GENE THERAPY OF SYSTEMIC LUPUS ERYTHEMATOSUS IN NZB/W F1 MICE

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In loving memory of my dear mother, Cuiyun Wang

Thank you for giving me life and being a great inspiration to me

I am proud to be your daughter and your special friend

I miss you so much

Xiao Xiao, PhD

GENE THERAPY OF SYSTEMIC LUPUS ERYTHEMATOSUS IN NZB/W F1 MICE

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Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by polyclonal B-cell activation, autoantibody production and immune complex-mediated glomerulonephritis (GN). NZB/W F1 mice spontaneously develop SLE-like symptoms and have been widely used as an animal model for SLE. Even though the etiologic cause of autoimmunity in both human and murine lupus is not clearly understood, mounting evidence indicates the involvement of autoreactive B cells and T cells. Blockade of costimulatory pathways using CTLA4Ig fusion protein and anti-CD40 ligand monoclonal antibody (mAb) has been able to suppress autoantibody production and inhibit lupus nephritis in NZB/W F1 mice. In spite of these successes, protein- or mAb-based therapies are expensive, deliveries are problematic, and bolus injections result in transient and toxic levels of these reagents. In comparison, gene therapy offers an advantageous solution by achieving long-term, stable transgene expression and convenient gene delivery. Among the vectors used in gene therapy, recombinant adeno-associated virus (rAAV) has been proven to be a promising one because of its non-pathogenicity and non-immunogenicity.

In this project, the hypothesis that rAAV serotype 8 (rAAV8)-mediated immunomodulator gene transfer was able to prevent and suppress lupus development in NZB/W F1 mice was tested. First, the transgene expression pattern following systemic delivery was studied. rAAV8-mediated gene delivery was able to achieve long-lasting and high-level

transgene expression following a single intraperitoneal or intrasplenic injection. Second, rAAV8-mediated costimulatory blockade gene transfer prevented autoantibody production, delayed proteinuria onset, prolonged survival and protected kidneys from immune-complex induced tissue damage. Moreover, it successfully suppressed $CD4^+$ T cell activation and also the transition from naïve to memory T cells. Among all early prevention strategies tested in newborn mice, the combination of murine CTLA4Ig (mCTLA4Ig) and murine CD40Ig (mCD40Ig) was more efficient than both single vectors, suggesting a synergistic effect between these two costimulatory blockades. Surprisingly, the late prevention in young lupus-prone mice achieved results similar to those observed in the early prevention. Furthermore, rAAV-mediated costimulatory blockade gene transfer did not suppress host humoral response to foreign antigens. Finally, in the treatment of lupus mice with high autoantibody titers, mCTLA4Ig alone, or in combination with mCD40Ig, was able to decrease autoantibody titers, delay proteinuria onset, and prolong survival. The therapeutic effects were evidenced to be dose-dependent. In general, this study indicates that rAAV8-mediated costimulatory blockade gene transfer is capable of preventing and suppressing lupus development, thereby suggesting the successful application of this autoimmune disease gene therapy both in research and in clinical settings to promote public health.

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1.1. Systemic lupus erythematosus

1.1.1. Clinical manifestation

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by autoantibody production and multi-system involvement. It affects 40 to 250 individuals out of 100,000 people, and mostly young women are affected with a female to male ratio of 9 to 1(1). There is a wide spectrum of clinical presentations, all resulting from inflammation in various organ systems, including skin, joints, kidney, brain, lung, heart and nervous system. The initial symptoms of SLE patients include constitutional complains such as malaise, overwhelming fatigue, fever, and weight loss. As the disease develops, patients suffer from organ-specific symptoms, such as multiple joints arthritis, proteinuria, pericarditis and seizure. The most recognized manifestation of SLE is the so-called butterfly rash in a malar distribution, but this is seen only occasionally. Because this disease has such a pleomorphic nature, there exists no classic disease pattern, and the diagnosis must be based on an overall view of the clinical picture combined with serologic and laboratory studies. In some cases, biopsies or other diagnostic procedures are necessary. Clinically, cyclophosphamide and methylprednisolone remain the gold standard treatment of SLE patients through nonspecific inhibition of the inflammatory immune response. With appropriate therapy, lupus patients go through flare-ups and remissions spanning a period of years or even decades. The most common causes of death are renal failure, infection and diffuse central nervous system disease, resulting from the disease progress, or the complication of its therapy.

