博士論文

論文題目　**Depletion of $CD4^+CD25^-LAG\text{-}3^+Egr2^+$ Regulatory T cells Using DNA Vaccination Results in Lupus-like Severe Systemic Autoimmunity**

（**DNA ワクチンによる LAG-3 陽性 Egr2 陽性制御性 T 細胞の除去と、ループス様自己免疫病態**）

氏　　名　タニタ　ヌール

Tanita　Noor

Depletion of $CD4^+CD25^-LAG\text{-}3^+Egr2^+$ Regulatory T cells Using DNA Vaccination Results in Lupus-like Severe Systemic Autoimmunity

（DNA ワクチンによる LAG-3 陽性 Egr2 陽性制御性 T 細胞の除去と、ループス様自己免疫病態）

Department of Allergy and Rheumatology

Professor Kazuhiko YAMAMOTO

タニタ　ヌール

Tanita Noor

Table of Contents

ACRONYMS

Ab	Antibody
ANAs	antinuclear antibodies
APCs	antigen presenting cells
Blimp-1	B lymphocyte-induced maturation protein-1
CAG	chicken β-actin promoter
cDNA	complementary DNA
CMV	cytomegalovirus
CP	Connecting Peptide
CTLs	cytotoxic T lymphocytes
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dsDNA	double-stranded DNA
Egr2	early growth response gene 2
Egr2-CKO	Egr2 conditional knockout mice

ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FoxP3	forkhead box P3
GWAS	genome-wide associated study
H_2PO_4	Phosphoric acid
H_2SO_4	Sulfuric acid
IFN-γ	interferon-γ
IgSF	immunoglobulin super family
IL-10	interleukin-10
IL-2	interleukin-2
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
iTregs	Induced Tregs
LAP	Latency associated protein
LAG-3	Lymphocyte activation gene-3
mAb	monoclonal antibody
MHC	major histocompatibility complex
mRNA	messenger RNA

NFAT	Nuclear factor of activated T cells
NK cells	natural killer cells
nTregs	naturally occurring Tregs
PBS	phosphate buffer solution
pDC	plasmacytoid dendritic cells
PP	Peyer's Patch
SLE	Systemic Lupus Erythematosus
TCR	T cell receptor
TGF-β	transforming growth factor-β
Th cells	helper T cells
Tregs	Regulatory T cells
Tr1 cells	Type 1 regulatory T cells

Abstract

$CD4^+CD25^-LAG\text{-}3^+$ T cells are newly reported regulatory T cell subset that plays a significant role in suppressing peripheral inflammation and specifically expresses the transcription factor Egr2. Although the lack of LAG-3 molecule is expected to induce autoimmunity, LAG-3-deficient mice present minimal immunopathological change. I tried to deplete $CD4^+CD25^-LAG\text{-}3^+$ regulatory T cells using immunological procedure in wild type mice and analyzed its sequelae *in vivo*.

In order to deplete $CD4^+CD25^-LAG\text{-}3^+$ T cells, I adopted DNA vaccination procedure and generated pCAGGS-LAG-3 vector construct that contained mouse LAG-3 cDNA sequence. I injected 100 μg pCAGGS-LAG-3 vector into C57BL/6 mice intravenously and observed their pathophysiological conditions.

After several months of vaccination, mice with pCAGGS-LAG-3 vector developed severe dermatitis, Proteinuria and high titer of anti-dsDNA antibody, while control mice were normal. Histopathological analysis revealed glomerulonephritis with IgG/C3 deposition and dermatitis with epidermal hyperplasia, hyperkeratosis and mononuclear cell infiltration. In FACS analysis, pCAGGS-LAG-3 injected mice with severe lupus-like lesion presented the depletion of $CD4^+CD25^-LAG\text{-}3^+Egr2^+$ T cells. Moreover, serum ELISA in these mice revealed high titer of anti-LAG-3 antibody.

DNA vaccination with pCAGGS-LAG-3 was considered to be the main cause of anti-LAG-3 antibody production and the depletion of $CD4^+CD25^-LAG\text{-}3^+Egr2^+$ T cells, which is

responsible for the development of lupus-like lesions. This is the first report about the procedure of efficient $CD4^{+}CD25^{-}LAG\text{-}3^{+}Egr2^{+}$ T cells depletion.

1. Introduction

1.1. Systemic Lupus Erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a common connective tissue disorder that is characterized by the breakdown of tolerance to self–antigen, the production of autoantibody and the formation of immune complexes. Circulating immune complexes lead to the complement activation and inflammation, and deposit on the basement membranes of multiple target organs including skin and kidneys. Serum antinuclear antibodies (ANAs) are found in nearly all individuals with SLE. Moreover, antibodies to double-stranded DNA (dsDNA) are specific for SLE and reflect the disease activity of SLE [1].

The pathogenesis of SLE involves immune disturbances which includes autoantibody production and hyperactivation of B cell and T cell. One widely proposed mechanism for the development of autoantibodies involves a dysregulation in apoptosis that causes increased cell death and a disturbance in immune tolerance [2, 3]. Regulatory T cells (Tregs) contribute to the maintenance of self-tolerance [4]. Tregs in MRL/lpr mice are not properly stimulated by antigen presenting cells (APCs) and are unable to suppress proinflammatory cytokine secretion from effector T cells [5].

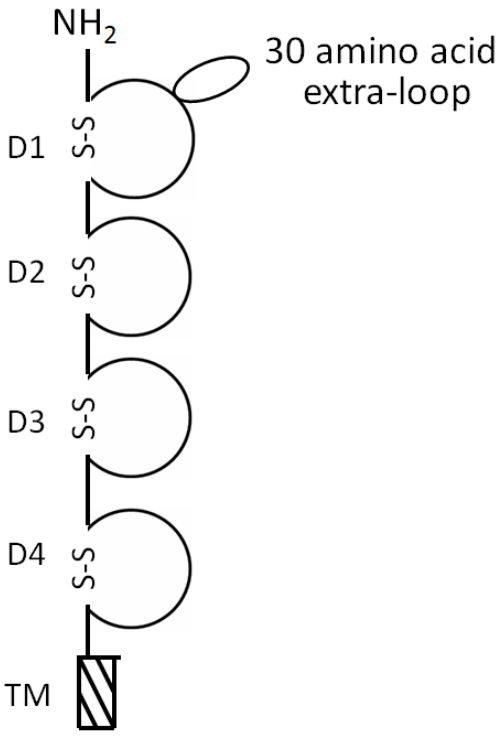
1.2. Lymphocyte Activation Gene-3 (LAG-3)

Our immune system is equipped with a number of mechanisms to fine-tune the immune responses, which helps to reject the invading pathogens efficiently with a little damage to the

host cells. Multiple regulatory pathways are working to maintain this equilibrium and one molecule which is taking part in this well-orchestrated mechanism is lymphocyte activation gene-3 (LAG-3).

LAG-3 is a transmembrane protein and a member of immunoglobulin super family (IgSF) with four extracellular Ig like domain and binds with major histocompatibility complex (MHC) class II molecule which is homologous to CD4 (Supplemental figure 1) [6, 7]. CD4 and LAG-3 both binds toMHC class II, however LAG-3 binds with 100 fold higher affinity than CD4 [8, 9]. Moreover, while CD4 acts as a positive co-stimulatory molecule, LAG-3 acts as a negative regulator of TCR-mediated signal transduction, proliferation and homeostasis [10, 11]. Unlike CD4, LAG-3 is expressed on multiple cells including plasmacytoid dendritic cells (pDC) [12], B cells [13], NK cells [14], γδ T cells [15], tumor-infiltrating lymphocytes [16], exhausted $CD8^+$ T cells [17], and activated $CD4^+$ T cells [18].

The inhibitory function of LAG-3 was observed on activated T cells, where the simultaneous cross-linking of the T cell receptor (TCR) and LAG-3 causes decreased calcium flux when compared to TCR cross-linking alone and reduce IL-2 production and Th1 polarization [19, 20]. In contrast, the blockade of LAG-3 with anti-LAG-3 antibody during antigen specific T cell stimulation led to increased T cell proliferation and function [21]. These studies illustrate that the interaction between MHC class II bearing antigen presenting cells and $CD4^+LAG\text{-}3^+$ T cells induces a T cell-intrinsic inhibitory signaling pathway, which indicates LAG-3 as a cell-intrinsic inhibitory molecule. However, LAG-3 knockout mice, which were first generated in the late 1990's [22], displayed apparently normal pathological condition and showed no defect on T cell function [23].



Supplemental figure 1. Structure of LAG-3

1.3. Regulatory T cell (Treg)

Regulatory T cell (Treg) is crucial to maintain immune homeostasis and avoid immune-mediated pathology by inhibiting the activity of effector T cells. There are two groups of Tregs: one termed as naturally occurring Tregs (nTregs) and other known as induced Tregs (iTregs). nTregs are normally generated in thymus, while iTregs developed in the periphery from naïve T cells following antigen-specific activation in an immunosuppressive condition. nTregs are mainly $CD4^+CD25^+$ T cells and characteristically express transcription factor forkhead box P3 (FoxP3) [24]. Scurfy mutant mice (having mutation in the FoxP3 gene) exhibits a fatal lymphoproliferation and severe inflammatory infiltration in multiple organs [25] which is similar to human disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked

syndrome) [26, 27]. This confirms that Treg is indispensable in maintaining self-tolerance and in the prevention of autoimmune diseases.

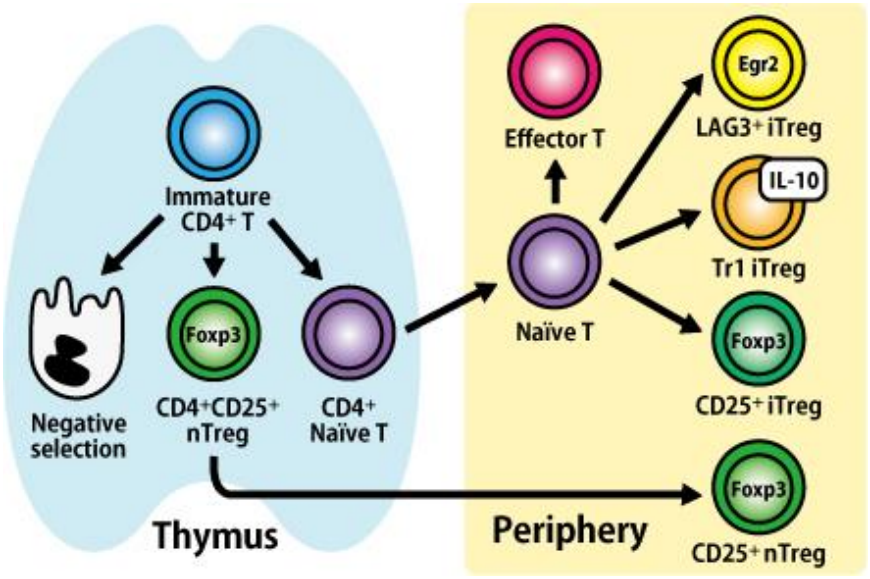
Regarding peripherally induced iTreg, there are several reports about regulatory T cell subsets other than conventional $CD4^+CD25^+Foxp3^+$ Treg. For example, Roncarolo et al. reported IL-10-secreting $CD4^+FoxP3^-$ T cells, named as type 1 Tregs (Tr1 cells) [28]. Ochi et al. reported $CD4^+CD25^-LAP^+$ T cells produce both IL-10 and TGF-β, and suppress autoimmune encephalomyelitis [29].

1.4. $CD4^+CD25^-LAG\text{-}3^+$Tregs

In naïve animals, LAG-3 expression on $CD4^+$ T cells is mainly restricted to the $CD4^+CD25^-CD^{45}RB^{low}$ memory T cells. In a recent study, Okamura et al. found that $CD4^+CD25^-LAG\text{-}3^+$ T cells are in high numbers in Payer's patch and in smaller number in the spleen, mesenteric lymph node and inguinal lymph node. These $CD4^+CD25^-LAG\text{-}3^+$ T cells produce elevated amounts of IL-10, Blimp-1 and LAG-3 mRNA than other T cell subsets and also express IFN-γ, but did not express the Treg marker Foxp3 [30]. Transcription factor Blimp-1 is necessary for the production of IL-10 by $CD4^+$ T cells [31] and also required for the formation of IL-10 producing effector Tregs [32]. $CD4^+CD25^-LAG\text{-}3^+$ T cells secret high amount of IL-10 upon antigenic stimulation and inhibit the development of colitis in an IL-10 dependent manner. As $CD4^+CD25^-LAG\text{-}3^+$ Tregs from scurfy mice also retained regulatory activity in the colitis model, the function of $CD4^+CD25^-LAG\text{-}3^+$ Tregs was independent of Foxp3.

