行政院國家科學委員會專題研究計畫 成果報告

提昇私校研發能量專案計畫-自體免疫疾病中心之建立--子計畫三:探討第二型轉麩胺胺酶在清除凋亡細胞時所扮演的角色(3/3)

研究成果報告(完整版)

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處 理 方 式 ： 本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 96 年 09 月 11 日

行政院國家科學委員會補助專題研究計畫

■ 成果報告
□ 期中進度報告

Role of TG2 in the clearance of apoptosis cells

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中　華　民　國　　96　年　　01　月　　31　日

中文摘要

紅班性狼瘡的致病原因已知和許多因子有關，但是其分子機制仍未被完全瞭解，目前相信凋亡細胞的清除不全，是自體抗原產生的重要因素。第二型殼胺醯胺轉移胺是一種具有轉胺活性的多功酵素，而且也被發現參與在細胞凋亡及後續之凋亡細胞的清除，本計劃就是要釐清第二型殼胺醯胺轉移胺在紅班性狼瘡的致病機轉中的角色。在第一年我們檢測第二型殼胺醯胺轉移胺的表現。兩種類似紅班性狼瘡症狀及一種對照組的老鼠將分為兩組，分別於 6 週齡及 24 週齡時採集巨嗜細胞及不同部位器官組織，包括心、肝、脾、肺、腎、肌肉。第二型殼胺醯胺轉移胺的表現將以反轉錄聚合胺鏈反應及免疫墨點法偵測。結果發現在狼瘡鼠中，雖然第二型殼胺醯胺轉移胺的表現在不同組織器官中的表現量不同，但是在紅班性狼瘡小鼠發病前後並無不同。第二型殼胺醯胺轉移胺，本身為一個需鈣離子之轉胺甲醯轉移胺，也被證實牽涉在紅斑性狼瘡的致病基轉中。所以在第二年我們分析狼瘡鼠之巨嗜細胞的吞噬能力，與第二型殼胺醯胺轉移胺活性。結果發現類狼瘡鼠之巨嗜細胞的吞噬能力，跟對照之正常小鼠比較，有明顯的下降。而且跟第二型殼胺醯胺轉移胺活性有關。為了找出更確切的原因，第三年度則針對狼瘡模式小鼠的巨嗜細胞，找出可能造成吞噬能力下降的原因。我們的實驗結果發現，粒線體相關路徑的細胞凋亡蛋白，在狼瘡模式小鼠有較明顯增加。這些發現也說明小鼠之巨嗜細胞的吞噬能力較低是因為自身凋亡所致，而不是第二型殼胺醯胺轉移胺表現上的缺失所致。

關鍵詞: 紅班性狼瘡，殼胺醯胺轉移胺，自體抗原，凋亡

Abstract

The pathogenesis of SLE is certainly determined by multiple factors, but the molecular mechanisms have not been completely characterized. In recent reports, incompetence in the clearance of apoptotic cells has been demonstrated as an important factor in autoantigen production, but the mechanism is still unclear. Transglutaminase 2 (TG2) is a multi-functional protein with transamidating activity, and has been reported involving in apoptosis and the subsequent clearance process. In this 3-years project, we intend to elucidate the association between TG2 and the clearance of apoptotic cells in SLE. The purpose of the first year is examining the expression localization of TG2 in cells derived from and NZB/W F1 mice. Mice were divided into two groups and various tissue samples including macrophage, heart, liver, spleen, lung, kidney and muscle were collected at age of 6 week and 24 week. The localization and expression of TG2 were determined by RT-PCR and immunoblotting. The phagocytic ability of macrophage was also examined and compared among Balb/C and NZB/W F1 mice at age of 6 and 24 week. Since the expression of TG2 protein in various organs from NNB/W F1 mice, there is no expression variation between mice at age of 6 week and 24 week. Transglutaminase 2 (TG2), an inducible transamidating acyltransferase that catalyzes calcium-dependent protein modification, is involved in the pathogenesis of SLE. In the second year, we designed to examine whether the known association of TG2 with apoptosis and apoptotic clearance might explain its role in SLE. Our experimental results demonstrated that decreased phagocytic ability in macrophages of NZB/W mice was not associated with changes in expression of TG2 (mRNA or protein), but was associated with increased TG2 activity. In the third year, we further identified the decreased phagocytic ability of macrophages from NZB/W F1 mice. Experimental results demonstrated that increased caspase activity, and increased expression of Bax and Apaf-1 was associated with the reduced uptake of apoptotic bodies. This finding may account for the decreased phagocytic ability of macrophages in SLE mice.

Key words: systemic lupus erythematosus (SLE), Transglutaminase 2 (TG2), autoantigen, apoptosis,

Aims

In this proposal, we will investigate the association between TG2 and SLE. Firstly, we will examine the expression, localization and activity of tissue transglutaminase (tTG) in macrophages derived from NZB/W F1 mice and their counterparts. Moreover, the capacity of proliferation, activation, adhesion, migration and phagocytosis of macrophages will also be evaluated. Eventually, we suppose to clarify the role of TG2 in pathogenesis of SLE.

Background

Tissue transglutaminase is a 75 kDa monomeric globular protein expressed in the majority of cells and tissues. Its subcellular localization is in the cytosolic fraction and no interactions with other specific subcellular compartments are described. It is translated as a fully active enzyme and there is no evidence for a proteolytic activation (1-2). Cells such as endothelial cells, vascular smooth muscle cells, platelets, and epithelial cells of the lens express the enzyme constitutively and accumulate high levels of active enzyme (3). In other cells, such as neurons and skeletal muscle cells, the tTG constitutive expression is very low and not easily detectable. Since tTG is expressed in the vast majority of cells and tissues, it has been also implicated in a wide variety of functions resulting in intra and/or extracellular structural alterations. These include modeling of the extracellular matrix (4), stimulus-secretion coupling (5), receptor mediated endocytosis (6), cell differentiation (7), tumor growth (8) and programmed cell death (9-10).

During apoptosis tTG expression is increased and its catalytic function is activated (11). The fast increase in the expression of the enzyme in cells undergoing programmed cell death may be due to a specific induction of the enzyme or rather to the loss of factors that normally suppress its expression. Cell death may serve to unmask the activity of the constitutive core-promoter of the tTG gene and in turn this may lead to the accumulation of the enzyme that occurs in many dying cells. Immunohistological studies together with the isolation of highly polymerized protein products from apoptotic cells have established tissue transglutaminase as an useful biochemical marker of programmed cell death. Its precise role in the death process, though, is still under discussion (12-13). The enzyme could participate to the death program as part of the killing mechanism or alternatively it could modulate the course of apoptosis by temporary stabilization of the dying cell. Mounting evidence has shown that persistence of apoptosis and impaired clearance may contribute to the pathogenensis of SLE, as residual apoptotic cells undergo secondary necrosis, leading to the spillage of potentially immunogenic macromolecules to the exterior. TG2 is a multi-functioned proteins involved in protein modification, signal transduction, cell adhesion and apoptosis. One emerging role is to regulate phagocytosis of apoptotic cells (14). $TG2^{-/-}$ mice develop autoantibodies and immune complex glomerulonephritis, strongly suggesting that deregulation of TG2 might play a role in the pathogenesis of SLE (15).