1.1.2. Epipathogenesis

Although the exact cause of SLE remains unknown, epidemiological studies of SLE etiology have identified three important factors: (1) genetic (2) environmental and (3) endocrine-metabolic, which lead to the body's failure to distinguish between self and non-self, and the production of autoantibodies. Of these three risk factors, epidemiology data strongly indicate genetic susceptibility in SLE. As an evidence of family clustering; the risk ratio of affected sib pair to population is estimated to be 10-20:1, which is similar to that of type I diabetes and multiple sclerosis (2). A second supporting evidence for genetic involvement is the disease prevalence, or concordance rate, in twins. The concordance rate of SLE is 24% in monozygotic twins versus 2% in dizygotic twins (3).

The origin of this disease is likely to be multigenic. Association studies of MHC class II alleles with SLE confirms that HLA-DR2 and HLA-DR3 separately confer 2-fold or 3-fold relative risks for the development of SLE in Caucasians (4, 5). In the NZB/W F1 hybrid, a murine model of SLE, H2 d/z heterozygosity is associated with enhanced IgG autoantibodies production (6-8), and the presence of these alleles confers a 2-fold to 5-fold risk for lupus. Moreover, studies on the NZB/W F1 mice have mapped three recessive loci responsible for SLE susceptibility, including sle1 on chromosome 1, sle2 on chromosome 4, and sle3 on chromosome 7. Since lupus is an autoimmune disease, several genes involving in the immune regulation have been the special interests of lupus genetic study. It is shown that CT60A/G dimorphism of CTLA4 gene, located in 2q33, is associated with SLE in Spanish patients (9). Another gene, CD40 located in 20q11-13, also is linked with lupus susceptibility (10). In conclusion, SLE is a complex genetic trait with contributions from MHC and multiple non-MHC genes. Instead of

being inherited in a typical Mendelian way, multiple genes behave in an additive fashion. Some act as lupus susceptibility genes, while others as disease-modifying genes.

In the setting of certain susceptibility genes, environmental factors probably trigger the pathogenic immune responses, which initiate or activate lupus. A typical example is that in many SLE patients, UV light provokes a photosensitive skin rash that may be followed by a generalized disease flare. The most investigated lupus-associated environmental factors are chemicals. The syndrome of drug-induced lupus by hydralazine, procainamide, and isoniazid is well established (11). Finally, the predominance of lupus in females suggests that hormones play a significant role in disease susceptibility. The female to male ratio in SLE patients peaks in young adults, and decreases at menopause (12). Experiments on lupus-prone NZB/W F1 mice have shown a disease-accelerating effect of estrogen and prolactin (13). Moreover, tamoxifen, an estrogen antagonist reduces autoantibody production and alleviates disease severity in lupus mice (14). Similar studies using pre-pubertal treatment with testosterone have shown a protective effect in B/W female mice (15).

At the pathogenic level, SLE is the prototypic immune complex disease, characterized by excessive autoantibody production, immune complex formation and consequential tissue injury. Besides forming immune complex, autoantibodies may cause direct damage by inducing cell lysis (16, 17), or by causing functional disturbances of mononuclear cells (18, 19). The pathology of skin lesions illustrates the basic pathophyiologic mechanisms in SLE: immune complex formation, acute vascular and perivascular inflammation and more chronic mononuclear cell infiltration. The most extensively studied organ in lupus is the kidney. Various pathologic changes have been found, including increase of the mesangial cells, basement membrane abnormalities, and immune complex deposition. These deposits within the kidney

include IgG, IgM, IgA and components of complement. The pathologic findings in the kidney are classified, and used to predict clinical outcomes. While many other organs can be affected by lupus, they usually display nonspecific inflammation because of the deposition of immune complexes.