$CD4^+CD25^-LAG\text{-}3^+$ Tregs characteristically express the transcription factor Early growth response gene 2 (Egr2), and intracellular staining of Egr2 revealed a strong correlation

between Egr2 and LAG-3 expression in $CD4^+$ T cells. Retroviral gene transfer of Egr2 gene makes $CD4^+$ naïve T cells to have regulatory activity and express LAG-3 and IL-10 [30]. Egr2 is considered as key transcription factor of $CD4^+CD25^-LAG\text{-}3^+$ Tregs (Supplemental figure 2).



Supplemental figure 2. Schematic view of regulatory T cell subsets

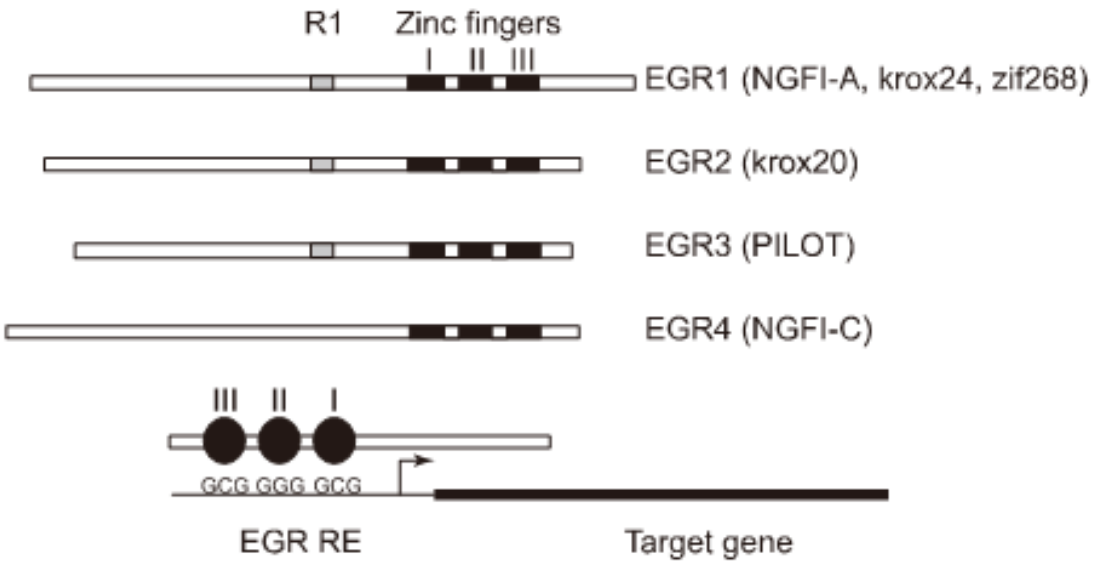
1.5. Early Growth Response Gene 2 (Egr2)

Early growth response (Egr) is a transcription factor that binds with DNA through a highly conserved Cys2His2-type zinc finger domain (Supplemental figure 3) [33]. There are four members of Egr family: Egr1, 2, 3 and 4. They play a key role in coordinating gene expression that underlie the long-term changes in various biological processes including proliferation, apoptosis, and differentiation [34]. Egr2 has been found to play an essential role in hindbrain development and myelination of the peripheral nervous system as null mutation of Egr2 resulted in perinatal or neonatal death due to respiratory and feeding deficits [35].

Egr2 plays a critical role in anergy induction in T cells. Egr2 is induced early upon TCR engagement in a nuclear factor of activated T cells (NFAT) dependent manner *in vitro*, which causes inhibition of T cell activation. Egr2 is also induced *in vivo* by the antigen stimulation in tolerant T cell and deletion of Egr2 prevents the induction of anergy [36, 37]. Another study also proposed that both Egr2 and Egr3 play role in the formation of T cell anergy by augmenting the expression of the E3 ligase Cbl-b and inhibiting IFN-γ and IL-2 secretion by T cells [38].

Egr2-deficient mice are perinatally lethal [35], and Egr2 conditional knockout mice (Egr2-CKO) in which the Egr2 gene was deleted specifically in T cells and B cells demonstrated massive infiltration of Th1 and Th17 cells in multiple organs and developed a lupus-like syndrome in later stage of their life. This Egr2-deficient T cells did not show the alteration in primary activation but highly proliferated in response to stimulation [39].

These previous observations strongly suggest the pivotal roles of Egr2, Egr3 and $CD4^+CD25^-LAG\text{-}3^+$ Treg in the control of autoimmunity. In order to understand the immunological function of $CD4^+CD25^-LAG\text{-}3^+$ Treg *in vivo* deeply, the analysis of mice without $CD4^+CD25^-LAG\text{-}3^+$ Treg is important. Since $CD4^+CD25^-LAG\text{-}3^+$ Tregs are regulated by Egr2, the depletion of $CD4^+CD25^-LAG\text{-}3^+$ Tregs in Egr2-CKO mice was expected. However, there were still IL-10 expressing $CD4^+CD25^-LAG\text{-}3^+$ Tregs, although they lost their suppressive function to B cells (unpublished data). I speculate that this is due to the compensatory function of other factors including Egr3. As mentioned above, the phenotype of LAG-3-deficient mice is almost normal [22, 40]. Moreover, administration of monoclonal antibody against mice LAG-3 did not deplete $CD4^+LAG\text{-}3^+$ T cells; just affected the function of $CD8^+$ T cells [16]. In order to produce an efficient anti-LAG-3antibody that enables depletion of $CD4^+CD25^-LAG\text{-}3^+$ Tregs, I adopted DNA vaccination procedure.



Supplemental figure 3. Early growth response (Egr) family [33]

1.6. DNA Vaccination

DNA vaccination was reported in the early 1990s as a time- and labor-saving unique method compared to the conventional vaccination using foreign protein [41-43]. DNA vaccines basically need the preparation of a plasmid in which eukaryotic and synthetic sequences administered by genetic engineering. Plasmids are circular double-stranded DNA that contains replication origin, drug resistance gene and a multiple cloning site for insertion of DNA fragment [44].

DNA vaccination can induce a wide range of immune response. Several reports showed that plasmid DNA elicits both humoral and cellular immunity (Supplemental figure 4) [45]. Iwasaki et al. proved that antigen presenting cells (APCs) presented the encoded antigen after immunizing with plasmid vector, which proves antigen uptake by APCs mainly dendritic cells [46]. These APCs are capable of activating both MHC class I and MHC class II pathways, resulting in the activation of all arms of the immune response including helper T cells, cytotoxic

T lymphocytes and B cells. By inducing $CD8^+$ and $CD4^+$ T cells, this vaccination mimics some aspects of natural infection of the hosts and differs with traditional antigen-based vaccines that generally induce only antibody response [47]. Till today this vaccination possibly applied for development of protective vaccinations, control of tumors and the management of allergies and autoimmune diseases [44].

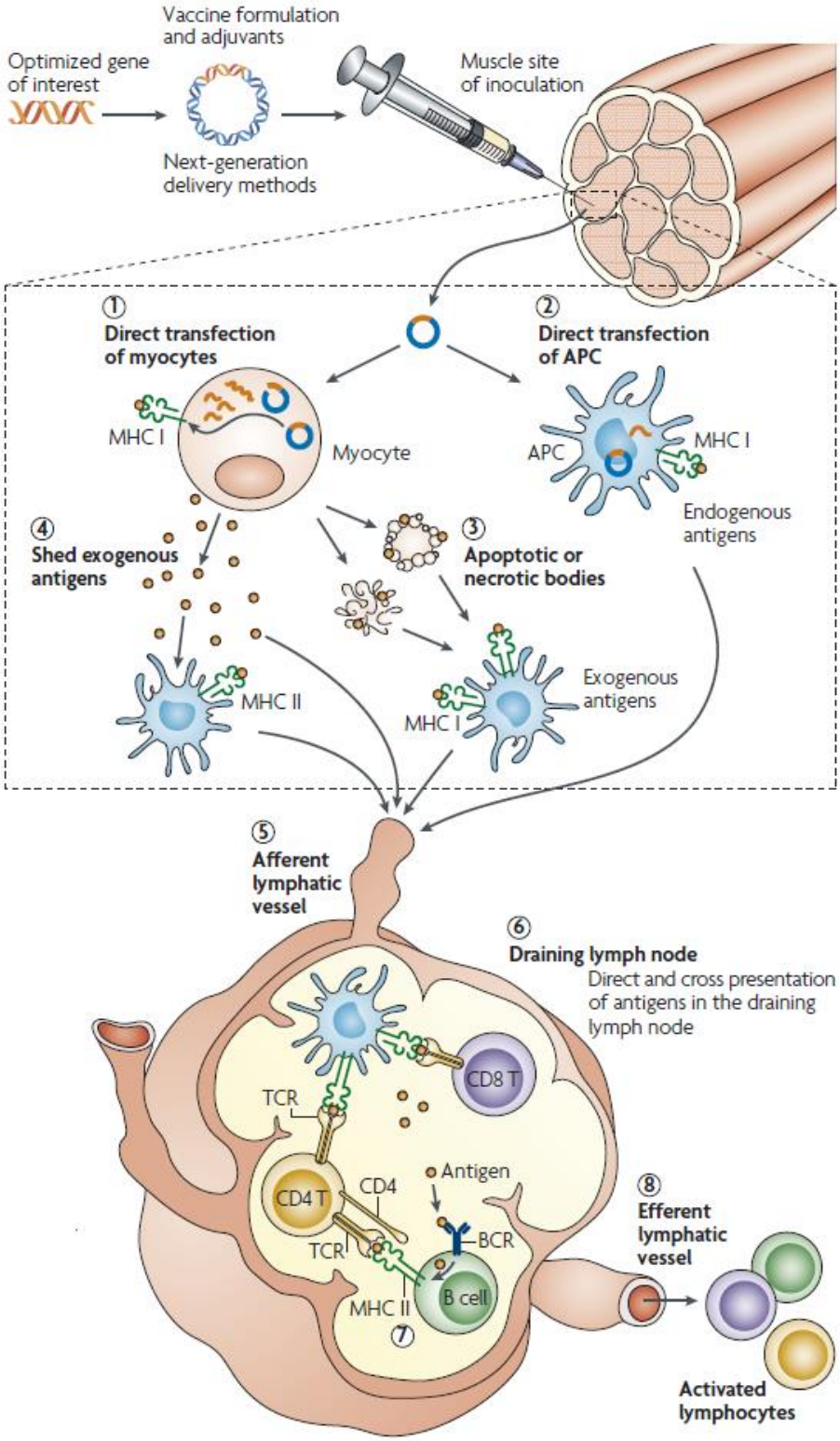
DNA vaccination is a simple, versatile and safe immunization platform. However, the main limitation is the relative impotency. DNA vaccination usually requires multiple boosts with high doses to raise responses comparable to that achieved from a single virus vaccination [48]. Multiple strategies had been taken in order to enhance the potency of DNA vaccine.

In order to increase gene expression, the human cytomegalovirus (CMV) immediate-early enhancer/promoter is most commonly used [49]. However, CMV promoter has been down regulated in the presence of IFN-γ, and inactive in the skin [50]. Consequently, because of the need for more consistent and stronger gene expression, vectors containing a hybrid CMV enhancer coupled with a modified chiken β-actin promoter (CAG) had been used for DNA vaccination [51].

Plasmid can be delivered by intramuscular [43], epidermal [52], intradermal [53], intravenous [54], intranasal [55]. Intramuscular and epidermal injection are the major inoculation route, however several reports described that the intravenous inoculation is also effective [43, 54]. Moreover, in order to increase the uptake of APCs, inoculation with gold colloids [54], employing gene gun [52], lidocaine administration before intramascular injection [56] had been reported.