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Material and methods

Mice and tissue sample

Twenty of Balb/C, and NZB/W F1 mice were purchased from Animal center, SINICA, Taiwan. Each strain of mice were divided into 2 groups (10 mice/group) for sampling at the age of 7 week and 26 week. Various tissue sample including heart, liver, spleen, lung, kidney, and muscle were collected for further analysis.

Macrophage isolation

Peritoneal macrophage was collected as previous reported. Mice were sacrificed with CO2. Sink a mouse in the 5% water solution of Dettol (Chloroxylenol) for 1 min, at RT. Dry the fur thoroughly. Inject i.p. 5 ml of ice-cold IDDM-FCS (5%, decomplimented). Gently massage the anterior and lateral walls of the abdomen. Accurately collect the peritoneum washes on to the Petri dish placed on ice. Filter the washes thorough a nylon filter into the 15 ml Falcon tube placed on ice. Centrifuge at 1200 rpm, at 4℃, for 5 min. Then resuspend in IMDM-FCS (5%, decomplimented) Count cells (do not count erythrocytes). Adjust the concentration of counted cells as 20x10^6/ml

RT-PCR

RNAs were isolated with TRIZOL (Invitrogen). RT-PCR was performed in 50 μl of reaction volume with 25 mM MgCl2The amplification parameters used were sequentially as follows: 50 ℃, 30 min for reverse transcription, then 30 s at 94℃, 30 s at 55℃, and 1 min at 72℃ for 25 cycles.

SDS-PAGE and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 12.5% acrylamide gel with 5% acrylamide stacking gel, was performed according to the method of Laemmli. Samples were reduced for 5 min in boiling water with 0.0625M Tris-HCl buffer, pH 6.8, containing 2.3% SDS, 5% 2-mercaptoethanol, and 10 % glycerol. Samples applied to the gel were run of 100-150V for 1.5hr and then electrophoretically transferred to nitrocellulose. The membrane was soaked in 5% nonfat dry milk in PBS, for 30 min at room temperature, to saturate irrelevant protein binding sites. Antiserum diluted with 5% nonfat dry milk in PBS were incubated for 1.5 hr at room temperature. The membranes were washed twice with PBS-Tween for 1hr and adding secondary antibody consisting of alkaline phosphatase conjugated goat anti-human or mouse IgG antibodies. The substrate NBT/BCIP (nitroblue tetrazolium/ 5-bromo-4-chloro-3 indolyl phosphate) was used to detect antigen-antibody complexes.

Assessment of Phagocytosis

After 24 hours, adherent cells were co-incubated with FITC-labeled resin (10 μL of a 2 mg/mL suspension, Sigma) in phosphate buffered saline (PBS). The cultures were then washed to remove particles that had not been internalized. Phagocytic ability was determined by observing engulfed particles in over 100 macrophages from each mouse, using phase contrast microscopy. The

percentage of macrophages with engulfed particles was calculated and cells with more than 10 internalized particles were considered positive.

TG activity

TG activity was determined by a modification of a previously described method (16). The detection of bound BP was performed as described previously.

Caspase 3 activity assay

A caspase 3 ELISA kit (Pharmingen, San Diego, CA, USA) was used for *in vitro* determination of caspase 3 enzymatic activity in cell lysates, according to the manufacturer's instructions.

Results and Discussion

First year

To elucidate the association of TG2 and SLE, mice were breed until sacrificed at age of 6 and 24 week. The SLE alike clinical phenomena were observed in MRL and NZB mice as shown in figure 1. Firstly, macrophage of these 3 kinds of mice was collected as described in materials and methods and the phagocytic ability was determined by stimulating with fluorescence labeled beads. Figure 1A and 1B represent the phagocytosis results of macrophage from mice at age of 6 and 24 week. Pictures of phase contrast, merge, and fluorescence were taken as shown in upper, medium, and bottom panels. Macrophage from mice at age of 6 week represented similar phagocytic percentages of 88.5%, 83.6% and 76.7%, respectively. Moreover, the phagocytic percentages were calculated as 74.5%, 53.9% and 39.7% while engulfed beads were taken into consideration (Table 1). Macrophage from mice at age of 24 week also represented similar phagocytic percentages of 92.1%, 80.2% and 75.3%, respectively. The phagocytic percentages were calculated as 77.2%, 62.3% and 47.7% while engulfed beads were taken into consideration (Table 2). Taken together, the macrophages of NZB showed decreased phagocytic ability as compared to their counter part, Balb/C.

To investigate the possibility whether TG2 is associated with this phenomenon, the expression of TG2 is compared among the macrophages from Balb/C, MRL and NZB mice at age of 24 week. Our result represented a slight decrease of TG2 amount in NZB as compared to Balb/C and MRL (Fig. 3). RT-PCR was performed for further investigate the expression of TG2. In addition, expression of various chemokines' receptors including mCCR7, mTNF-alpha, mCP1R, mCCR3, mCCR5, mCCR2 and mCCR1 was also examined. Figure 4 and 5 show the results of RT-PCR of macrophage from mice at age of 6 and 24 week. A slight decrease was observed in TG2 mRNA expression in NZB mice at both 6 and 24 week. However, no significant difference in mRNA expression of chemokines was detected (Fig 4 and 5). More works will be performed to clarify the association between these cytokines' receptor and TG2.

Although previous study has indicated that TG2 is associated with clearance of apoptotic cells and production of autoantigen in a knockout transgenic mice model, no further investigation was reported. To assess other possible molecules or routes involving in the mechanism of clearance of apoptotic cells, various organs of these 3 kinds of mice were also collected for analysis of TG2 expression. Heart, liver, spleen, lung, kidney and muscle sample were taken and separated on SDS-PAGE (Fig 6A). The expression of TG2 protein in various organs was detected with immunoblot (Fig 6B). Among these tissue sample, liver expressed the lowest amount of TG2 but the expression of TG2 abounded in muscle. Since no significance difference in expression of TG2 was observed among Balb/C, MRL and NZB at age of 6 week, further study will be done by analyzing the tissue sample of Balb/C, MRL and NZB at age of 24 week. Moreover, TG2 activity will also be detected in consequent work.