1.1.3. Immune abnormalities

There are several immune abnormalities common in both SLE human patients and murine models. The most pervasive are: (1) the ability to produce pathogenic autoantibodies, (2) lack of T- and B-lymphocyte regulation, (3) defective clearance of autoantigens and immune complexes and (4) imbalance of cytokine synthesis. These hallmarks of immune deregulation are closely interrelated and it has been difficult to determine which, if any comes first. They are described in detail as follows.

1.1.3.1. Autoantibodies and T-dependent autoantibody production

Lupus is characterized by a plethora of different autoantibodies, which are directed at self molecules found in the nucleus, cytoplasm or cell surface. It is well documented that antibodies to chromatin target DNA and DNA-associated proteins, such as histones (20); whereas antibodies to ribosome target RNA and/or ribosomal proteins, such as P proteins (21). Studies in lupus mice suggest that the most important antigens initiating anti-DNA autoantibodies are derived from nucleosomes (22, 23), and that these autoantibodies themselves can be a source of peptides that activate pathogenic T cells (24). All combined, nucleosome and anti-nucleosome antibodies make interesting targets for future therapies, such as using derived peptide as tolerogen for autoreactive T cells (25). In addition, antiphospholipid antibodies (aPL), including anticardiolipin and lupus anticoagulant, are also used for the diagnosis of lupus and linked with

thrombosis and miscarriage in lupus patients (26). Clinical study shows that aPL helps to predict early organ damage in SLE patients (27).

High titer anti-nuclear antibodies are able to induce immune complex (IC)-mediated glomerulonephritis (GN) and renal injury. Among all IgGs commonly found in IC, it is the anti-dsDNA IgG that is thought to be pathogenic. About 60-70% of lupus patients produce anti-dsDNA IgG, and numerous lines of evidence have suggested that some of these antibodies are nephrotoxic (28). Furthermore, in the transgenic mouse model, anti-DNA IgG directly cause lupus (29).

Mounting evidence indicates that the production of pathologic autoantibodies by B cells is dependent on stimulatory influences from $CD4^+$ T cells (30, 31). The isotype and specificity of the autoantibodies in lupus mice suggest that they are under T-cell control (32). In addition, lupus nephritis does not occur in athymic B/W mice (33). Finally, monoclonal antibodies against $CD4^+$ T cells can prevent murine lupus in B/W mice (34, 35). All this evidence supports the essential role of T helper cells in the activation and differentiation of autoreactive B cells, and also in autoantibody production.

1.1.3.2. T lymphocytes and natural killer cells

T cell hyperactivation is probably one of the most important features observed in all the strains of spontaneous SLE murine models (36-38). In murine lupus, polyclonal T cell activation is seen in the early stage of disease. As a result, T lymphocyte-dependent autoantibodies are produced at high levels, and subsets of these autoantibodies, and the immune complexes they form, mediate tissue damage. The presence of autoreactive T lymphocytes is not thought to reflect a defect in central tolerance. Instead, it reflects a breakdown of peripheral tolerance (39). T cells in the circulation of patients with active SLE are in activated state, and express activation marker HLA-

DR and DP antigens (40, 41). Consistently, there is a dramatic decrease of $CD4^+$ cells with a naïve phenotype (42, 43). When SLE T lymphocytes are isolated, however, their *in vitro* response to mitogen stimulation is usually deficient (44, 45). Moreover, diminished T helper function in response to exogenous antigen is seen both *in vivo* and *in vitro*, and it correlates with disease activity (46-48). SLE patients have functionally defective $Fc(IgG)^+$ T cells and a defective response to allogeneic and autologous MHC II molecules (49). In human patients of lupus, decreased numbers of T, B, and NK cells are common (50). In addition to the decrease in the numbers of these cells, functional abnormalities have also been observed. Delayed hypersensitivity responses *in vivo* and *in vitro* are generally impaired, even in patients not receiving immunosuppressive drugs (51). Both $CD8^+$ T cells and NK cells have decreased cytotoxic activity (52, 53), and these two cell subpopulations enhance IgG production in lupus patients (54, 55). This specific abnormality has been attributed, at least partly, to defective production of TGF-β (55).