Based on these findings, I adopted DNA vaccination procedure for successful immunization. I selected pCAGGS vector consist of CAG promoter that is mostly used for DNA

vaccination. I immunized C57BL/6 mice with pCAGGS-LAG-3 vector, and found that the mice developed severe lupus-like systemic autoimmunity including dermatitis, antibody production and deposition of immune-complexes in organs. Anti-LAG-3 antibody production was also found, and depletion of $CD4^{+}CD25^{-}LAG\text{-}3^{+}$ Tregs in mice was detected. Although the detailed mechanism of the depletion is still to be considered, DNA vaccination and antibody production is estimated to be related closely. This procedure is the first report about the efficient depletion of $CD4^{+}CD25^{-}LAG3^{+}Egr\text{-}2^{+}$ T cells.



Supplemental figure 4. Schematic view of DNA vaccination [45]

2. Materials and Methods

2.1 Plasmid DNA

The Plasmid pCAGGS-LAG-3 vector was constructed by cloning the PCR product of mouse LAG-3 D1-D3 portion and then this portion was inserted into pCAGGS vector. Plasmid DNA encoding LAG-3 gene was amplified in Escherichia coli JM 109 bacteria and purified by using Qiagen Plasmid Purification Kit (QiagenPlasmid Maxi Kit). The quantity and quality of the purified plasmid DNA were assessed from the optical density at 260 and 280 nm. The insertion sites of the plasmid DNA was confirmed using restriction enzyme EcoRI. The empty plasmid pCAGGS was used as control.

2.2 Detection of protein in transfected cells

To conform the expression of protein mediated by the vector, 293T human embryonic kidney cells were plated in 60 mm diameter tissue culture dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) 24 hours prior to transfection. They were then transfected with the plasmid DNA after mixing them with FUGEN6 Transfection Reagent (Roche Applied Science) according to the manufacturer's instruction. 48 hours after transfection, the cells were lysed. The lysates were separated by 7.5% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane by using an electroblotting apparatus. The membrane was then blocked by using 5% skim milk and incubated with anti mouse LAG-3 antibody (R&D Systems. cat. No. AF3328) followed by a goat anti-rabbit IgG antibody (BETHYL Laboratories.INC) and finally detected with ECL.

2.3 Animals

9 to 16 weeks C57BL/6 mice were used in these experiments (purchased from Japan SLC) and maintained under specific pathogen-free conditions. All animal procedures were conducted according to the guidelines of the University of Tokyo Institutional Animal Care and under an approved protocol. For all experiment female mice were used.

2.4 DNA vaccination

Different routes of administration were used for DNA inoculation. Mice were immunized with plasmid DNA encoding LAG-3 protein intravenously, intradermally and intramuscularly. 9-16 weeks C57BL/6 mice received either single or three repeated vaccination with 100 μg of each vector. For the intravenous inoculation, tail vein was used by taking all aseptic precaution. For intramuscular inoculation, the mice were pretreated with 50 μl 0.25% Lidocaine hydrochloride [57] in each quadriceps muscle one day before the first vaccination in order to enhance the muscle cell uptake of plasmid DNA. 100 μg of pCAGGS-LAG-3 vector was used three times at three weeks intervals. Mice immunized with empty pCAAGS vector were served as negative control.

2.5 Anti-dsDNA antibody ELISA

Mice blood was collected by retrobulbar puncture using glass pipettes or by tail bleeding at different time points. Serum samples were collected from the blood and used for antibody assays. The titers of anti-dsDNA antibody were measured by enzyme-linked immunosorbent

assay (ELISA) using Shibayagi's mouse anti-dsDNA antibody ELISA kit (code. No. AKRDD-061). ELISA was performed sequentially by following the maker's protocol. In brief, strips pretreated with antigen were washed and 100 µl serum samples were applied after diluting with the supplied buffer. Dilution rate was 100x and 500x. After two hours of incubation at room temperature, the plates were washed three times with wash buffer. 100 µl of diluted secondary antibody was added and incubated for another 2 hours at room temperature. Next, the plate was washed for three times and color was developed by adding TMB solution following 20 minutes incubation. The reaction was stopped by adding 1M H_2SO_4. The absorbance was measured at 450 nm on a microplate reader.

2.6 Anti-LAG-3 antibody ELISA

To detect anti-LAG-3 antibody, mice serum samples were collected as described above. Then the titers of antibody against LAG-3 were evaluated using ELISA. Briefly, mouse LAG-3-Fc (IgG2a) chimera protein (Enzo ALX-522-099) was used as antigen. The 96-well Nunc plates were coated overnight at 4°C. After washing three times with phosphate buffer solution (PBS) containing 0.05% Tween 20 (PBST), plates were blocked by 300 µl of 3% skim milk in PBS tween (0.05%) at room temperature for three hrs. Next, 100 µl of diluted serum sample were added to each well and incubated overnight at 4°C. Next day the plates were washed three times with wash buffer and 100 µl of diluted horseradish peroxidase (HRP)-conjugated rat-anti-mouse IgG1 (invitrogen) was added to each well followed by 30 minutes incubation at room temperature. Then, the plates were washed with PBST for five times and color was generated by the addition of substrate solution (KPL) for 20 minutes. The reaction was stopped by using 1M H_2PO_4. The absorbance was measured at 450 nm on a microplate reader.

2.7 Histopathology

Mice were anaesthetized with pentobarbital sodium intraperitoneally. Then, they were incised ventrally along the median line from the xiphoid process to the point of chin. For hematoxylin and eosin staining, skin, spleen and kidney were taken out and fixed with 4% paraformaldehyde phosphate buffer solution. For immunofluorescent staining with rabbit anti-mouse IgG and C3d was performed on frozen kidney sections. IgG and C3 deposition was visualized by incubation with anti-rabbit ATTO488 antibody. About kidney histopathological scoring, glomerular score and interstitial inflammation scores were taken. Glomerular scores is the sum of scores for glomerular inflammation, proliferation, crescent formation, and necrosis (each scored from 0 to 4). Interstitial inflammation was also scored 0-4 [58]. For Lung injury score, alveolar and periluminal injury scores were taken based on the former report [59]. Ten fields at ×400 magnification were viewed for each lung section and scored for alveolar infiltration as follows: 0= no infiltrate was present, 1= the infiltrate could be visualized easily only at ×400 magnification, 2= infiltrates were readily visible, and 3= consolidation. Similarly, each section was scored for periluminal damage (airway or blood vessel) at ×100 magnification as follows: 0= there was no infiltrate, 1= the infiltrate was 1–3 cell layers thick, 2= the infiltrate was 4–10 cell layers thick, and 3= the infiltrate was >10 cell layers thick. On the basis of the overall involvement of the section, a severity score was calculated as follows: 1 for 0–25% involvement, 2 for 25–50% involvement, and 3 for >50% involvement. For calculation of the total lung injury score, the means of alveolar and periluminal scores for each section were summed and multiplied by the severity score, which gave a final score of 0–18.

2.8 Cell Isolation and FACS analysis

Cell suspension was prepared from spleen of pCAGGS-LAG-3 immunized and control mice. Splenocytes were harvested from mice after 8 months of vaccination and treated with hemolysis buffer (ACK) to remove red blood cells. 1×10^7 cells from each mouse were Fc blocked (antimouse CD16/CD32 mAb) for 10 minutes and stained with the surface and intracellular staining. Indicated mAbs for surface staining: FITC anti-CD45RB, PE anti-LAG-3, APC-Cy7 Anti-CD4, APC anti-CD25 and for the intracellular staining: Alex488 anti-LAG-3, APC-Cy7 anti-CD25, APC anti-CD4, PE anti-Egr2. After staining, cells were kept on ice for 30 minutes. The antibodies were purchased from e-Bioscience. Then, cells were washed with PBS twice. For intracellular attaining with anti-Egr2 antibody, cells were stained using the FoxP3 staining buffer set (e-Bioscience). Analysis and cell sorting was performed by using FACSVantage (BD). Data were processed with FlowJo software (Tree Star Inc.).

2.9 Statistical analysis

Statistical analysis was performed using GraphPad PRISM5. For parametric data, unpaired two group were compared using student's t-test. For non-parametric data, unpaired two group were compared using Mann-Whitney U-test. Survival analysis was tested using Log-rank test. Pearson's correlation coefficient was analyzed for the correlation between the two groups. A p-value of <0.05 was considered to indicate a significant difference. *, $p<0.05$ and **, $p < 0.01$.

Results

2.10 Construction of pCAGGS vector

To develop the efficient way to deplete $CD4^+CD25^-LAG\text{-}3^+$ regulatory T cells in mice model, I used commercially available monoclonal anti-LAG-3 antibody for intravenous administration at first. However, that did not deplete $CD4^+CD25^-LAG\text{-}3^+$ T cells (data not shown). This follows the result of former report for the administration of monoclonal anti-LAG-3 antibody that did not deplete $CD4^+LAG\text{-}3^+$ T cells, and just affected the function of $CD8^+$ T cells [16].

In order to develop an antibody against LAG-3 that can deplete $CD4^+CD25^-LAG\text{-}3^+$ regulatory T cells efficiently, I adopted DNA vaccination procedure. Following former reports [49], I selected a plasmid vector named pCAGGS to induce DNA vaccination (Figure 1A). The D1-D3 portion of murine LAG-3 cDNA sequence was inserted into the cloning region of pCAGGS vector, and pCAGGS-LAG-3 vector was constructed. After inserting the sequence, expression of the cloned LAG-3 gene was confirmed by western blot analysis using 293 T cells transfected with pCAGGS-LAG-3 construct (data not shown). Insertion of cDNA was confirmed by the enzymatic digestion with restriction enzyme EcoRI and PCR (Figure 1B).

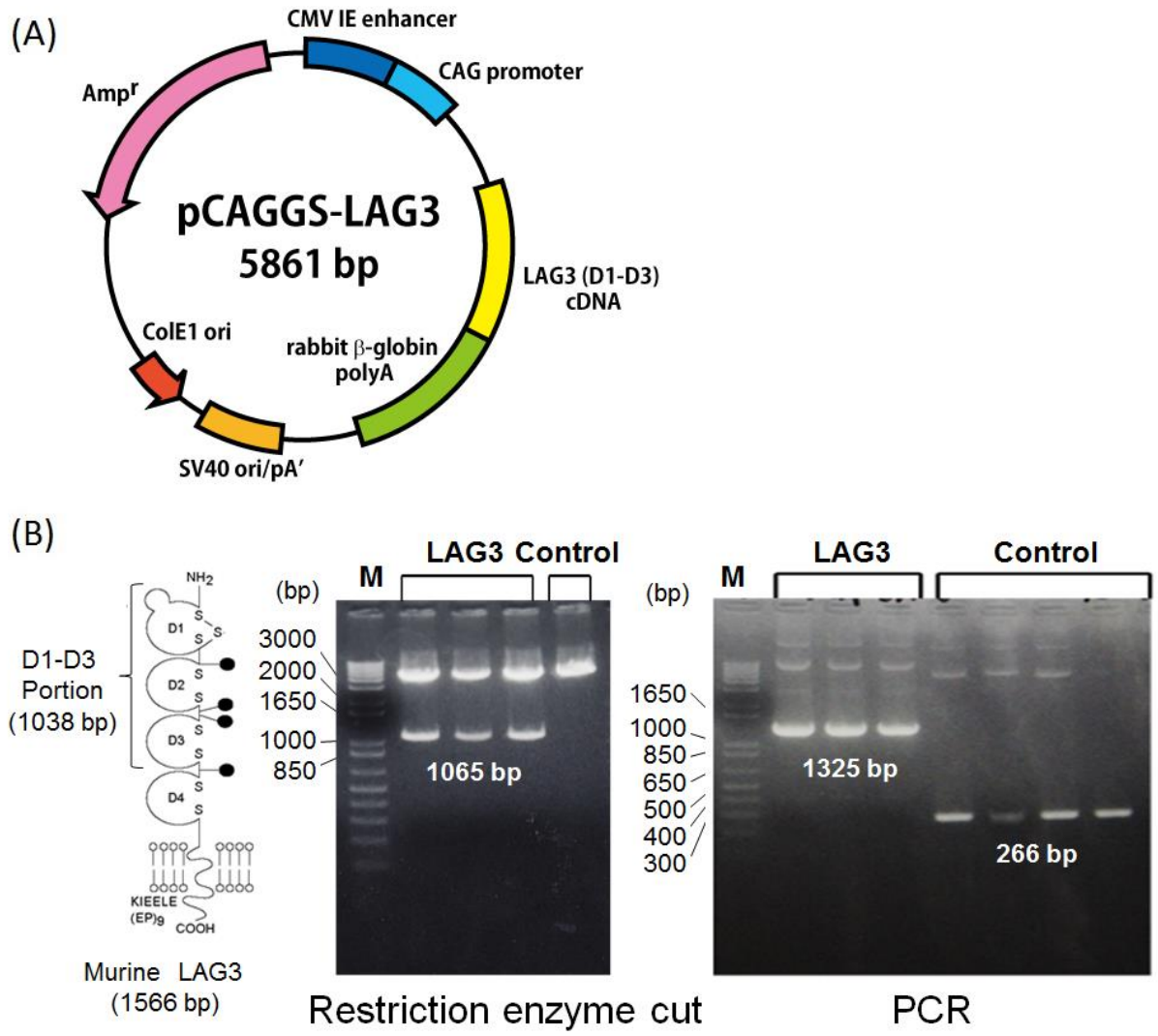


Figure 1. Construction of pCAGGS-LAG-3 vector. (A) Structure of pCAGGS vector. (B) Confirmation of subcloned gene by EcoRI and PCR.