Second year

A representative photography of phagocytosis was shown as figure7. To assess phagoctyic ability we incubated macrophages with FITC-labeled beads and quantified the percentage of macrophages with greater than 10 internalized particles. The phagocytic ability of macrophages from NZB/W mice at both 6 and 28 weeks of age was significantly lower compared to macrophages isolated from age matched BALB/c controls (Table 3). Notably, at 6 weeks of age, macrophages derived from both SLE prone MRL-lpr/lpr and NZB/W mice exhibited a significantly lower phagocytic ability than macrophages derived from BALB/c mice. However, at 28 weeks of age, only macrophages derived from NZB/W mice had significantly lower phagocytic ability.

To address whether reduced phagocytic ability in NZB/W mice was due to differences in chemokine receptor expression, we quantified mRNA levels of MCP1R, CCR1, CCR2, CCR3, CCR5 and CCR7 by RT-PCR using GAPDH as an internal control. Notably, we found no difference in the mRNA expression levels of these chemokine receptors in the three strains of mice (Figure 8).

Next, we evaluated the expression levels and activity of TG2 in macrohages isolated from the three strains of mice. TG2 mRNA was quantified by RT-PCR using GAPDH as an internal control (Figure 9). TG2 proteins levels were measured by SDS-PAGE and immunoblotting using actin as an internal control (Figure 10). Notably, there was no significant difference in the expression of TG2 mRNA (Figure 9) or protein (Figure 10) in macrophages from the three strains of mice at both 6 and 28 weeks. However, although protein levels were similar, we did observe that TG2 activity was significantly increased in macrophages isolated from NZB/W mice at 28 weeks of age compared to macrophages from age-matched MRL-lpr/lpr and BALB-C mice (Figure 11).

Third year

We also compared expression levels of the pro-apoptotic proteins, Bax and Apaf-1, as well as caspase 3 activity in macrophages isolated from the three lines of mice. Protein expression was evaluated by SDS-PAGE and immunoblotting using actin as an internal control. Bax and Apaf-1 expression were significantly higher in macrophages isolated from 28-week old NZB/W mice compared to macrophages isolated from age-matched MRL-lpr/lpr and BALB-C mice (Figure 12). Caspase activity was also significantly higher in macrophages from NZB/W mice at 28 weeks compared to macrophages from MRL-lpr/lpr and BALB-C mice (Figure 13). In immunohistology analysis, the kidney showed raised immunocomplex as previous found (Figure 14), however, no significant TG2 expression was observed as shown in our previous experimental results. On the other hand, increased TNF-alpha and TGF-beta mRNA expression but not IL-10

and IL-19 were observed in macrophages of lupus-prone mice. The zymorgraphy analysis was also performed to investigate MMPs activity variation in various tissues of lupus-prone mice. Here we found the mmp9 activity was lower in spleen of NZB/W F1 mice. These experimental results may provide clues in understanding the pathogenesis of SLE.