1.1.3.3. B lymphocytes

The most characteristic abnormality in both SLE patients and in murine lupus is B lymphocyte hyperactivity, which results in hypergammaglobulinaemia (31, 56). Numerous defects in B cell phenotype and function have been described. Phenotypic changes include increased cell surface expression of CD40L (57, 58), CD80 and CD86 (59, 60). With respect to functional abnormality, B cells from SLE patients exhibit higher intracellular calcium concentrations and phosphorylated tyrosine residues after stimulation with an anti-IgM Ab (61). Furthermore, B cells from B/W mice are hyperresponsive to T cell-derived stimuli, such as IL-5, IL-6 and signals generated through CD40 (62-64). In addition, B cells from both lupus patients and B/W mice spontaneously proliferate and secrete large amounts of antibodies *in vitro* (65). However, when

the cultured B lymphocytes from SLE patients are challenged with mitogens, they are found to be markedly deficient in their ability to proliferate further, or to increase immunoglobulin synthesis, both of which suggest prior activation (66, 67). Another evidence of the prior activation of B cells is the decreased antibody responses to immunization in lupus patients (46, 68). Finally, another feature of SLE is the uncontrolled differentiation of B cells into plasmacytes that secrete IgG autoantibodies (69). Normally, these autoantibody-producing plasmacytes are deleted, anergized, or undergo receptor editing to create a non-self reacting Ig receptor. In both SLE patients and lupus-prone mice, a predisposition to B cell hyperactivity appears to override these tolerance mechanisms and change the composition of B cell compartments (70-72).

Intriguingly, accumulating evidence suggests that B cells serve not only as precursors of autoantibody-producing cells but also as antigen presenting cells (APCs) for autoreactive T cells, probably via over-expression of costimulatory molecules (73, 74). Studies show that B cells can process and present self-antigens to naïve T cells (75) and activate memory T cells (76). In MRL/lpr mice, the expansion of activated and memory T cells is also highly dependent on B cells (77). Strong evidence lies in transgenic MRL/lpr mice that have B cells expressing surface Ig, but lack circulating antibodies. These mice develop nephritis characterized by cellular infiltration within the kidneys (78). These data indicate that, in the absence of serum antibodies, B cells can exert a pathogenic role. Since B cells are not considered major producers of cytokines, they most likely serve as APCs for autoreactive T cells, or contribute directly to local inflammation. It is even possible that autoantigen-presenting B cells might promote the breakdown of peripheral T cell tolerance (79). Therefore, there is an antibody-independent mechanism for renal and vascular disease in murine SLE.

1.1.3.4. Complement

It has been well noted that complement deficiency is associated with SLE. The increased susceptibility of SLE, associated with C1q, C1r, C1s, and C2 deficiency, is thought to be because of impairment of immune complex clearance and solubilization (80, 81). Partial C4a deficiency is present in 50-80% of Caucasian SLE patients, but in only 10-20% of controls (82). Even though complement deficiency accounts for only 5% of all lupus patients, at least 50% of patients with homozygous deficiencies in the early classical complement pathway develop a lupus-like disease (83). In addition, the level of complement receptors, CR1 and CR2 are also altered in SLE patients (84, 85). CR1, which binds to C3b and C4b fragments, is decreased on erythrocytes in lupus patients compared with controls, and its level inversely correlates with disease activity (86, 87). In spite of its role in B cell hyper-responsiveness, CR2 expression on B-lymphocytes of patients with active lupus is reduced to half of that of control (88).