2.11 Development of DNA vaccination protocol

There are several reports for the route of administration of DNA vaccination. Based on the comparison study [49], I set a pilot study in 16 weeks female C57BL/6 mice in order to compare different routes of administration including intravenous, intramuscular and intradermal (Figure 2A). Control mice were immunized with empty pCAGGS vector. I also tried booster vaccination protocols thrice in three weeks apart by following a former report [52]. Surprisingly, several mice developed alopecia and dermatitis, and production of anti-dsDNA antibody. Among these administration protocols, the 100 μg intravenous administration protocol showed typical lupus-like pathological condition repeatedly (Figure 2B). 50 μg and 100 μg intramuscular vaccinations also showed pathological condition, however they took significantly prolonged time. And the comparison of anti-LAG-3 antibody production between intravenous and intramuscular administration showed; 8 months after vaccination only one intramuscular administered mice developed high titer of anti-LAG-3 antibody in their serum while the number in intravenous administration was more than half of the total immunized mice (Figure 2C). Therefore I decided to adopt 100 μg intravenous administration protocol.

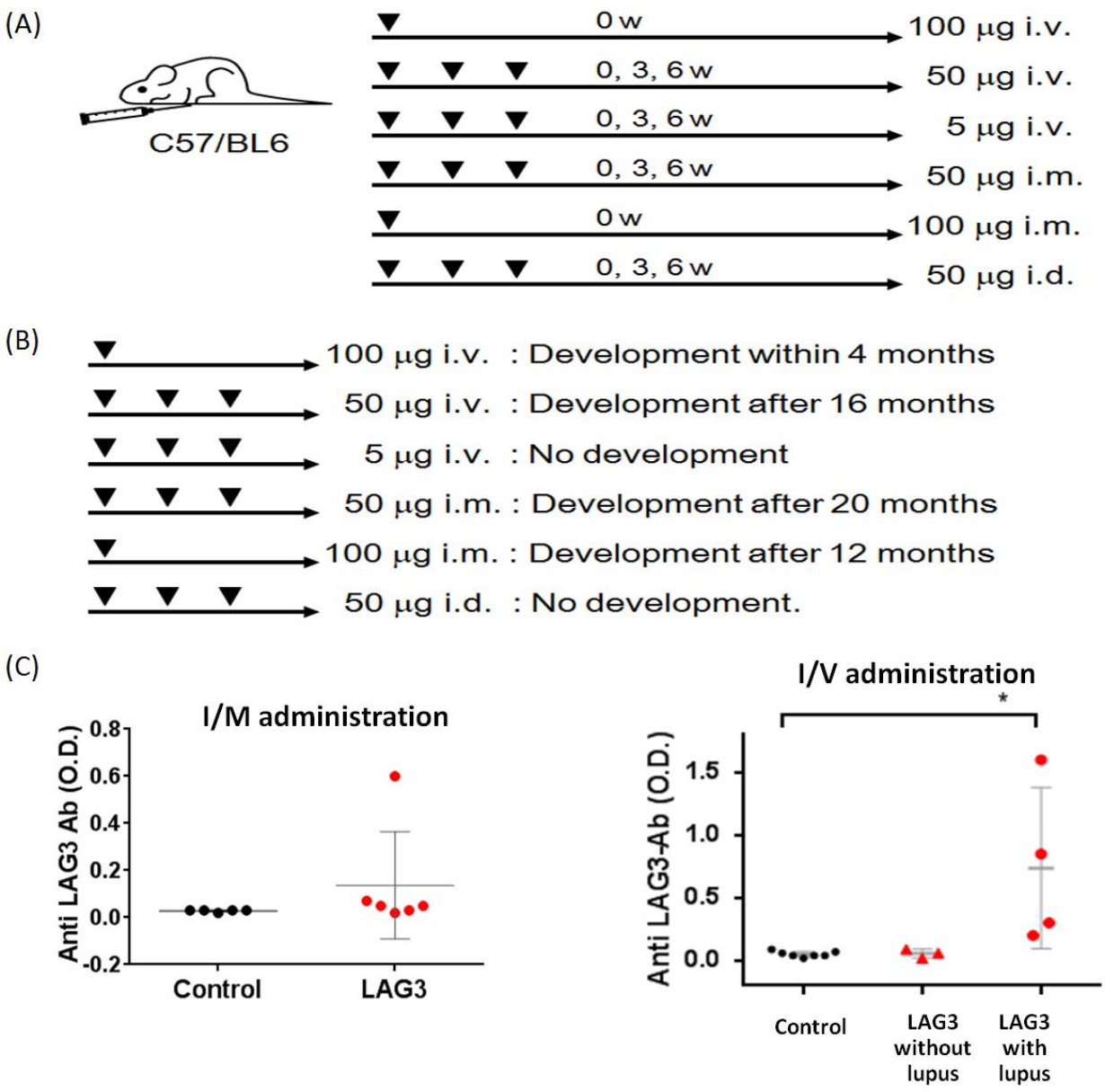


Figure 2. Development of DNA vaccination protocol. (A) Pilot study protocol to compare inoculation route and dose of DNA vaccination. Five to seven mice were used for each group. (B) Result of pilot study. (C) Comparison of Anti-LAG-3 antibody production by intramuscular and intravenous administration. *, P <0.05 (Unpaired t-test).

2.12 DNA vaccination causing proteinuria and anti-dsDNA antibody production

After immunization with pCAGGS-LAG-3 vector, mice were observed for possible outcome, and urine was monitored monthly for any change. In one experiment group I immunized 7 mice for each vector. Among the vaccinated mice, 4 mice immunized with vaccinated with pCAGGS-LAG-3 vector developed typical pathological condition including alopecia, dermatitis, loss of activity, weight loss and proteinuria within eight month of vaccination. At first, the mice started showing hair loss from back and increased protein level in urine while the control mice were normal and healthy. Within three to five months after vaccination, pCAGGS-LAG-3 immunized mice showed significant proteinuria which paralleled with the exacerbation of skin lesion (Figure 3A). The severity of proteinuria of pCAGGS-LAG-3 immunized mice was higher than the control mice (Figure 3B). Furthermore, pCAGGS-LAG-3 immunized mice with lupus-like skin lesion showed detectable level of serum anti-dsDNA antibody in comparison with the control vector immunized mice (Figure 3C). This high production of anti-dsDNA antibody in the serum of pCAGGS-LAG-3-immunized mice may be associated with the development of lupus-like skin lesion. In agreement with the lupus-like pathological condition, survival rate of pCAGGS-LAG-3-immunized mice is significantly low in comparison with the mice immunized with only pCAGGS vector (Figure 3D).

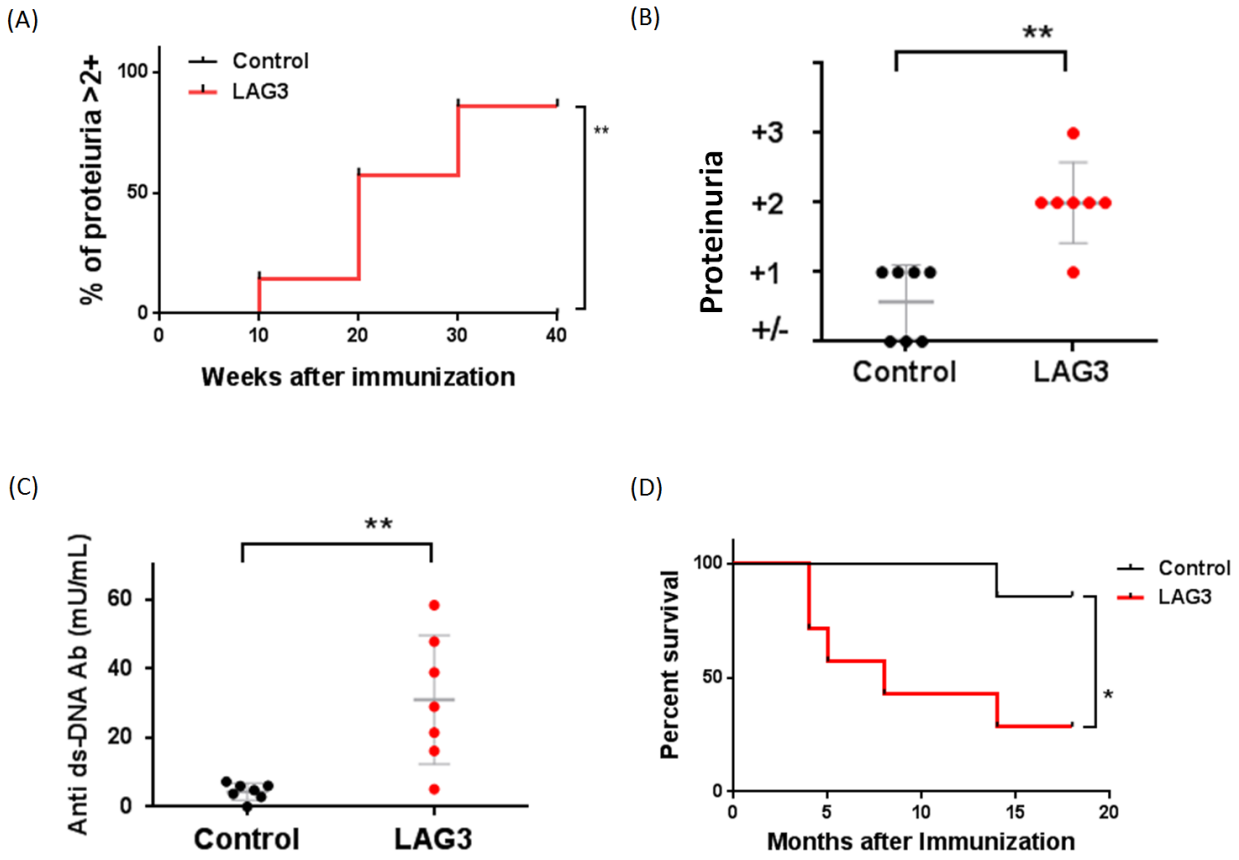
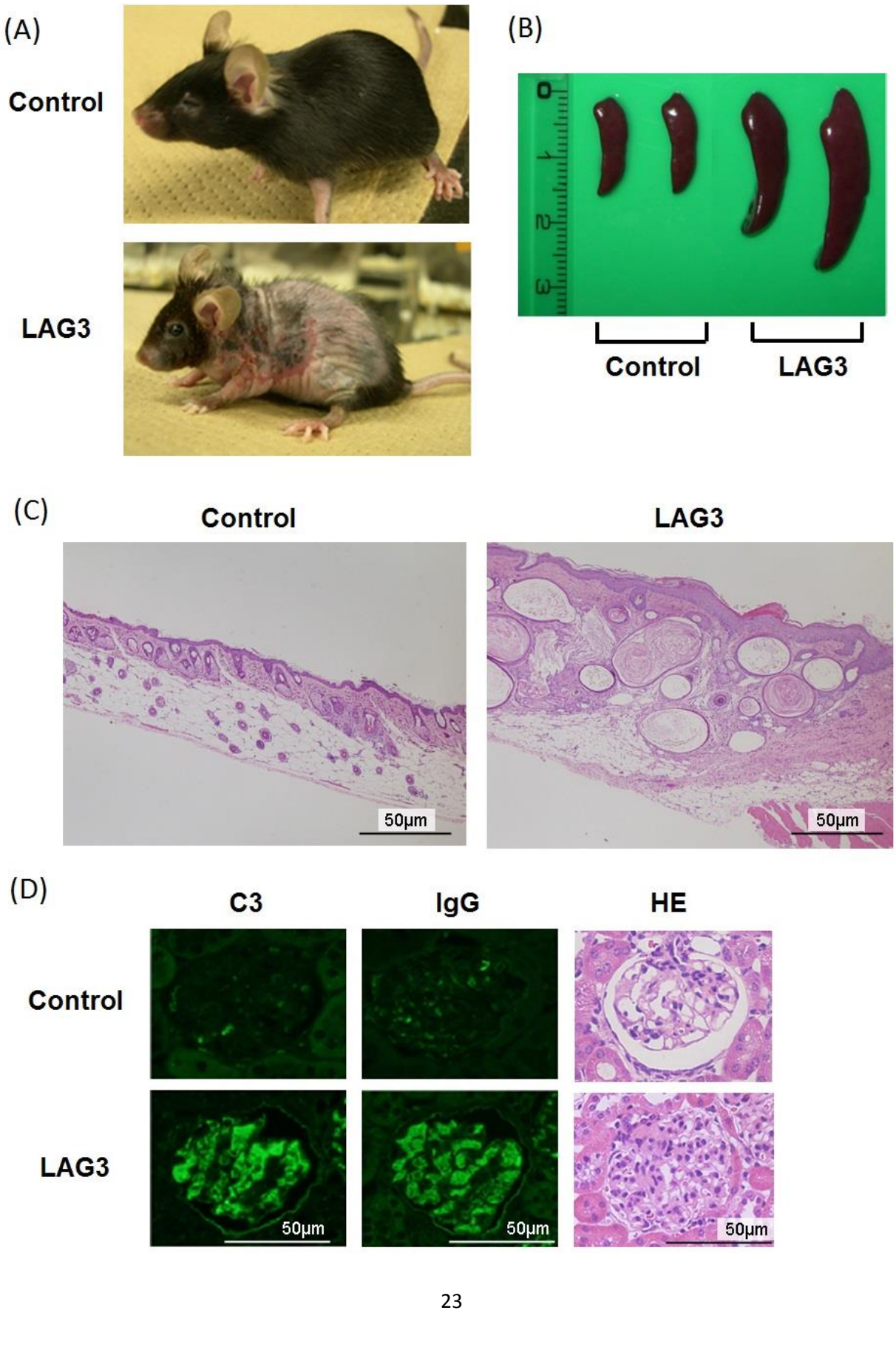


