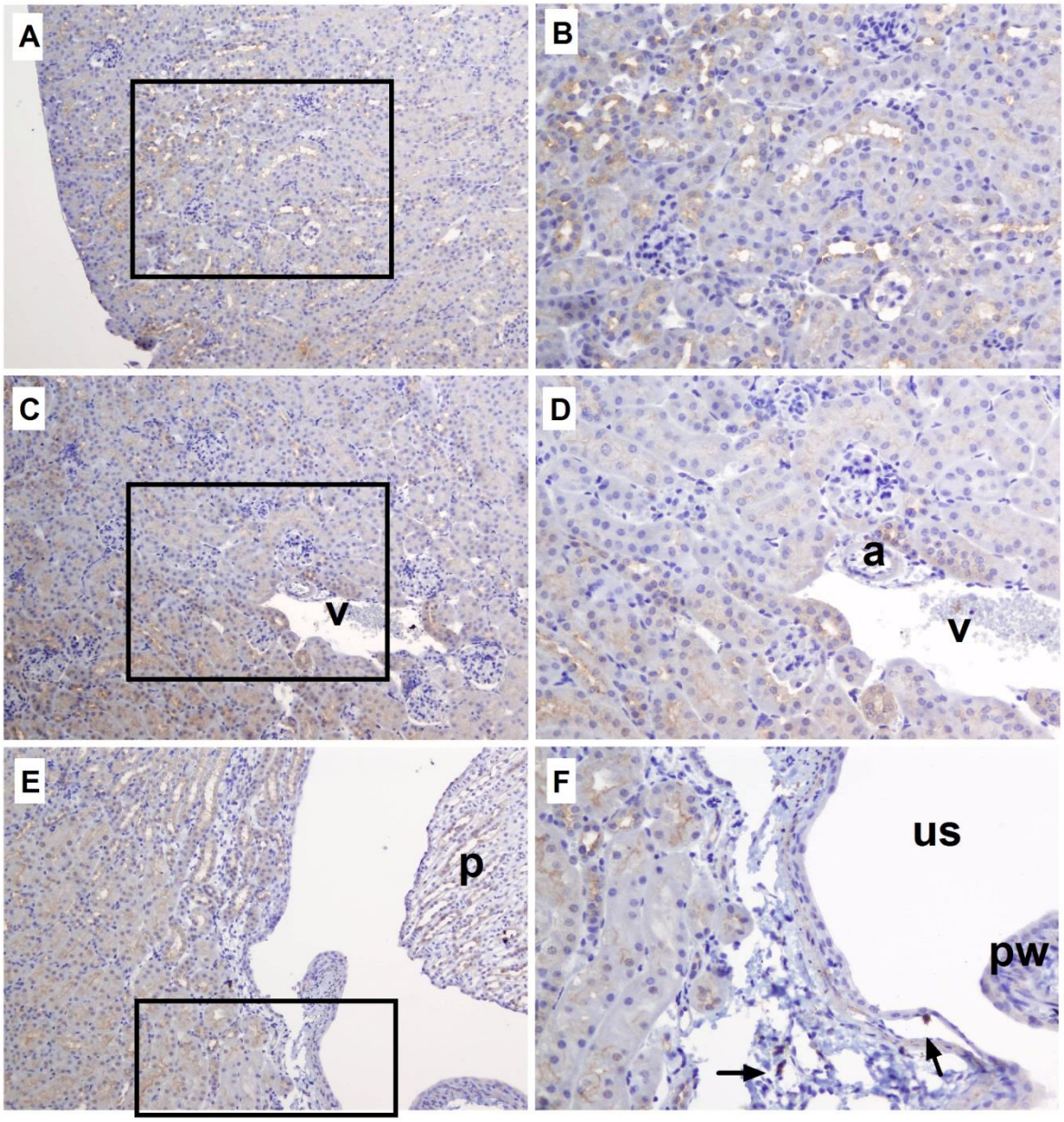

Figure 4.8: IHC detecting IL7R in formalin fixed kidney sections from a 4 w.o. NZB/W mouse (A and C), a 16 w.o. NZB/W mouse (C-F) The receptor is weekly expressed in tubular cells in the cortex. There are some positive cells near the pelvic wall in the 16 w.o. mouse (F, arrows).a, artery; p, pelvis; Pw, pelvic wall; us, urinary space; v, vein. A, C and D taken at 10 x magnification. B, D and F taken at 20 x magnification.