Appendant Figures

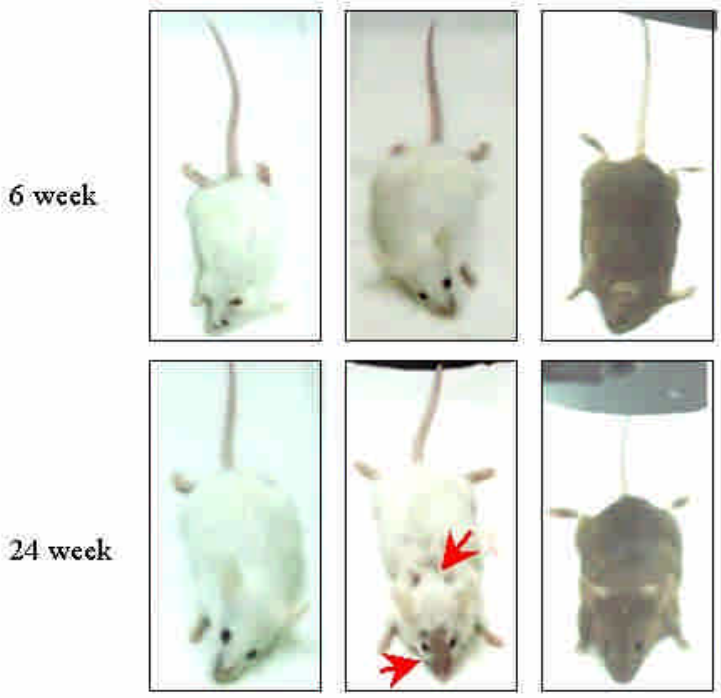


Fig. 1

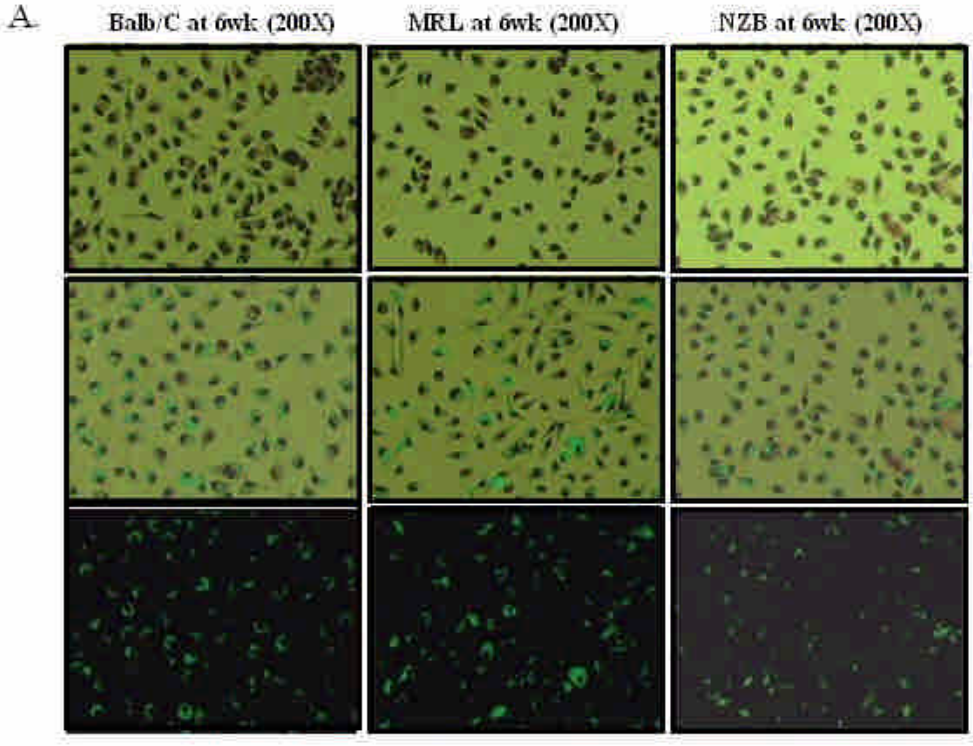


Fig. 2A

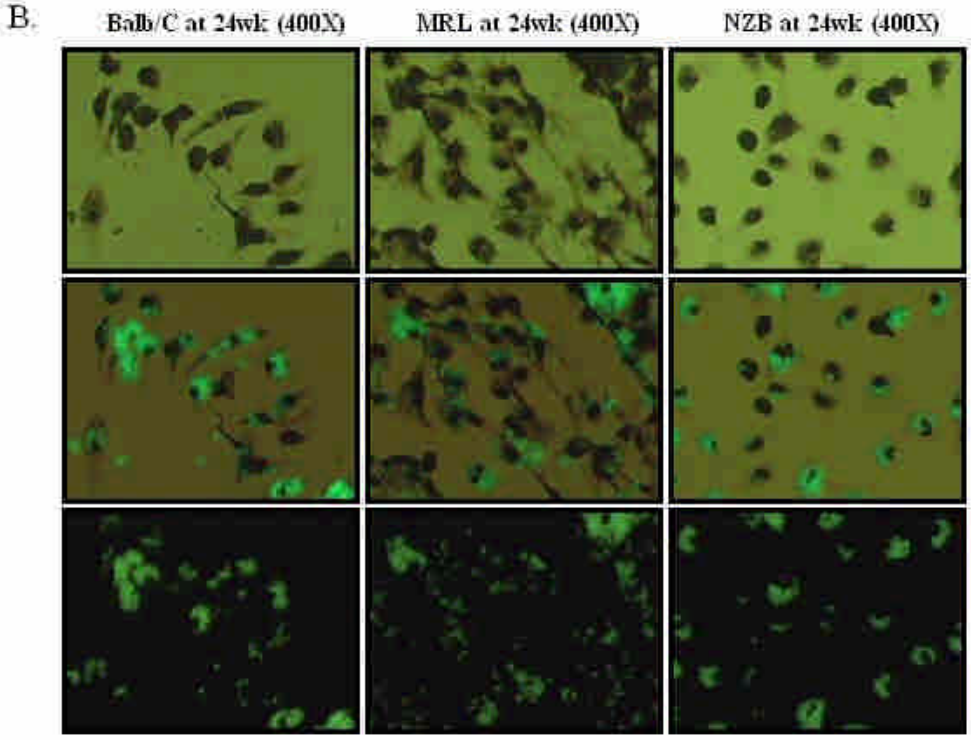


Fig. 2B

Expression of TG2 of macrophages at the age of 24 week

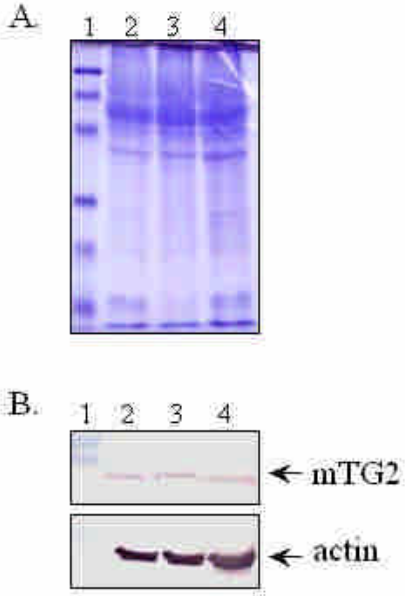
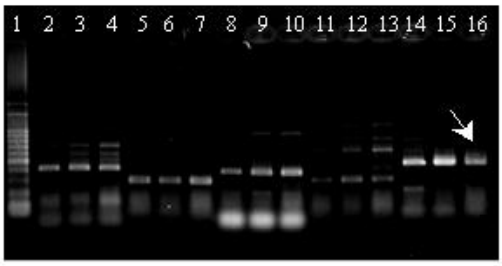


Fig. 3

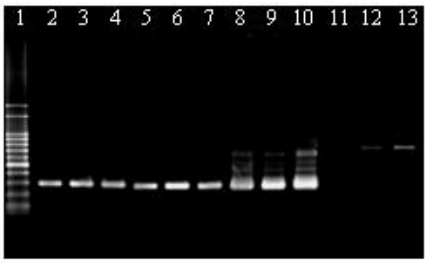
RT-PCR of macrophage at age of 6 week

A.



1. .Marker
2-4. CCR7
5-7. TNF-a
8-10. CP1R
11-13. CCR3
14-16. mTG2

B.



1. .Marker
2-4. CCR5
5-7. CCR2
8-10. GAPDH
11-13. CCR1

Fig. 4

RT-PCR of macrophage at age of 24 week

A.

1. Marker
2-4. mCCR7
5-7. mTNF-alpha
8-10. mCP1R
11-13. mCCR3
14-16. mCCR5

B.

1. Marker
2-4. mCCR2
5-7. mTG2
8-10. mGAPDH
11-13. mCCR1

Fig. 5

Different tissues of BALB/C, NZB and MRL (6wk old)

A.

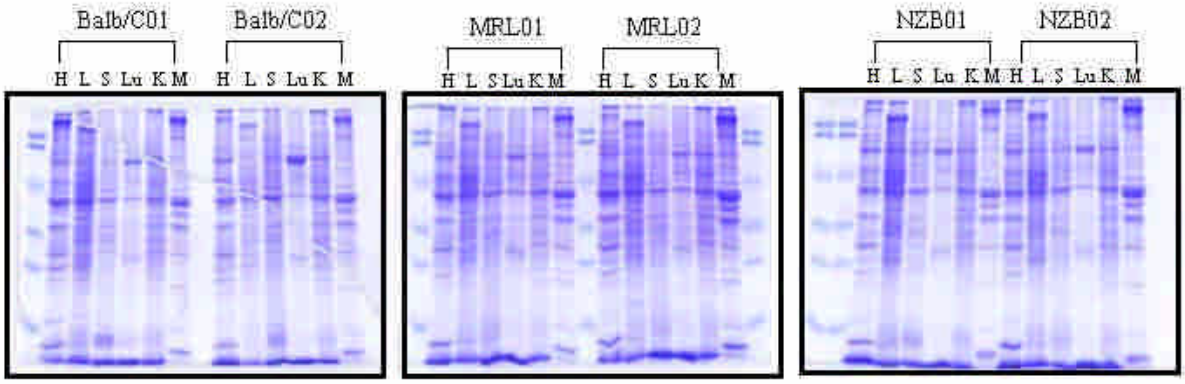


Fig. 6A

B.