Figure 3. pCAGGS-LAG-3 immunized mice developed proteinuria and anti-dsDNA antibody production. (A) Incidence of proteinuria and (B) Severity of proteinuria in C57BL/6 mice immunized with pCAGGS-LAG-3 vector and empty pCAGGS vector. Each symbol represents an individual mouse. Horizontal line indicates the mean ± s.d. The difference was significant ((A) Log-rank test, (B) Mann-Whitney U-test). **, P <0.01. (C) Serum concentration of anti-ds-DNA antibody in pCAGGS-LAG-3 and empty pCAGGS vector immunized mice (n= 7). Each symbol indicates an individual mouse. Horizontal line shows the mean value. **, P <0.01 (Unpaired t-test). (D) Survival rate of pCAGGS-LAG-3 and empty pCAGGS vector immunized mice (n= 7). The difference was significant (Log-rank test). *, P <0.05.

2.13 DNA vaccination with pCAGGS-LAG-3 vector was associated with lupus-like inflammatory changes in multiple organs

pCAAGS-LAG-3 immunized mice developed hair loss within 3-4 months of vaccination and displayed the features of dermatitis within eight months. Dermatitis developed from the back at the level of ears to the whole back and abdominal region. The severity of the inflammation exacerbated until eight months of vaccination (Figure 4A). On sacrifice, pCAAGS-LAG-3 immunized mice showed obvious splenomegaly compared to the control mice (Figure 4B). Histopathological analysis of skin from pCAAGS-LAG-3-immunized mice revealed prominent epidermal hyperplasia with hyperkeratosis, liquefaction and mononuclear cell infiltration in dermis (Figure 4C). Pathological analysis of kidney showed C3 and IgG deposition in the glomeruli of pCAGGS-LAG-3-immunized mice, whereas the control mice were normal (Figure 4D). Histopathological score of renal disease revealed the glomerulonephritis of mice immunized with pCAAGS-LAG-3 vector with lupus-like lesion (Figure 4E). Reflecting the fulminant infiltration of lymphocyte in pCAAGS-LAG-3-immunized mice, pathological analysis of lung presented lymphocyte infiltration around bronchiole (Figure 4F), and lung injury score shows remarkable tissue damage in pGAGGS-LAG-3 immunized mice with lupus lesion (Figure 4G).


(A)
Control
LAG3
(B)
Control
LAG3
(C)
Control
LAG3
50μm
50μm
(D)
C3
IgG
HE
Control
LAG3
50μm
50μm
50μm

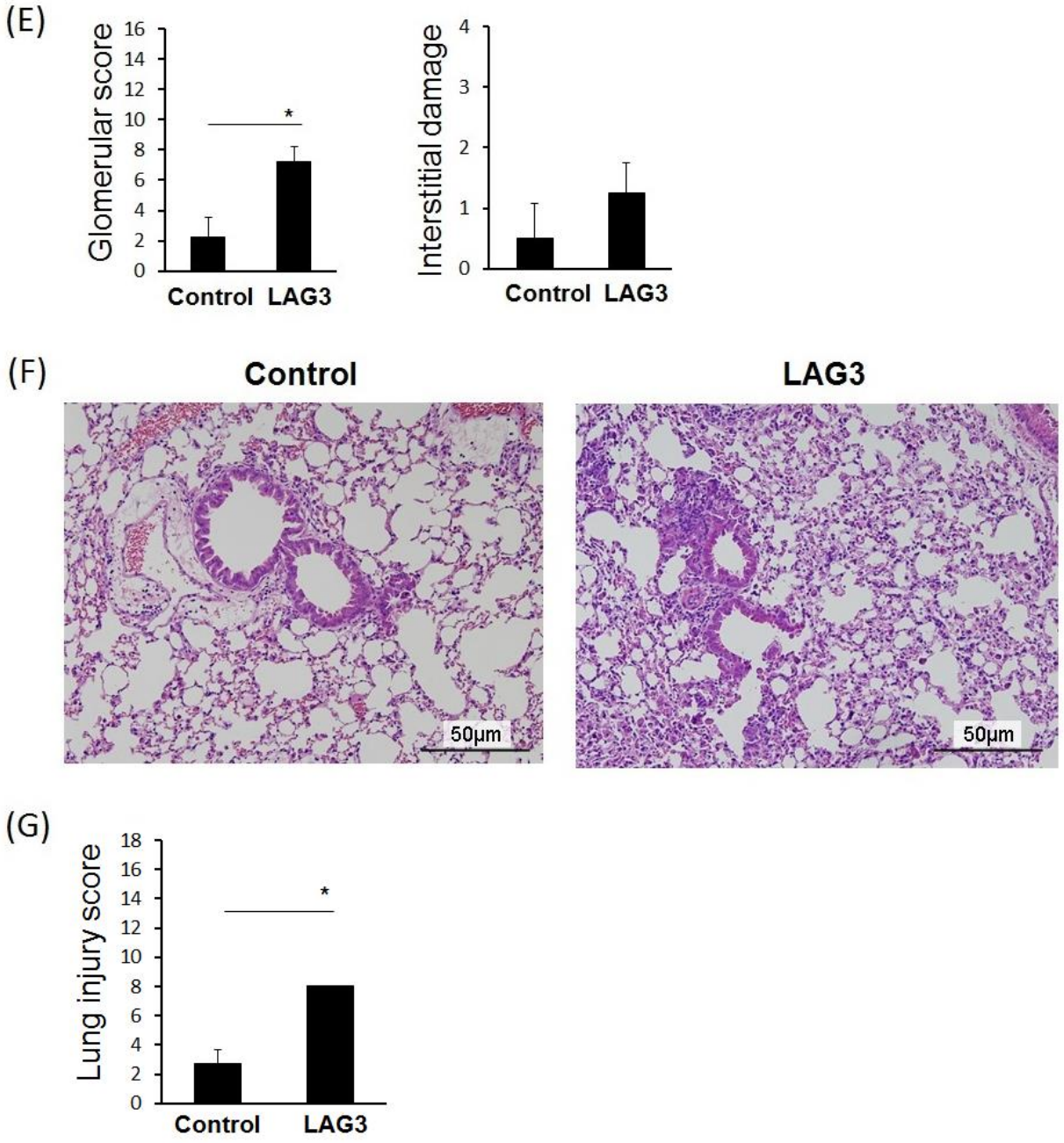


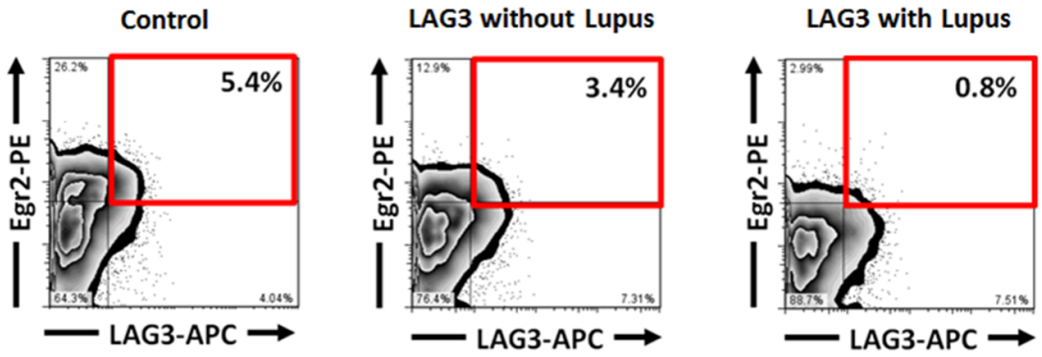
Figure 4. Pathological change in mice after DNA vaccination. (A) Representative image of mice immunized with pCAGGS-LAG-3 after developing skin lesion compared with age-matched control mice. (B) Spleen size of pCAGGS-LAG-3 immunized mice 8 months after vaccination compared with control mice. (C) Pathological analysis of skin of mice immunized with pCAGGS-LAG-3 vector after 8 month of vaccination compared with control mice. Section of the

indicated tissue was stained with Hematoxylin and eosin (HE). (D) Pathological analysis of kidney presenting C3/IgG immune complex deposition in glomeruli and HEs staining. (E) Histopathological score of renal disease. A total of four mice in each group. *, $P < 0.05$ (Mann-Whitney U-test). (F) Pathological analysis of lung presenting lymphocyte infiltration around bronchiole stained with HE. (G) Lung injury score. A total of four mice in each group. *, $P < 0.05$ (Mann-Whitney U-test).