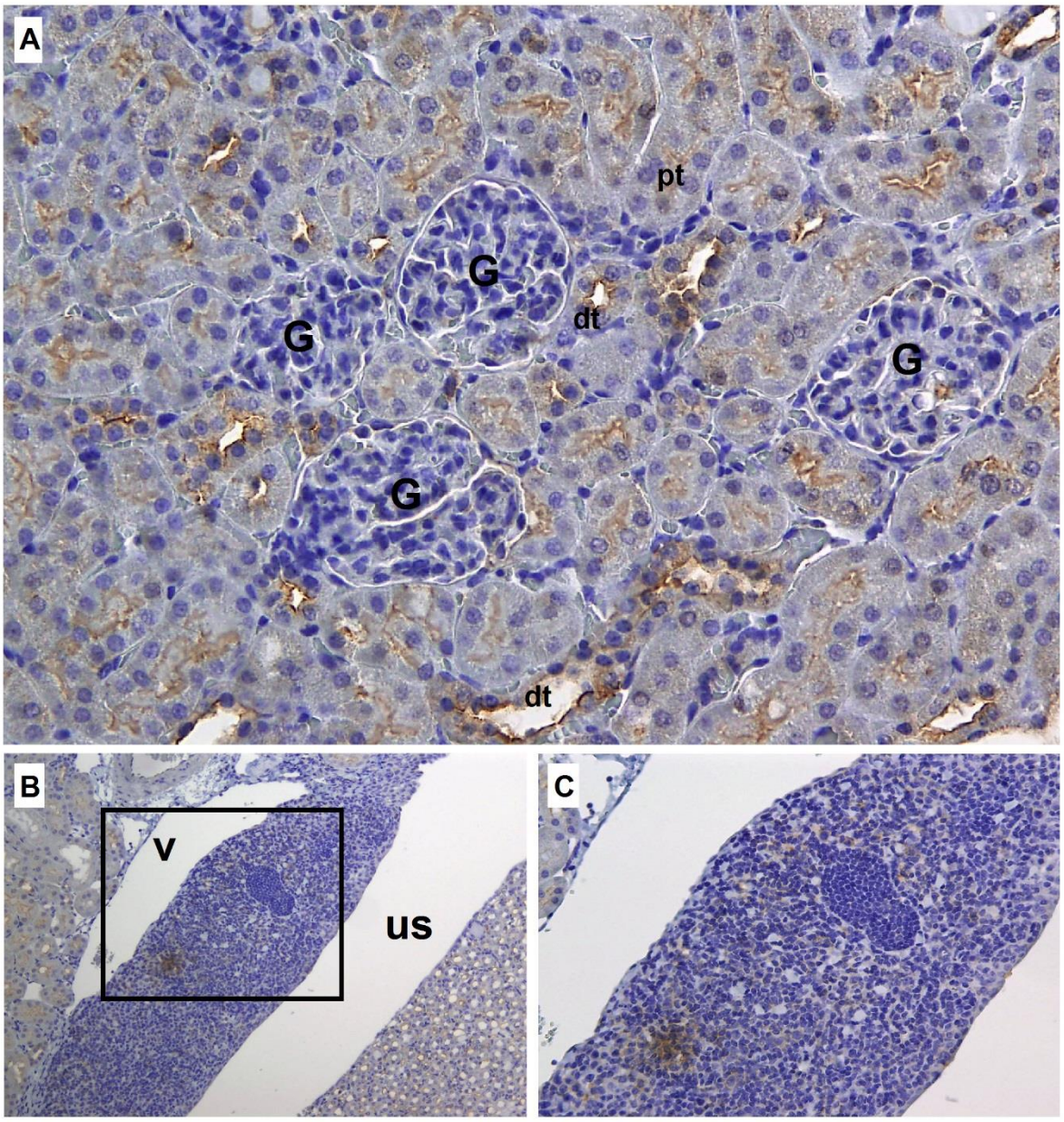

Figure 4.9: IHC detecting IL7R in kidneys of a 32 w.o. mouse with anti-dsDNA ab production and proteinuria. An increased staining of IL7R is observed in proximal and distal tubules (A). The glomeruli is not stained by the anti-IL7R ab. Stromal looking like cells in the TLS is weakly stained by the anti-IL7R ab (B and C). G, glomerulus; dt, distal tubule; v, vein; us, urinary space; pt, proximal tubule.