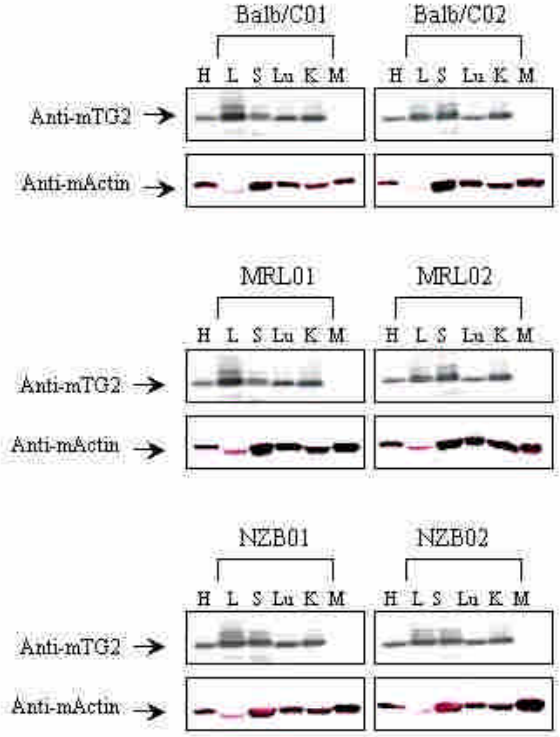


Fig. 6B

Table 1. Phagocytic ability of macrophage

Beads engulfed	Macrophages isolated at age of 6 week		
	Balb/C	MRL	NZB
< 10	139/157 (88.5)	107/128 (83.6)	112/146 (76.7)
> 10	117/157 (74.5)	69/128 (53.9)	57/146 (39.7)

* Number in parentheses are percentages.

Table 2. Phagocytic ability of macrophage

Beads engulfed	Macrophages isolated at age of 24 week		
	Balb/C	MRL	NZB
< 10	105/114 (92.1)	101/126 (80.2)	82/109 (75.3)
> 10	88/114 (77.2)	79/126 (62.3)	52/109 (47.7)

* Number in parentheses are percentages.

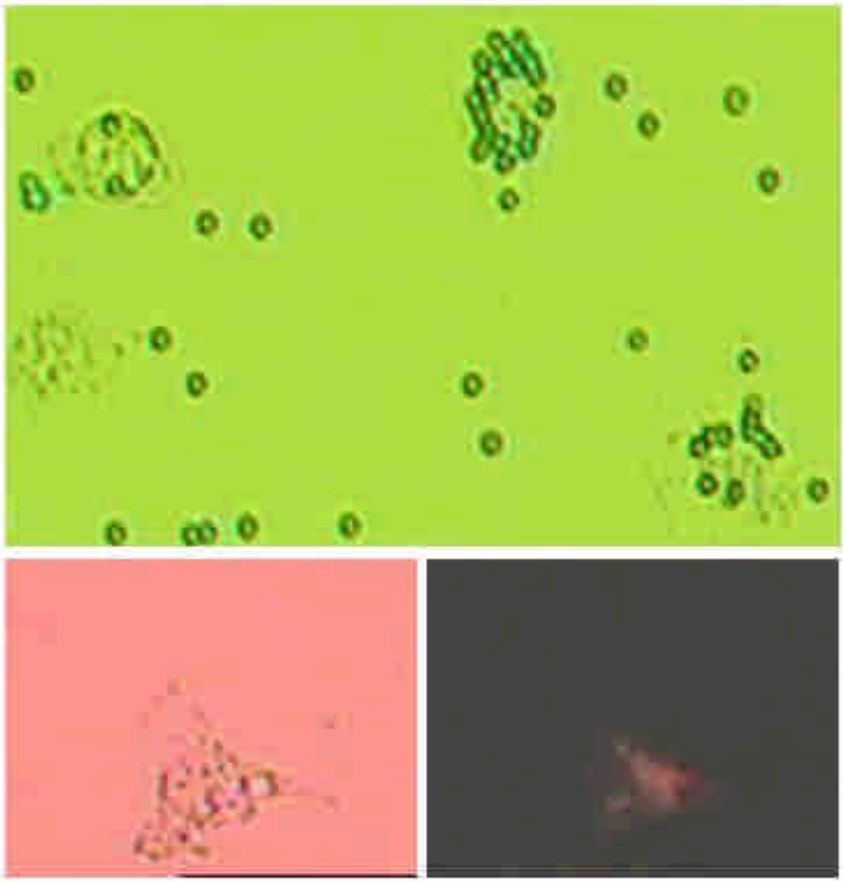


Figure. 7A Phagocytosis of fluorescein labeled beads and apoptotic body. A representative photo that fluorescein labeled beads were internalized by macrophages (upper panel). The photography of macrophages internalized apoptotic bodies was shown in lower panel.

Table 3 Phagocytic ability of macrophages

Age of mice (weeks)	Macrophages		
	BALB/c	MRL(lpr/lpr)	NZB/W
6	74.5 ± 19.7	53.9 ± 12.3*	39.7 ± 9.8*
28	70.2 ± 26.1	62.3 ± 19.8	47.7 ± 14.5*

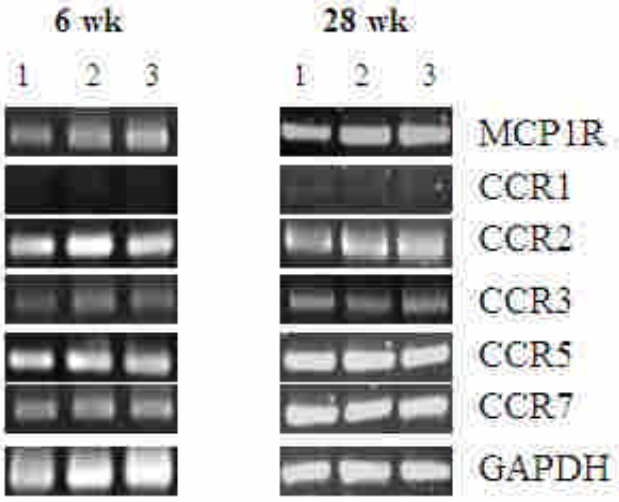


Figure. 8 Expression of various chemokine receptors mRNA. GAPDH was used as internal control