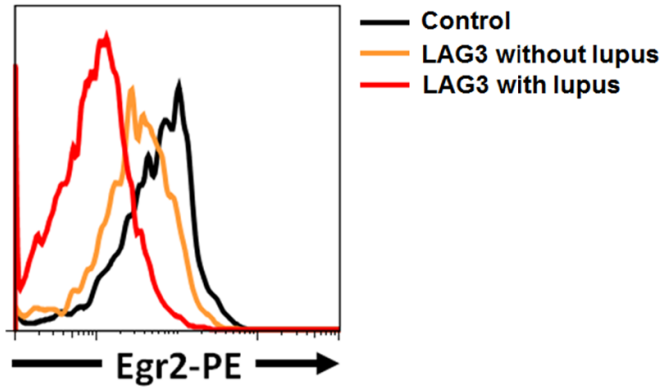
DNA vaccination with pCAGGS-LAG-3 vector induced the depletion of $CD4^+CD25^-LAG\text{-}3^+Egr2^+$ T cells

In order to evaluate the cause of lupus-like pathological condition in pCAGGS-LAG-3 immunized mice, I analyzed the splenocytes by FACS analysis. Mice splenocytes were stained for LAG-3 and Egr2 both extracellularly and intracellularly. Interestingly, the pCAGGS-LAG-3-immunized mice with lupus-like pathological condition showed significant depletion of $CD4^+CD25^-LAG\text{-}3^+Egr2^+$ T cells in comparison with the control mice and pCAGGS-LAG-3-immunized mice without lupus-like pathological condition (Figure 5A). The expressions of Egr2 among $CD4^+CD25^-LAG\text{-}3^+$ T cells showed a significant decrease in pCAGGS-LAG-3-immunized mice (Figure 5B). Percentage of $CD4^+CD25^-LAG\text{-}3^+Egr2^+$ T cells in pCAGGS-LAG-3 immunized mice displayed a significant reduction compared to the control mice while the percentage of other T cell subsets like $CD4^+CD25^+$T cells, $CD4^+CD25^-LAG\text{-}3^+Egr2^-$ T cells and memory T cells were comparable in both vector immunized mice (Figure 5C).

(A)


Control
LAG3 without Lupus
LAG3 with Lupus
5.4%
3.4%
0.8%
Egr2-PE
LAG3-APC

(B)


Control
LAG3 without lupus
LAG3 with lupus
Egr2-PE

(C)

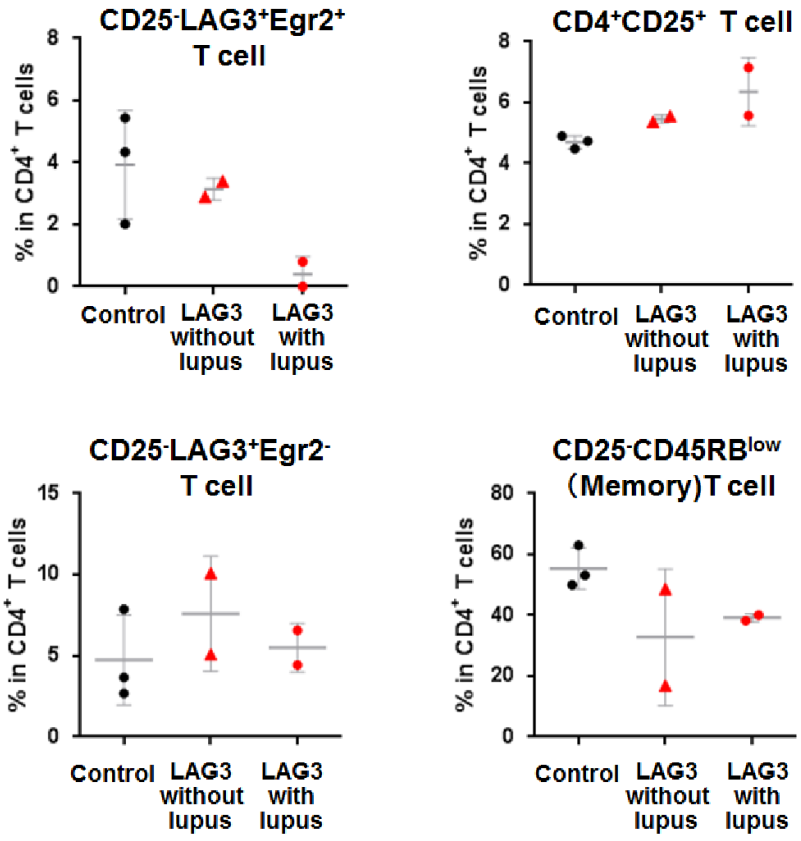
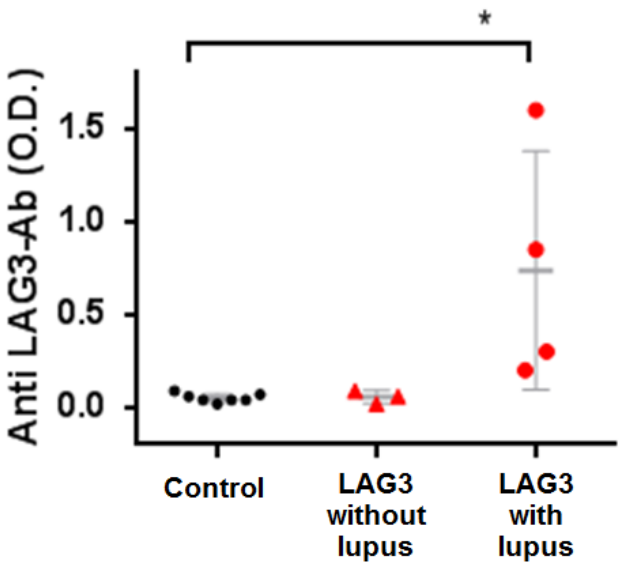

CD25-LAG3+Egr2+ T cell
CD4+CD25+ T cell
CD25-LAG3+Egr2- T cell
CD25-CD45RBlow (Memory)T cell
% in CD4+ T cells
Control
LAG3 without lupus
LAG3 with lupus

Figure 5. pCAGGS-LAG-3 vaccination induced depletion of $CD4^+LAG\text{-}3^+Egr2^+$ Treg cells: (A) Flow cytometric analysis of splenocytes in pCAGGS-LAG-3 immunized mice with lupus pathological condition, pCAGGS-LAG-3 immunized mice without lupus and control mice. Number adjacent to outlined areas indicate percent of $LAG\text{-}3^+Egr2^+$ T cells shown in all three mice; $CD4^+CD25^-$ cells were gated. (B) Isolated splenocytes were stained and $CD4^+CD25^-LAG\text{-}3^+$ cells were gated for histogram comparison of Egr2 expression in all three groups. (C) Summary of the percentage of $CD4^+CD25^-LAG\text{-}3^+Egr2^+$ T cells, $CD4^+CD25^+$T cells, $CD4^+CD25^-LAG\text{-}3^+Egr2^-$ T cells and memory T cells in mice immunized with pCAGGS-LAG-3 vector and control vector. Each symbol indicates an individual mouse.

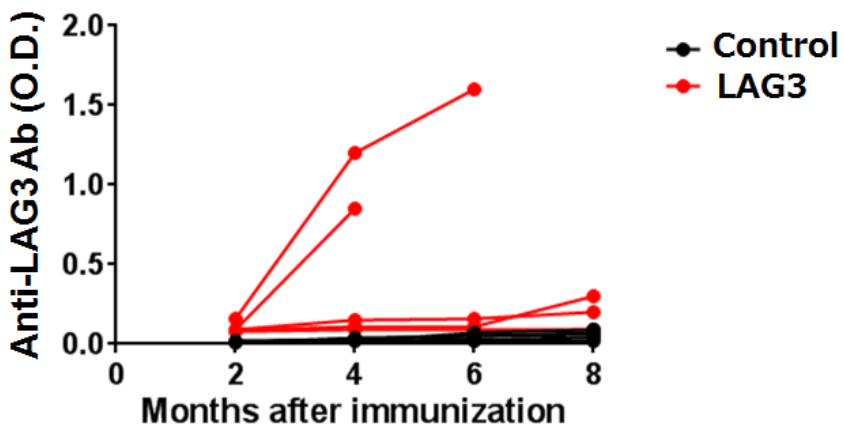
2.14 Anti-LAG-3 antibody in the mice with DNA vaccination

As DNA vaccination causes protein expression which induces immune response, I hypothesized that after vaccination the mice injected with pCAGGS-LAG-3 vector produce anti-LAG-3 antibody which might be the main cause of the depletion $CD4^+CD25^-LAG\text{-}3^+Egr2^+$ T cells and the severe lupus-like pathological condition. ELISA of anti-LAG-3 antibody revealed that the titer of anti-LAG-3 antibody significantly increased in pCAGGS-LAG-3 immunized mice with lupus-like symptoms compared with control mice (Figure 6A). Then, I tried to trace the time course of anti-LAG-3 antibody production using a part of mice serum in same experimental group. Here the mice immunized with pCAGGS-LAG-3 vector showed tendency to develop anti-LAG-3 antibody titer in their serum within 4 months of immunization and the titer increased later (Figure 6B). The correlation analysis found a negative correlation between the percentage of $CD4^+CD25^-LAG\text{-}3^+Egr2^+$ T cells and anti-LAG-3 antibody titers (Figure 6C). This result implied a working hypothesis that the DNA vaccination with pCAGGS-LAG-3 vector induced the production of anti-LAG-3 antibody, and anti-LAG-3 antibody depleted $CD4^+LAG\text{-}3^+Egr2^+$ T cells. Then, the depletion of $CD4^+LAG\text{-}3^+Egr2^+$ T cells might induce lupus-like systemic autoimmunity.

(A)


*
Anti LAG3-Ab (O.D.)
1.5
1.0
0.5
0.0
Control
LAG3 without lupus
LAG3 with lupus

(B)


Anti-LAG3 Ab (O.D.)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
Months after immunization
Control
LAG3

(C)

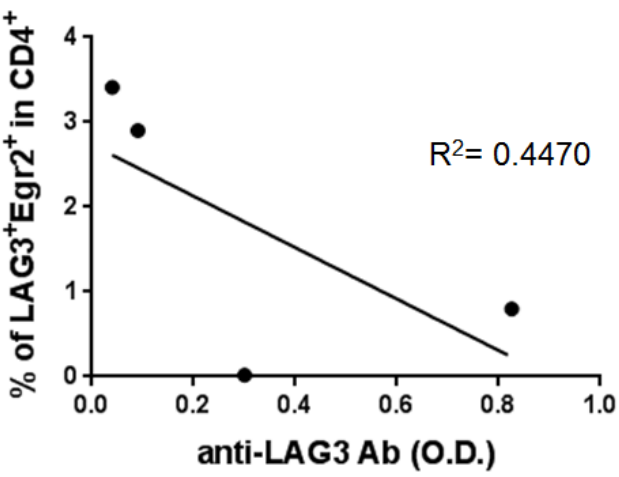

% of LAG3+Egr2+ in CD4+
4
3
2
1
0
0.0
0.2
0.4
0.6
0.8
1.0
anti-LAG3 Ab (O.D.)
R2= 0.4470

Figure 6. pCAGGS-LAG-3 immunized mice with lupus pathological condition have high titer of anti-LAG-3 antibody. A) Serum concentration of anti-LAG-3 antibody in pCAGGS-LAG-3 immunized mice compared with control mice (n= 7). Each symbol indicates an individual mouse. Horizontal line indicates the mean ± s.d *, $P < 0.05$ (Unpaired t-test). (B) Time course of anti LAG-3 antibody production compared with control mice (n= 4). (C) Association between anti-LAG-3 antibody titer and $CD4^{+}CD25^{-}LAG\text{-}3^{+}Egr2^{+}$ cells in pCAGGS-LAG-3 immunized mice with lupus pathological condition.

3. Discussion

$CD4^+CD25^-LAG\text{-}3^+$ Tregs are reported as a new subset that play a significant role in suppressing peripheral inflammatory reaction, and their function is IL-10 dependent and FoxP3 independent [30, 60, 61]. LAG-3 on $CD4^+$ T cell has been reported as a suppressive surface marker of conventional $CD4^+CD25^+$ Treg [62], however most $CD4^+LAG\text{-}3^+$ T cells are actually CD25 negative. Intracellular staining of $CD4^+CD25^-LAG\text{-}3^+$ Tregs revealed a strong correlation with transcriptional factor Egr2, which was reported as a negative regulator of T cell activation and necessary for clonal anergy induction [38]. Transduction of Egr2 confers LAG-3 expression and regulatory activity on $CD4^+$ naïve T cells [30]. Therefore, it is speculated that suppressive activity of $CD4^+CD25^-LAG\text{-}3^+$ Treg is regulated by Egr2. In this experiment, I attempted to deplete $CD4^+CD25^-LAG\text{-}3^+$ Tregs.