4.4 Analyzes of gene expression using quantitative real-time PCR (qPCR)

To investigate if there were any differences between the IL7 and IL7R gene expression in different groups of NZB/W mice, RNA was isolated from kidneys, spleen and lymph nodes and analysed by qPCR. The results from the qPCR were processed using excel to calculate fold change in the different mice. The fold change results were then inserted to the GraphPad program which was used to calculate statistics. The mice were divided in different groups by age, anti-dsDNA antibody positive or not and if the mice had proteinuria as shown in Table 4.1Table 4.1. A Kruskal-Wallis tests was performed followed with Dunn's Multiple Comparison Test as a post-test. Post-test was performed if there was any statistical difference.

Table 4.1 Grouping of the mice used in the real-time qPCR. The mice are grouped after age, antibody positive and proteinuria.

Group 1	Group 2	Group 3	Group 4	Group 5
Young 4-12 weeks old(wo) without anti-dsDNA antibody(ab)	Older 12-28 wo without anti-dsDNA ab	18-38 wo with anti-dsDNA ab	23-29 wo with anti-dsDNA ab and proteinuria	30+ wo with anti-dsDNA ab and proteinuria

4.4.1 IL-7 expression in kidneys, spleen and LN of different NZB/W mice.

The gene expression of IL7 in kidney samples increased in mice as they got older and developed lupus nephritis (Figure 4.10Figure 4.3), while IL7 expression in spleen and LN seemed to be decreased in groups' 2-5 mice (Figure 4.10). The Kruskal-Wallis test gave a P value of 0.0121 for kidney. 0.0463 For LN (Figure 4.10) and 0.3299 for the spleen (Figure 4.10). The P value summary showed that there was a statistical difference for the kidney and LN. A post-test was performed comparing all groups using Dunn's Multiple Comparison Test. The test was used separately for the different organs. There were a significantly difference in the expression of IL7 in kidneys from group 5 compared to young mice in group 1 (Figure 4.10).

The test showed that there was no significant difference in IL7 gene expression in LN between the groups of mice tested.