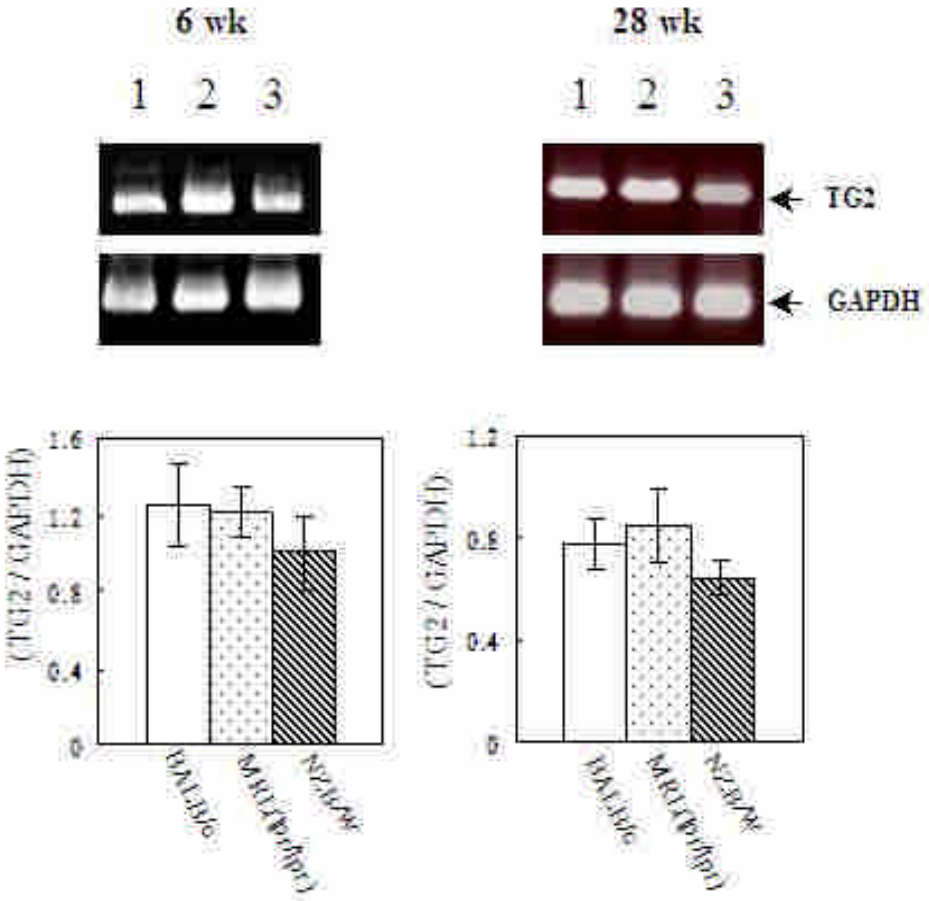


Figure. 9 Expression of TG2 mRNA in macrophage from mice at different age. Macrophages derived from mice at age of 6 (left panel) and 28weeks (right panel) were assessed for the TG2 mRNA expression. The quantified results were shown in bottom panel.

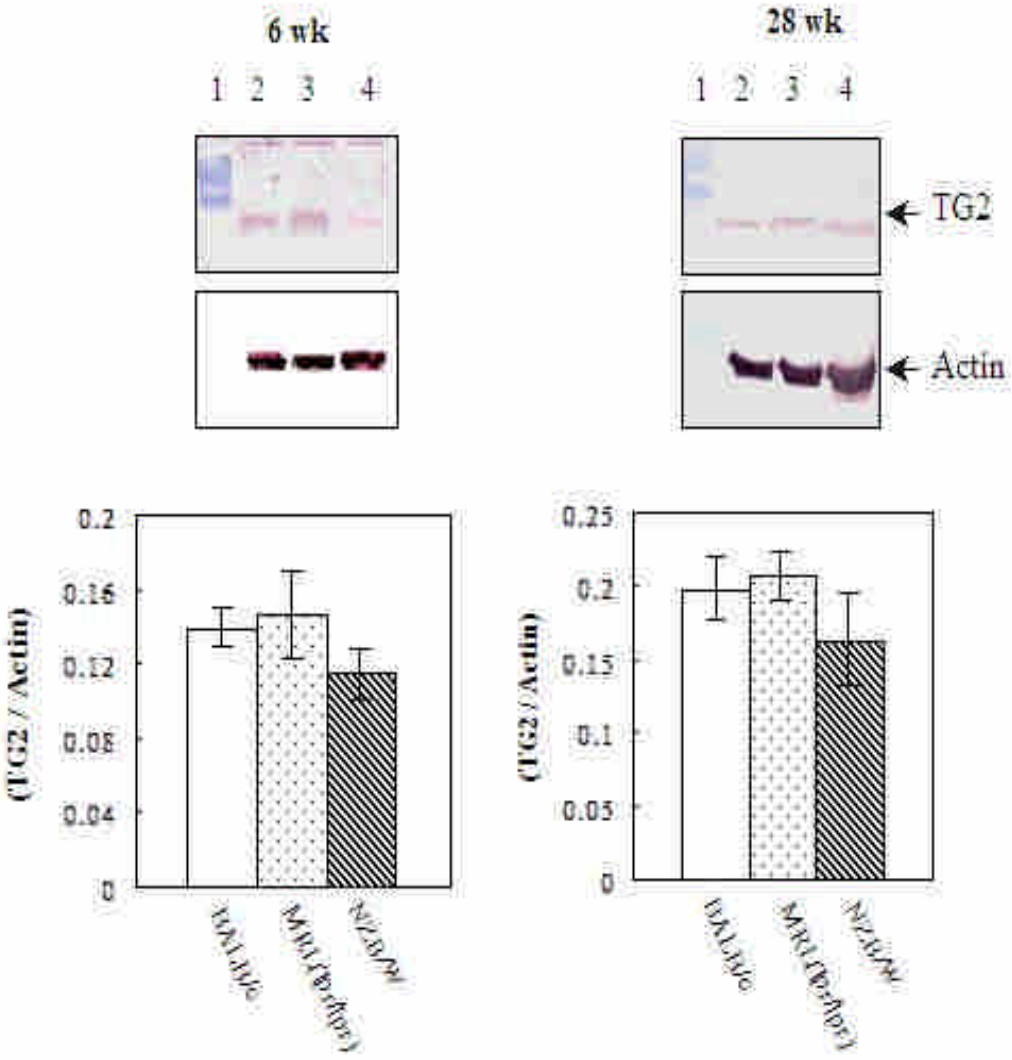


Figure. 10 . Expression of TG2 protein in macrophage from mice at different age. Macrophages derived from mice at age of 6 (left panel) and 28weeks (right panel) were assessed for the TG2 protein expression. The quantified results were shown in bottom panel.