I adopted DNA vaccination procedure, and constructed pCAGGS-LAG3 vector containing mouse LAG-3 cDNA. For DNA vaccination, several routes including intravenous, intramuscular and intradermal were practiced, however the most efficient result was obtained by intravenous administration. Within five months of vaccination, a part of mice immunized with pCAAGS-LAG-3 vector had developed lupus-like histological and functional abnormalities including alopecia, dermatitis and proteinuria while control mice did not. Intriguingly, only the pCAGGS-LAG-3 immunized mice with lupus-like lesion had high titer of anti-dsDNA antibody in the serum. On sacrifice, these mice had huge splenomegaly. Histopathological analysis of skin and kidney shows dermatitis with hydrophic degeneration of basal cells and Glomerulonephritis with IgG/C3 deposition in glomeruli. Thus, mice immunized with pCAGGS-LAG-3 vector leads to systemic autoimmunity in C57BL/6 mice.

FACS analysis of splenocytes revealed that $CD4^+LAG\text{-}3^+Egr2^+$ T cells were depleted in pCAGGS-LAG-3 immunized mice with severe lupus-like lesions. Control mice or pCAGGS-LAG-3 immunized mice without lupus-like lesions did not show the depletion of $CD4^+LAG\text{-}3^+Egr2^+$ T cells. Moreover, ELISA of anti-LAG-3 antibody revealed that $CD4^+LAG\text{-}3^+Egr2^+$ T cells depleted mice developed high titer of anti-LAG-3 antibody. I hypothesized that the DNA vaccination with pCAGGS-LAG-3 vector induced the production of anti-LAG-3 antibody, and anti-LAG-3 antibody depleted $CD4^+LAG\text{-}3^+Egr2^+$ T cells. The depletion of $CD4^+LAG\text{-}3^+Egr2^+$ T cells might lead to lupus-like systemic autoimmunity (Figure 7).

However, more experiment should be done. Although the production of anti-LAG-3 antibody was certainly induced by pCAGGS-LAG-3 vaccination, it still needs to be elucidated whether this anti-LAG-3 antibody actually works for the depletion of $LAG\text{-}3^+$ T cells. In order to verify the direct effect of anti-LAG-3 antibody, making monoclonal antibody using hybridoma technique from efficient antibody producing mouse is a rational plan. *In vivo* depletion of LAG-3 Treg using monoclonal antibody can confirm the association between $CD4^+LAG\text{-}3^+Egr2^+$ T cell depletion and anti-LAG-3 antibody formation. If these mice repeated the lupus-like pathological condition, our working hypothesis would be verified.

This study inevitably raises a question why depletion of $Egr2^+LAG\text{-}3^+$ T cells results in lupus-like systemic autoimmunity in pCAGGS-LAG-3 vector-immunized mice. Previous report demonstrated that LAG-3-deficient mice displayed apparently normal pathological condition with no defect in T cells and B cells functions and no gross T cell abnormalities [22]. One may speculate that these LAG-3-deficient mice develop some compensative systems to avoid autoimmunity; whereas Egr2-cKO mice whose Egr2 gene was conditionally knocked out in T cells and B cells developed lupus-like systemic autoimmunity that is similar as our mice with

$CD4^+LAG\text{-}3^+Egr2^+$ T cells depletion [39]. Several genome-wide associated study (GWAS) also showed there is a link between Egr2 and autoimmunity in human. Two independent GWAS study identified a strong association between Egr2 and Crohn's disease, the most common form of chronic inflammatory bowel disease [63, 64], which is consistent with the study by Okamura et al. [30]. Another report demonstrated that Egr2 polymorphism influence SLE susceptibility in human [65]. However, further experiment is needed in order to confirm the role of $CD4^+LAG\text{-}3^+Egr2^+$ T cells depletion in the induction of lupus like pathology in mouse model. Adoptive transfer of wild type $CD4^+LAG\text{-}3^+Egr2^+$ T cells in depletion model mice is a rational plan.

The intricate part of this experiment is that the anti-LAG-3 antibody production reduced only $CD4^+LAG\text{-}3^+Egr2^+$ T cells, but not $CD4^+LAG\text{-}3^+Egr2^-$ T cells. The reason why only $CD4^+LAG\text{-}3^+Egr2^+$ T cells were depleted needs further investigation. One reason could be that the epitope expression of $CD4^+LAG\text{-}3^+Egr2^+$ T cells might be suitable for depletion with anti-LAG-3 antibody. LAG-3 is a transmembrane protein and consists of superficial D1, D2, D3, D4 domain, connecting peptide (CP), transmembrane domain and cytoplasmic domain [66]. LAG-3 was cleaved within the D4 transmembrane domain and connecting peptide, and generated both the truncated p54 LAG-3 fragment and soluble form LAG-3 (monomer). On the other hand, full-length LAG-3 on the cell surface form LAG-3 dimer via the D1 domain which possesses high affinity for MHC class II molecule [8, 23]. To confirm that LAG-3 is expressed as dimer, Li et al. performed immunoblotting assay with anti-LAG-3 monoclonal antibody [66]. Interestingly, not all LAG-3 was cross linked in these experiments, then they hypothesized that momomeric form of LAG-3 might compete with membrane-associated LAG-3 for ligand binding. Here in my experiments, I used monomeric form of LAG-3 for the construction of DNA vaccination vector, therefore anti-LAG3 antibody induced by pCAGGS-LAG-3 might recognize monometric form

of LAG-3 more efficiently. If Egr2 positivity affects the expression of each form of LAG-3, the discrepancy between the number of LAG-3^{+}Egr2^{+} T cells and LAG-3^{+}Egr2^{-} T cells might be explained by the difference of epitope recognition.

A main drawback in our experiment is the low incidence rate of lupus-like lesions. Generally, induction of immune response after DNA vaccination considered as a slow process. The vector-encoded gene hardly reaches the lymphoid tissue under intravenous, intradermal or intramuscular administration. Instead, the encoded gene is expressed in some cells including hepatocytes and DCs that has limited access to T cells in lymphoid tissue [67]. Further modification of DNA vaccination protocol might solve this problem.

This is the first report for the depletion of $CD4^{+}CD25^{-}$LAG-3^{+} Tregs in mouse model. I believe that this depletion model will help to analyze how LAG-3 Treg exert regulatory activity *in vivo* and will clarify what cells are important to develop lupus-like pathological condition in mouse model.

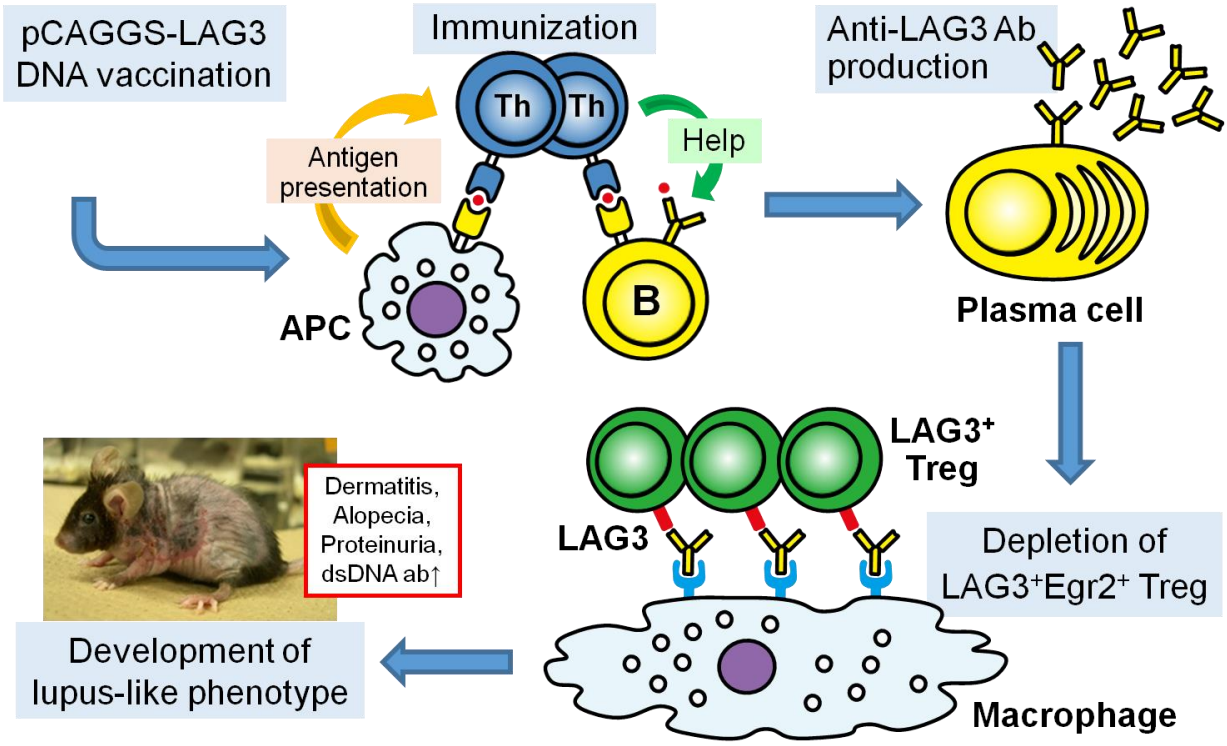


Figure 7. Working hypothesis of $CD4^{+}CD25^{-}LAG\text{-}3^{+}$ T cell depletion with DNA vaccination.

4. Acknowledgement

I owe a great debt of gratitude to many people who have given their time, energy, insights, and support during my graduate study and research. First, I express my sincere thanks and gratitude to my supervisor, Professor Kazuhiko YAMAMOTO, for his guidance and support. I am also deeply grateful to Dr. Keishi FUJIO and Dr. Shuji SUMITOMO for consistent guidance and advice throughout this research.

I would also like to express my appreciation to all my laboratory members and laboratory assistants' for their help. My special thanks to Prof. Jun-ichi MIYAZAKI [Osaka University Graduate School of Medicine] for his kind permission of using pCAGGS vector.

Besides, my deepest thanks go to all my family members, especially to my husband Dr. Shanawez Hossain.

Finally, I want to acknowledge the University of Tokyo for offering me 'The Tokyo University Fellowship' and the Japanese Government's Ministry of Education for the 'MEXT scholarship' which made my study possible. Without these supports, this research could not have been realized.

5. References

1. Hanly JG, Urowitz MB, Su L, Bae SC, Gordon C, Clarke A, et al. Autoantibodies as biomarkers for the prediction of neuropsychiatric events in systemic lupus erythematosus. *Ann Rheum Dis*. 2011;70(10):1726-32.
2. Andrade F, Casciola-Rosen L, Rosen A. Apoptosis in systemic lupus erythematosus. Clinical implications. *Rheum Dis Clin North Am*. 2000;26(2):215-27, v.
3. Munoz LE, Gaipl US, Franz S, Sheriff A, Voll RE, Kalden JR, et al. SLE--a disease of clearance deficiency? *Rheumatology (Oxford)*. 2005;44(9):1101-7.
4. Okamoto A, Fujio K, Okamura T, Yamamoto K. Regulatory T-cell-associated cytokines in systemic lupus erythematosus. *J Biomed Biotechnol*. 2011;2011:463412.
5. Parietti V, Monneaux F, Decossas M, Muller S. Function of CD4+,CD25+ Treg cells in MRL/lpr mice is compromised by intrinsic defects in antigen-presenting cells and effector T cells. *Arthritis Rheum*. 2008;58(6):1751-61.
6. Baixeras E, Huard B, Miossec C, Jitsukawa S, Martin M, Hercend T, et al. Characterization of the lymphocyte activation gene 3-encoded protein. A new ligand for human leukocyte antigen class II antigens. *J Exp Med*. 1992;176(2):327-37.
7. Workman CJ, Vignali DA. The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. *Eur J Immunol*. 2003;33(4):970-9.
8. Huard B, Mastrangeli R, Prigent P, Bruniquel D, Donini S, El-Tayar N, et al. Characterization of the major histocompatibility complex class II binding site on LAG-3 protein. *Proc Natl Acad Sci U S A*. 1997;94(11):5744-9.
9. Huard B, Prigent P, Tournier M, Bruniquel D, Triebel F. CD4/major histocompatibility

complex class II interaction analyzed with CD4- and lymphocyte activation gene-3 (LAG-3)-Ig fusion proteins. *Eur J Immunol*. 1995;25(9):2718-21.