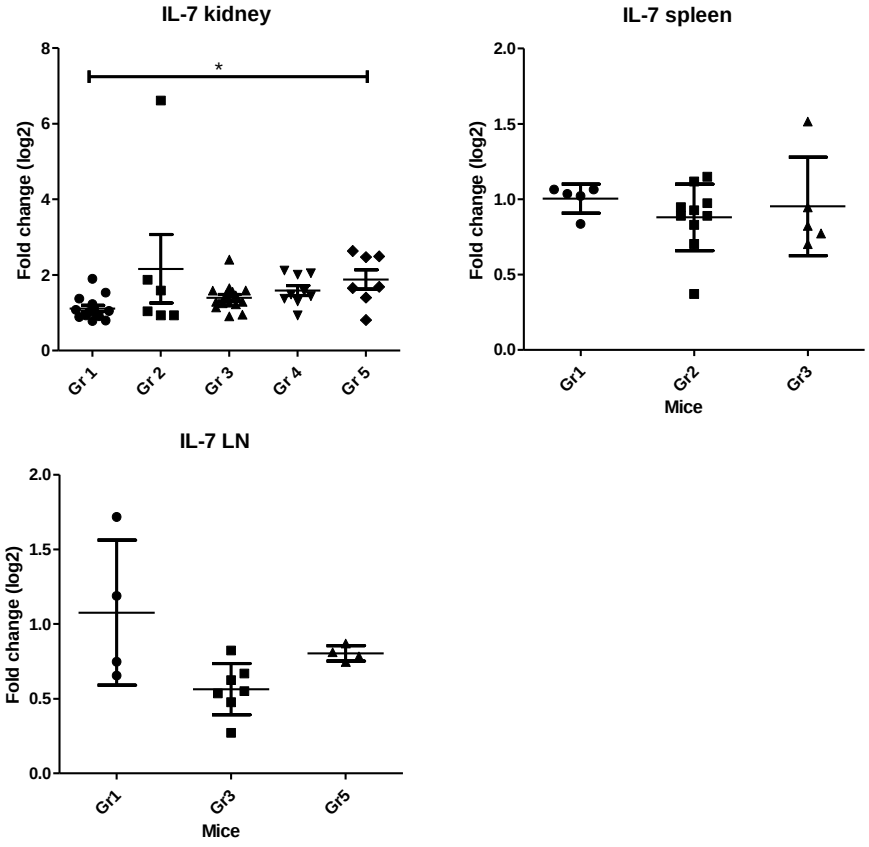

Figure 4.10: Gene expression of IL-7 in kidneys, spleen and LN of NZB/W mice at different ages and disease stages. qPCR detecting IL7 was performed on RNA isolated form kidneys, spleen and LN. Expression levels for each gene were normalized against the expression level of TBP. Data is given as Log 2 of mean ± SEM of fold change values normalized against 4-week-old mice (n=3) and are representative for at least two independently analysis. The p values are calculated based on Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. *, $p<0.01$, ***, $p<0.001$.

4.4.2 IL-7R gene expression in kidneys and spleen of NZB/W mice at different ages

IL7R gene expression seemed to be increased in group 2-5 mice compared to groups 1 mice. The Kruskal-Wallis test demonstrated significant variance of IL7R gene expression in kidneys of the different groups of mice, but Dunn's test showed that there was no significance between the different groups (Figure 4.11). For the spleen the Kruskal-Wallis test showed that there was a significant variance between the IL7R gene expression in spleen of the different groups of mice. According to Dunn's there was a significant difference between group 1 and 3 and a very significant difference between group 1 and 5 (p=0.05 and p=0.001, respectively). The graph also demonstrated that the fold change was higher in the spleen from the young mice compared to the older mice (Figure 4.11). For the kidney it is the complete opposite with the group 5 having the highest fold change and group 1 having the lowest (Figure 4.11).

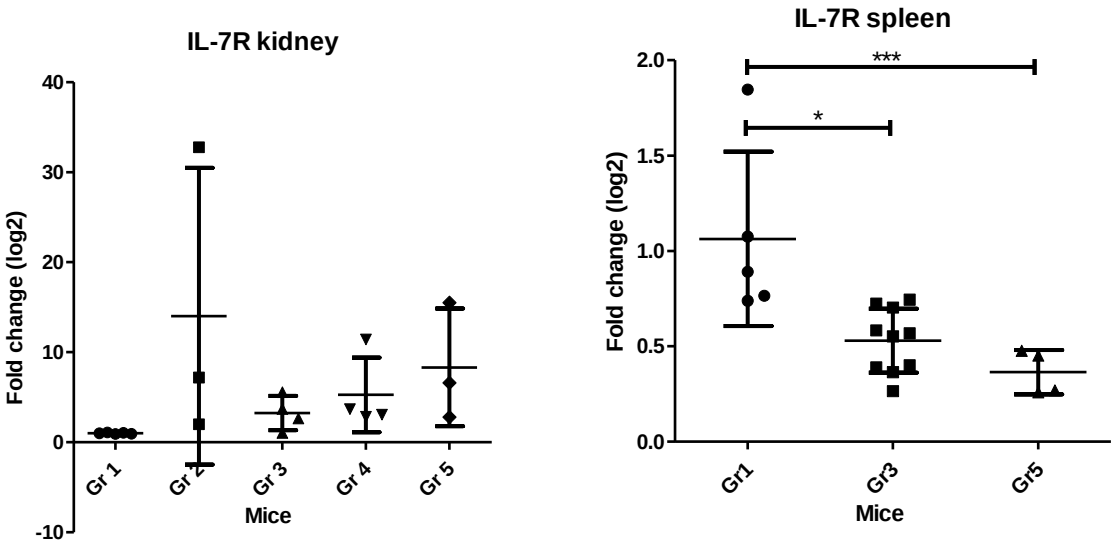

Figure 4.11: Gene expression of IL-7R in kidneys and spleen of NZB/W mice at different ages and disease stages. qPCR detecting IL7R was performed on RNA isolated form kidneys and spleen. Expression levels for each gene were normalized against the expression level of TBP. Data is given as Log 2 of mean ± SEM of fold change values normalized against 4-week-old mice (n=3) and are representative for at least two independently analysis. The p values are calculated based on Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. *, $p<0.05$, ***, $p<0.001$.