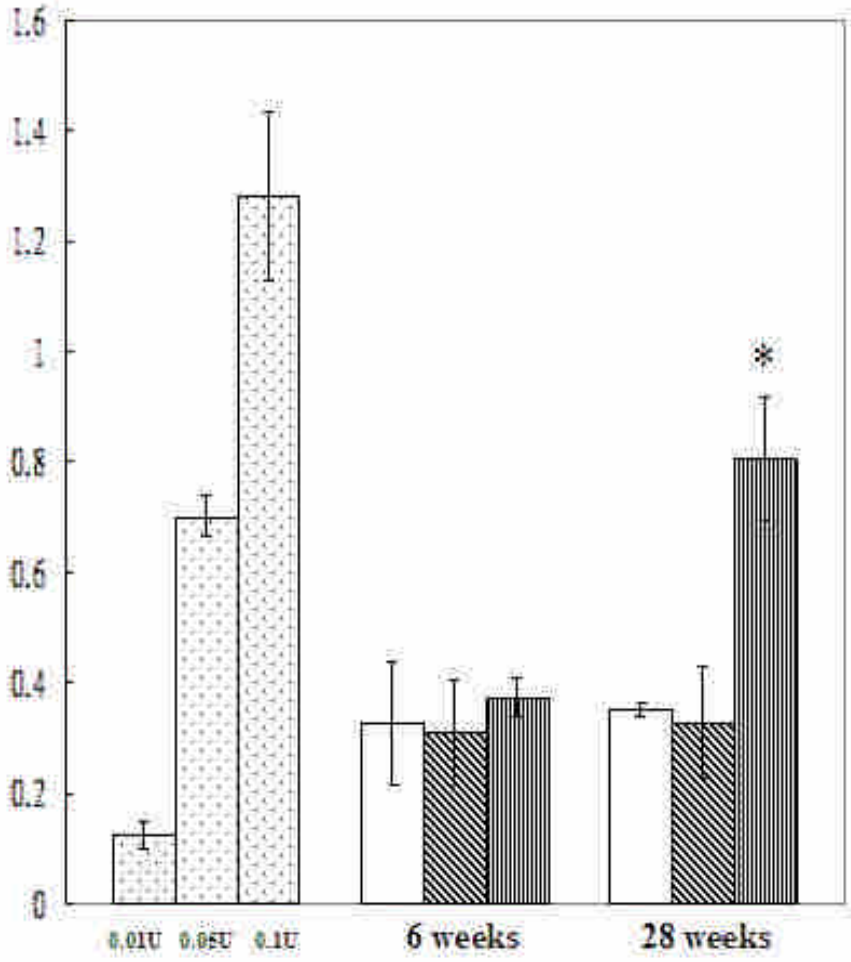


Figure. 11 TG2 activity of macrophages from various mice at age of 6 and 28 weeks. Protein in macrophage from mice at different age were obtained and the TG2 activities were analyzed.

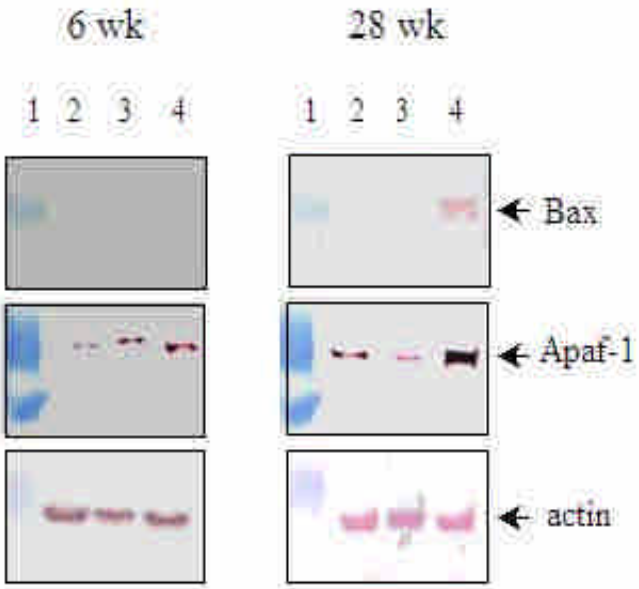


Figure. 12 Detection of pro-apoptotic protein in macrophage derived from different mice at age of 6 and 28 weeks.

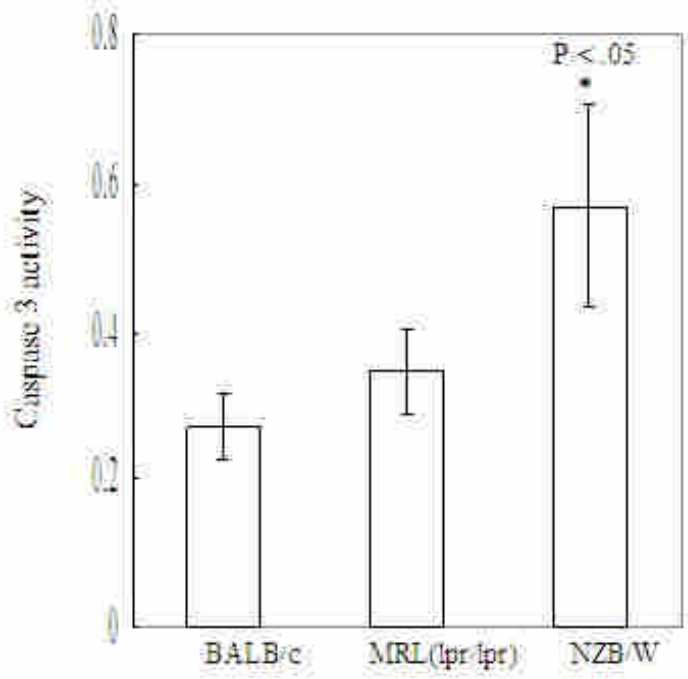


Figure. 13 Activity of Caspase 3. The macrophages from various mice at age of 6 and 28 weeks were isolated and assessed for the caspase 3 activity.

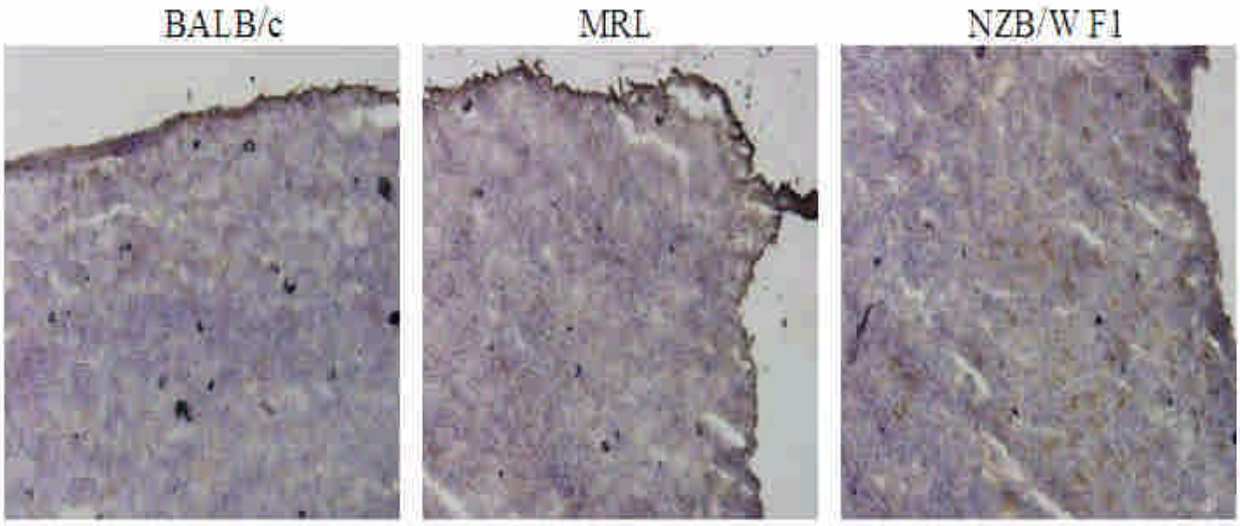


Fig 14 Immunohistology

Kidney tissues were embedded in OCT at -80°C and sectioned at 5 um. Sections were stained with HRP labeled anti-Immunoglobulin antibodies to examine the IC complex. Substrate development and electron microscopic analysis were performed.