10. Woo SR, Li N, Bruno TC, Forbes K, Brown S, Workman C, et al. Differential subcellular localization of the regulatory T-cell protein LAG-3 and the coreceptor CD4. *Eur J Immunol*. 2010;40(6):1768-77.
11. Crawford A, Wherry EJ. The diversity of costimulatory and inhibitory receptor pathways and the regulation of antiviral T cell responses. *Curr Opin Immunol*. 2009;21(2):179-86.
12. Workman CJ, Wang Y, El Kasmi KC, Pardoll DM, Murray PJ, Drake CG, et al. LAG-3 regulates plasmacytoid dendritic cell homeostasis. *J Immunol*. 2009;182(4):1885-91.
13. Kisielow M, Kisielow J, Capoferri-Sollami G, Karjalainen K. Expression of lymphocyte activation gene 3 (LAG-3) on B cells is induced by T cells. *Eur J Immunol*. 2005;35(7):2081-8.
14. Byun HJ, Jung WW, Lee DS, Kim S, Kim SJ, Park CG, et al. Proliferation of activated CD1d-restricted NKT cells is down-modulated by lymphocyte activation gene-3 signaling via cell cycle arrest in S phase. *Cell Biol Int*. 2007;31(3):257-62.
15. Workman CJ, Rice DS, Dugger KJ, Kurschner C, Vignali DA. Phenotypic analysis of the murine CD4-related glycoprotein, CD223 (LAG-3). *Eur J Immunol*. 2002;32(8):2255-63.
16. Grosso JF, Kelleher CC, Harris TJ, Maris CH, Hipkiss EL, De Marzo A, et al. LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems. *J Clin Invest*. 2007;117(11):3383-92.
17. Shin H, Blackburn SD, Intlekofer AM, Kao C, Angelosanto JM, Reiner SL, et al. A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. *Immunity*. 2009;31(2):309-20.

18. Triebel F, Jitsukawa S, Baixeras E, Roman-Roman S, Genevee C, Viegas-Pequignot E, et al. LAG-3, a novel lymphocyte activation gene closely related to CD4. *J Exp Med*. 1990;171(5):1393-405.

19. Huard B, Tournier M, Hercend T, Triebel F, Faure F. Lymphocyte-activation gene 3/major histocompatibility complex class II interaction modulates the antigenic response of CD4+ T lymphocytes. *Eur J Immunol*. 1994;24(12):3216-21.

20. Hannier S, Tournier M, Bismuth G, Triebel F. CD3/TCR complex-associated lymphocyte activation gene-3 molecules inhibit CD3/TCR signaling. *J Immunol*. 1998;161(8):4058-65.

21. Macon-Lemaitre L, Triebel F. The negative regulatory function of the lymphocyte-activation gene-3 co-receptor (CD223) on human T cells. *Immunology*. 2005;115(2):170-8.

22. Miyazaki T, Dierich A, Benoist C, Mathis D. Independent modes of natural killing distinguished in mice lacking Lag3. *Science*. 1996;272(5260):405-8.

23. Li N, Wang Y, Forbes K, Vignali KM, Heale BS, Saftig P, et al. Metalloproteases regulate T-cell proliferation and effector function via LAG-3. *EMBO J*. 2007;26(2):494-504.

24. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299(5609):1057-61.

25. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet*. 2001;27(1):68-73.

26. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The

immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*. 2001;27(1):20-1.

27. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet*. 2001;27(1):18-20.

28. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat Rev Immunol*. 2007;7(8):585-98.

29. Ochi H, Abraham M, Ishikawa H, Frenkel D, Yang K, Basso AS, et al. Oral CD3-specific antibody suppresses autoimmune encephalomyelitis by inducing CD4+ CD25- LAP+ T cells. *Nat Med*. 2006;12(6):627-35.

30. Okamura T, Fujio K, Shibuya M, Sumitomo S, Shoda H, Sakaguchi S, et al. CD4+CD25-LAG3+ regulatory T cells controlled by the transcription factor Egr-2. *Proc Natl Acad Sci U S A*. 2009;106(33):13974-9.

31. Martins GA, Cimmino L, Shapiro-Shelef M, Szabolcs M, Herron A, Magnusdottir E, et al. Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. *Nat Immunol*. 2006;7(5):457-65.

32. Cretney E, Xin A, Shi W, Minnich M, Masson F, Miasari M, et al. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat Immunol*. 2011;12(4):304-11.

33. Ehrengruber MU, Muhlebach SG, Sohrman S, Leutenegger CM, Lester HA, Davidson N. Modulation of early growth response (EGR) transcription factor-dependent gene expression by using recombinant adenovirus. *Gene*. 2000;258(1-2):63-9.

34. Gilardi P, Schneider-Maunoury S, Charnay P. Krox-20: a candidate gene for the

regulation of pattern formation in the hindbrain. *Biochimie*. 1991;73(1):85-91.

35. Topilko P, Schneider-Maunoury S, Levi G, Baron-Van Evercooren A, Chennoufi AB, Seitanidou T, et al. Krox-20 controls myelination in the peripheral nervous system. *Nature*. 1994;371(6500):796-9.

36. Harris JE, Bishop KD, Phillips NE, Mordes JP, Greiner DL, Rossini AA, et al. Early growth response gene-2, a zinc-finger transcription factor, is required for full induction of clonal anergy in CD4+ T cells. *J Immunol*. 2004;173(12):7331-8.

37. Zheng Y, Zha Y, Driessens G, Locke F, Gajewski TF. Transcriptional regulator early growth response gene 2 (Egr2) is required for T cell anergy in vitro and in vivo. *J Exp Med*. 2012;209(12):2157-63.

38. Safford M, Collins S, Lutz MA, Allen A, Huang CT, Kowalski J, et al. Egr-2 and Egr-3 are negative regulators of T cell activation. *Nat Immunol*. 2005;6(5):472-80.

39. Zhu B, Symonds AL, Martin JE, Kioussis D, Wraith DC, Li S, et al. Early growth response gene 2 (Egr-2) controls the self-tolerance of T cells and prevents the development of lupuslike autoimmune disease. *J Exp Med*. 2008;205(10):2295-307.

40. Miyazaki T, Dierich A, Benoist C, Mathis D. LAG-3 is not responsible for selecting T helper cells in CD4-deficient mice. *Int Immunol*. 1996;8(5):725-9.

41. Tang DC, DeVit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. *Nature*. 1992;356(6365):152-4.

42. Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science*. 1993;259(5102):1745-9.

43. Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL. DNA

vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc Natl Acad Sci U S A*. 1993;90(24):11478-82.

44. Iezzi M, Quaglino E, Amici A, Lollini PL, Forni G, Cavallo F. DNA vaccination against oncoantigens: A promise. *Oncoimmunology*. 2012;1(3):316-25.

45. Kutzler MA, Weiner DB. DNA vaccines: ready for prime time? *Nat Rev Genet*. 2008;9(10):776-88.

46. Iwasaki A, Torres CA, Ohashi PS, Robinson HL, Barber BH. The dominant role of bone marrow-derived cells in CTL induction following plasmid DNA immunization at different sites. *J Immunol*. 1997;159(1):11-4.

47. Lai WC, Bennett M. DNA vaccines. *Crit Rev Immunol*. 1998;18(5):449-84.

48. Gurunathan S, Klinman DM, Seder RA. DNA vaccines: immunology, application, and optimization*. *Annu Rev Immunol*. 2000;18:927-74.

49. Garg S, Oran AE, Hon H, Jacob J. The hybrid cytomegalovirus enhancer/chicken beta-actin promoter along with woodchuck hepatitis virus posttranscriptional regulatory element enhances the protective efficacy of DNA vaccines. *J Immunol*. 2004;173(1):550-8.

50. Harms JS, Splitter GA. Interferon-gamma inhibits transgene expression driven by SV40 or CMV promoters but augments expression driven by the mammalian MHC I promoter. *Hum Gene Ther*. 1995;6(10):1291-7.

51. Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*. 1991;108(2):193-9.

52. Chen Z, Sahashi Y, Matsuo K, Asanuma H, Takahashi H, Iwasaki T, et al. Comparison of the ability of viral protein-expressing plasmid DNAs to protect against influenza. *Vaccine*.

1998;16(16):1544-9.

53. Bins AD, Jorritsma A, Wolkers MC, Hung CF, Wu TC, Schumacher TN, et al. A rapid and potent DNA vaccination strategy defined by in vivo monitoring of antigen expression. *Nat Med*. 2005;11(8):899-904.

54. Zhao Z, Wakita T, Yasui K. Inoculation of plasmids encoding Japanese encephalitis virus PrM-E proteins with colloidal gold elicits a protective immune response in BALB/c mice. *J Virol*. 2003;77(7):4248-60.

55. Huang JL, Yin YX, Pan ZM, Zhang G, Zhu AP, Liu XF, et al. Intranasal immunization with chitosan/pCAGGS-flaA nanoparticles inhibits Campylobacter jejuni in a White Leghorn model. *J Biomed Biotechnol*. 2010;2010.

56. Zheng Q, Fan D, Gao N, Chen H, Wang J, Ming Y, et al. Evaluation of a DNA vaccine candidate expressing prM-E-NS1 antigens of dengue virus serotype 1 with or without granulocyte-macrophage colony-stimulating factor (GM-CSF) in immunogenicity and protection. *Vaccine*. 2011;29(4):763-71.

57. Yang B, Lan X, Li X, Yin X, Li B, Han X, et al. A novel bi-functional DNA vaccine expressing VP1 protein and producing antisense RNA targeted to 5'UTR of foot-and-mouth disease virus can induce both rapid inhibitory effect and specific immune response in mice. *Vaccine*. 2008;26(43):5477-83.

58. Atkinson C, Qiao F, Song H, Gilkeson GS, Tomlinson S. Low-dose targeted complement inhibition protects against renal disease and other manifestations of autoimmune disease in MRL/lpr mice. *J Immunol*. 2008;180(2):1231-8.

59. Ioannou A, Lieberman LA, Dalle Lucca JJ, Tsokos GC. Complement depletion protects lupus-prone mice from ischemia-reperfusion-initiated organ injury. *Am J Physiol*

Gastrointest Liver Physiol.304(3):G283-92.

60. Okamura T, Fujio K, Sumitomo S, Yamamoto K. Roles of LAG3 and EGR2 in regulatory T cells. *Ann Rheum Dis*. 2012;71 Suppl 2:i96-100.

61. Fujio K, Okamura T, Sumitomo S, Yamamoto K. Regulatory cell subsets in the control of autoantibody production related to systemic autoimmunity. *Ann Rheum Dis*. 2013;72 Suppl 2:ii85-9.

62. Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, et al. Role of LAG-3 in regulatory T cells. *Immunity*. 2004;21(4):503-13.

63. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature*. 2007;447(7145):661-78.

64. Rioux JD, Xavier RJ, Taylor KD, Silverberg MS, Goyette P, Huett A, et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet*. 2007;39(5):596-604.

65. Myouzen K, Kochi Y, Shimane K, Fujio K, Okamura T, Okada Y, et al. Regulatory polymorphisms in EGR2 are associated with susceptibility to systemic lupus erythematosus. *Hum Mol Genet*. 2010;19(11):2313-20.

66. Li N, Workman CJ, Martin SM, Vignali DA. Biochemical analysis of the regulatory T cell protein lymphocyte activation gene-3 (LAG-3; CD223). *J Immunol*. 2004;173(11):6806-12.

67. Lindquist RL, Shakhar G, Dudziak D, Wardemann H, Eisenreich T, Dustin ML, et al. Visualizing dendritic cell networks in vivo. *Nat Immunol*. 2004;5(12):1243-50.