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4.4.3 IL-1β gene expression in kidneys and spleen of NZB/W mice at different ages

IL-1β expression in both kidneys and spleen samples were increased in group 5 mice compared to group 1 mice. Kruskal-Wallis test showed that there was a significant variance in kidney gene expression of IL-1β between the tested groups of mice (Figure 4.12).This was also true for the spleen. Dunn's test showed that group 1 and 4 and group 1 and 5 has a significant difference for the kidney (Figure 4.12). The Fold change is much higher in the older mice with anti-dsDNA ab and proteinuria compared to the young mice. Dunn's test showed that for the spleen there was a significant difference between group 1 and 5. The fold change increased in group 3 and group 5 mice as the mice started to produce anti-dsDNA antibodies and got proteinuric.

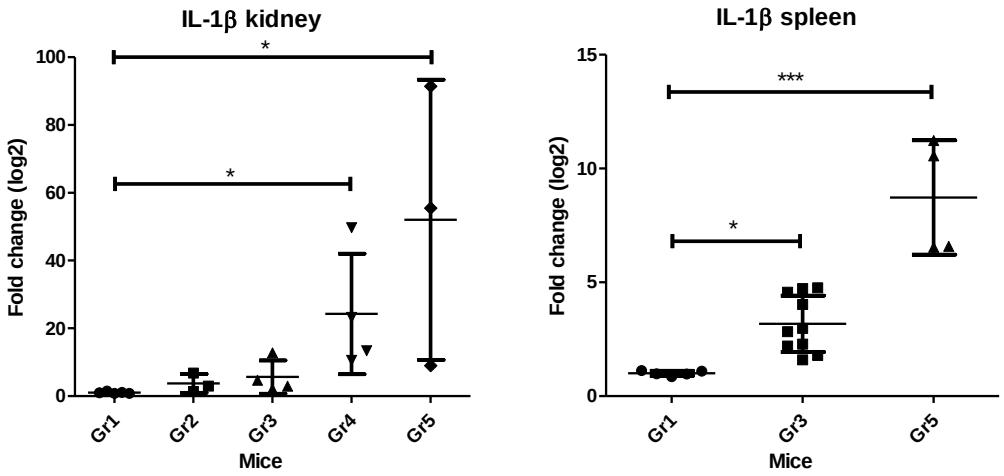

Figure 4.12: Gene expression of IL-1β in kidneys and spleens of NZB/W mice at different ages and disease stages. qPCR detecting IL-1β was performed on RNA isolated form kidneys and spleen. Expression levels for each gene were normalized against the expression level of TBP. Data is given as Log 2 of mean ± SEM of fold change values normalized against 4-week-old mice (n=3) and are representative for at least two independently analysis. The p values are calculated based on Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. *, $p<0.05$, ***, $p<0.001$.

4.4.4 TNFα gene expression in kidneys and spleen of NZB/W mice at different ages.

The relative gene expression of TNFα in kidneys increased as the mice got older (Figure 4.13) Kruskal-Wallis test showed that there was a significant variance in TNFα gene expression in the kidney and spleen of the different groups. Dunn's test demonstrated that the only significant difference for the kidney expression was between group 1 and 5. As seen in (Figure 4.13) the fold change was higher in group 5 compared to group 1.For the TNFα gene expression in spleen samples the significant difference was between group 3 and , but the relative gene expression of TNFα was low in the spleen samples compared to the kidney samples (Figure 4.13).