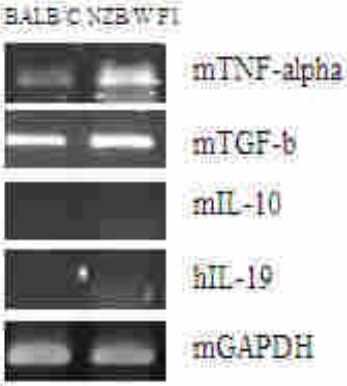


Fig. 15 Various cytokines mRNA expression in macrophages from Mice. The expression of TNF-a and TGF-b is significant higher in macropahges from NZB/W F1 mice.

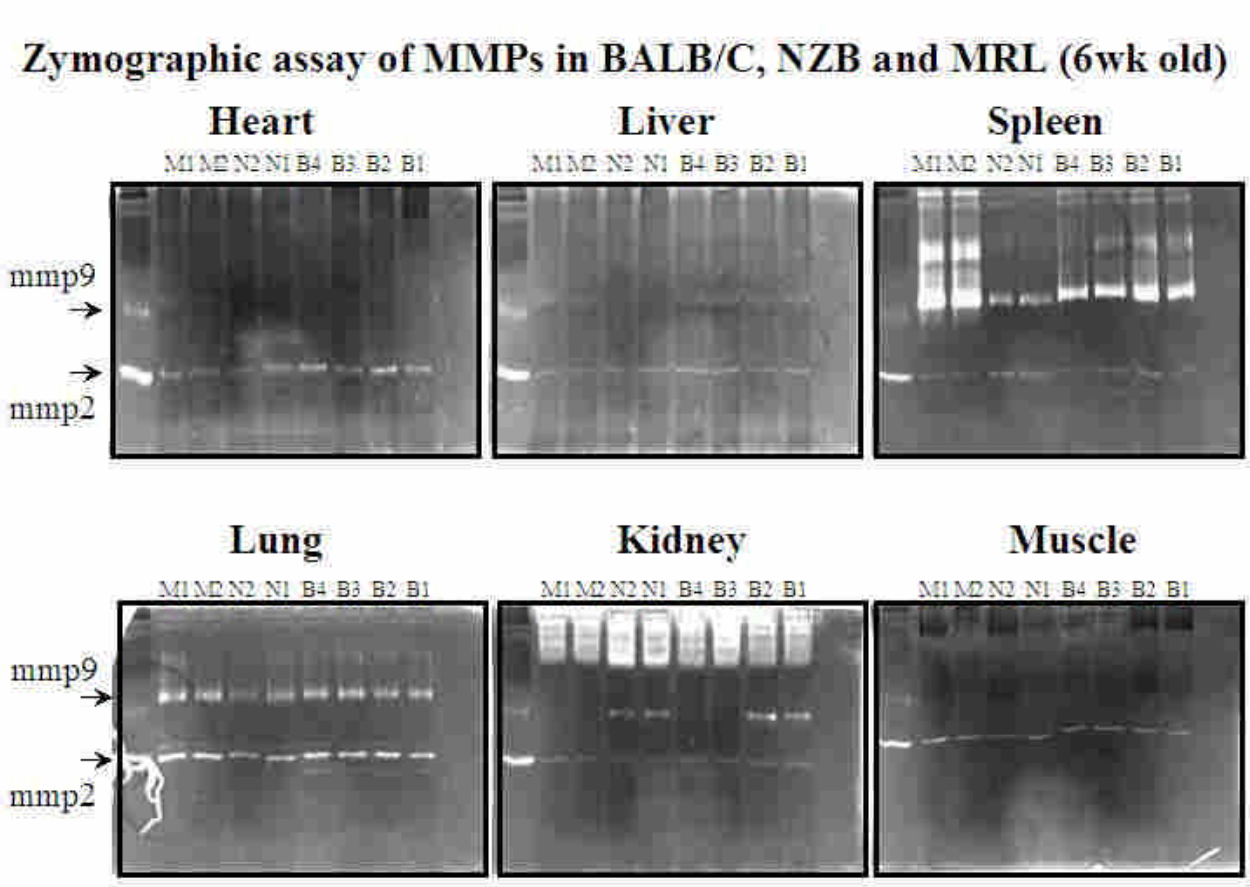


Fig 16. Zymorgraphy analysis of MMP9 and MMP2 activity in various tissues from BALB/c, MRL, and NZB/W F1 mice.

計畫成果自評

本子計劃設定在動物模式的研究，釐清第二型殼胺醯胺轉移胺與狼瘡致病之關聯性。首先實驗設計包含第二型殼胺醯胺轉移胺在各組織樣本的表現量之比較、狼瘡小鼠發病前後之巨嗜細胞吞噬能力的比較、以及巨嗜細胞中第二型殼胺醯胺轉移胺的表現。另外，為了更了解第二型殼胺醯胺轉移胺與吞噬能力間可能的分子機制，我們設計了細胞激素接受器基因之引子，以偵測其 mRNA 之表現。在我們反覆的實驗中，發現類紅班性狼瘡小鼠巨嗜細胞吞噬能力比起對照小鼠稍弱；另一方面，也發現其第二型殼胺醯胺轉移胺呈現表現減弱的現象，不過卻無明顯的變化。這現象雖然顯示狼瘡自體抗原產生，和巨嗜細胞吞噬能力有關，但仍缺乏一個能解釋巨嗜細胞吞噬能力下降的原因。另外在細胞激素接受器基因表現上，亦無差異。但是狼瘡模式小鼠的巨嗜細胞的第二型殼胺醯胺轉移胺活性卻有明顯增高現象。為了找出更確切的原因，本計劃針對於狼瘡模式小鼠的巨嗜細胞，分析其內在變化，找出可能造成吞噬能力下降的原因。在我們的實驗結果中發現，粒線體相關路徑的細胞凋亡蛋白，在狼瘡模式小鼠有較明顯增加，包括 Bax and Apaf-1。這些發現也說明小鼠之巨嗜細胞的吞噬能力較低是因為自身凋亡所致。在其他分析中也發現，狼瘡鼠的 TNF-alpha 及 TGF-beta 的 mRNA 表現量比正常高，可能與 TG2 活性的調節有關，當然此部分需要更進一步探討。另外我們也發現狼瘡鼠脾臟的 MMP9 表現較低，而這與 TG2 與 MMP 的相互間關係仍需要深入研究。相信這些發現及研究結果也將提供研究狼瘡之自體免疫失衡的重要參考。