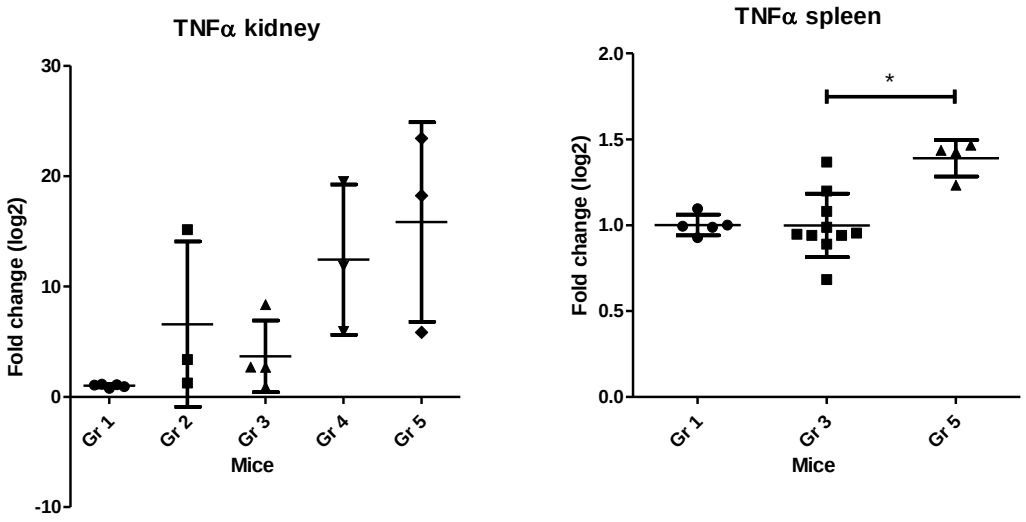

Figure 4.13: Gene expression of TNFα in kidneys, spleen of NZB/W mice at different ages and disease stages. qPCR detecting TNFα was performed on RNA isolated form kidneys and spleen. Expression levels for each gene were normalized against the expression level of TBP. Data is given as Log 2 of mean ± SEM of fold change values normalized against 4-week-old mice (n=3) and are representative for at least two independently analysis. The p values are calculated based on Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. *, $p<0.05$

5 Discussion

The development of anti-dsDNA ab is a hallmark of lupus nephritis. We have recently discovered the formation of TLS within the kidney of lupus prone (NZBxNZW) F1 mice. These structures seem to start to develop before anti-dsDNA IgG abs are produced. We therefore wanted to investigate if the production of anti-dsDNA IgM antibodies preceded the anti-dsDNA IgG ab production and if it could be linked to the formation of TLS.

The aim of this project was to follow the gene and protein expression of IL7 and IL7R in the kidneys, spleen and LN from lupus prone (NZBxNZW) F1 mice. The expression of IL7 and it corresponding receptor has been linked to the formation of TLS.(21)

The ELISA showed that anti-dsDNA IgM antibody production might precede the production of anti-dsDNA IgG. It is then possible that anti-dsDNA IgM might be present when TLS is developed. According to (Figure 4.1, D), anti-dsDNA IgM is present early while anti-dsDNA IgG starts increasing later on.

The cells expressing IL7 and IL7R in TLS as seen in the results. The titer experiment showed if the protein concentration was too low the anti-antibody would not bind well. The 6 w.o. mouse did not have a band and the 26 w.o. mouse had a weak band at 2µg protein per sample. It also showed that the younger mouse had something that the anti-IL7R ab bound to at 100kDa but that protein was not present in the older mouse since there was no band at 100 kDa at 2µg protein per sample. The band at 100 kDa was always present in the rest of the western blots with anti-IL7R. The bands around 50kDa was expected for the IL-7R since the molecular weight for IL-7R was 52 kDa. But there were also bands around 20kDa and 100kDa. The bands around 100kDa might be the IL-7Rα and γc both together. The γc has a molecular weight around 40 kDa and together with the weight of IL-7R it would be around 92 kDa. The 20kDa bands might be that the anti-IL-7R ab has bound to IL-7 since the molecular weight for IL-7 is 20kDa.The 20 kDa bands were also quite weak and barely visible for the protein extracts from spleen but stronger for kidney protein extracts. The 20 kDa band got more visible for the older mice. The bands around 50 kDa also got thicker for the older mice compared to the young (Figure 4.6). This showed that there is more expression of IL-7R in the older mice then in the young. There is also more expression in the kidneys then in the spleen. Which means that older mice that had TLS and were anti-dsDNA ab positive had more expression of IL-7R.

Figure 4.3 showed that there were bands below 30 kDa and above 40 kDa, but nothing at 20 kDa where the IL-7 ab was supposed to bind. The reason might be due to extensive glycosylation of the IL-7, which gives a molecular weight of 25 kDa.(22) With a molecular weight around 25 kDa the bands below 30 kDa might apply. The bands above 40 kDa might be un-specified binding by the anti-IL-7 ab, the reason might be that the membrane was not blocked long enough but then there would be other bands all over the membrane. Further testing has to be done to check if those bands disappear or stay.

The immunohistochemistry showed that IL-7 did not work well with zinc fixed section. There was too much background staining so it was difficult to see if there was other relevant staining. The formalin sections worked out better. It might be that the anti-IL7 ab was not suited for zinc fixated tissue. There was staining of IL-7 in the pelvic wall in a mice which had not yet developed TLS and there was also staining in the walls of the TLS of an older mouse. This means that IL-7 is present in the kidney before the development of TLS. There was also staining of IL-7 inside the TLS, which means that it might be like with the ectopic lymphoid follicle samples from RA patients that had increased expression of IL-7 and IL-7Rα.(22). IL-7R staining was weaker than IL-7. There might not be as much expression of IL-7R as there is of IL-7. The other reason might be that a higher concentration of the anti-IL-7R antibody should have been used. It remains to see if IL-7 and IL-7R have a role in TLS formation in lupus nephritis like in RA.(21)

The gene expression analysis showed that there was a difference in expression of the different genes in the different organs and mice. IL-7 expression in spleen and LN was not statistically significant but that was expected since those are the organs which produce IL-7. A study has shown that the T lymphocyte zones in LNs produce the most IL-7 mRNA.(50) As seen in the results, the kidneys from older mice with proteinuria and anti-dsDNA ab had more expression of IL-7 compared to the young mice. The reason for higher IL-7 expression in older mice could be because of TLS and chronic inflammation. It might be relevant if there was some expression at an earlier stage before the development of TLS. Even if there is IL-7 mRNA, it might not lead to production of IL-7 cytokines. (51)

There was more expression of IL-7R in the kidney then in the spleen but the expression in the kidney was not significant. The fold change numbers were much higher in the kidney but it was the spleen that had the significant values. It might be that there was not so much

expression of the IL7R in the kidney, that it was only IL-7 that was more expressed. It was also the younger mice that expressed the IL-7R in the spleen and not the old mice. Expression seems to be downregulated in the older mice and upregulated in the young mice in the kidney, which means that there might be more expression of IL-7R in the younger mice. IL-7R signaling is important for SLO development and it happens in a really early stage.(52) It might be why the younger mice have more expression.

Both of the proinflammatory cytokines, TNFα and IL-1β are expressed more in the kidneys then in the spleen and more expression in the older mice. The expression of TNFα was not significant in the kidney, but there was significant difference in the spleen although the expression was much lower. The reason might be that there were more mice in each group for the spleen compared to the kidney and thereby became significant. TNFα has been seen remodelling blood vessels and causing lymphangiogenesis in sustained airway inflammation in mice with *M. pulmonis* infection.(53) It is likely that it could also cause lymphoid neogenesis in kidneys.

For the IL-1β there was a significance in both of the organs. There was a more significant difference in the spleen but the expression was lower. There was also a really big gap between the three mice in group 5 that can affect the results. The spleen group was more collected which can have affected the statistical calculations. The higher expression in the kidneys for the two proinflammatory cytokines might be as expected in mice that have TLS. Rats that were injected with anti-GBM and got nephritis showed there was excess amount of IL-1 in the glomeruli.(54, 55)

Both the immunohistochemistry and gene expression results show that there is less expression of IL-7R in the kidney compared to the IL-7. The western blot results showed on the other hand that there was expression of IL-7R in the kidneys but it did not show how much expression there was, the bands only got thicker and clearer. It looks like there is more expression in the older mice.

Concluding remarks

This study indicates that there is some difference in expression of IL-7 and IL-7R in the different organs and mice. More research has to be done to see how relevant the difference is. For the future, bigger sets of mice and more parallels can be done to get a more accurate set of results.

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