1.1.3.5. Cytokines

Cytokines regulate the immune system and may be split into two groups: T helper type 1 (Th1) cytokines and T helper type 2 (Th2) cytokines. Th1 cytokines, such as IL-2, IFNγ, TNFα and IL-12 mainly activate the cellular arm of the immune system, while as Th2 cytokines, such as IL-4, IL-6 and IL-10 mainly activate the humoral arm of the immune system. While high levels of Th2 cytokine gene expression is observed in peripheral blood mononuclear cells (PBMCs) in SLE patients (89), other evidence indicates a direct correlation between Th1 and Th2 cytokines (90), suggesting a mutual Th1-Th2 participation in lupus.

IL-10 may be of pivotal importance in this imbalance because it inhibits the production of Th1 cytokines (91). There are numerous studies showing increases of IL-10 production by PBMCs, as well as increased serum IL-10 levels in SLE patients (92, 93). Interestingly,

monocytes and B cells, rather than T cells, are responsible for this increase. The elevated serum IL-10 level correlates with disease activity and with anti-DNA antibody production (94-97). Similar observations are also reported in lupus mice, and repeated injection of anti-IL-10 mAb substantially delays onset of autoimmunity in B/W mice (98). Studies show that human IL-10 (hIL-10) is a potent growth and activation factor for B cells. It stimulates the expression of HLA class II molecules on resting B cells, induces the proliferation of activated B lymphocytes and their differentiation in IgA-, M- or G-secreting plasmocytes (99). In contrast to its positive effects on B cell proliferation and activation, IL-10 displays strong inhibitory activities on the function of T lymphocytes. For instance, IL-10 inhibits $CD4^+$ T cell proliferation and $CD8^+$ T cell allospecific cytotoxic activity (100, 101). Interestingly, IL-10 does not act directly on T cells, but via down-regulation of monocytes/macrophages function (102).

Increased serum IL-6 levels have also been demonstrated in both patients with active SLE and lupus mice (103-105). Interestingly, while IL-10 normally inhibits monocytes IL-6 production, this is not the case in lupus, implying an intrinsic defect in IL-10-mediated feedback (106). In addition to SLE, high serum levels of IL-6 are found in other inflammatory conditions, such as rheumatoid arthritis, bacterial infections, burns, and alcoholic cirrhosis (107). Thus, increased IL-6 in SLE could be a consequence of apoptosis, rather than a causative factor (108).

The earliest identified defect in lupus cytokine networks is the reduced production of, and response to, IL-2 (109). Subsequent studies of lymphocytes from lupus patients also show reduced IL-2 production *in vitro* following stimulation with antigen or mitogen (110). Nevertheless, the relation between the observed IL-2 defect in lupus patients and the disease process remains unclear.

One of the most consistent cytokine abnormalities observed in SLE has been the high expression of IFNγ (111). In spontaneous SLE murine models, IFNγ plays a crucial role in permitting and promoting disease. In B/W mice, treatment with anti- IFNγ mAb, or soluble IFNγ receptor, during early life significantly delays disease progression (112, 113). Furthermore, administration of IFNγ to a patient with rheumatoid arthritis induces SLE (114).

IL-12, which promotes the expansion and survival of Th1 and NK cells, has been reported to be elevated with a great variability in the serum of SLE patients (115). However, most investigators have found impairment in IL-12 production in SLE patients (116, 117). In lupus mice, an intrinsic defect in the *in vitro* production of IL-12 by endotoxin-activated macrophages of MRL+/+ and B/W mice has been reported (118).

The production of lymphocyte-derived TGFβ is decreased in SLE patients, which is inversely correlated with disease activity (55, 119). Studies of TGFβ regulation reveal that IL-2 and TNFα increase the production of active TGFβ, whereas IL-10 has the opposite effect. TNFα, a cytokine well known for both its proinflammatory and immunoregulatory effects, is elevated in SLE patients and correlates with disease activity (120, 121). High levels of TNFα expression are observed in the inflamed kidneys of MRL/lpr mice (122).

In general, PBMCs from SLE patients show a decreased Th1 and an increased Th2 profile. Similarly, in the patients there is a general decrease of cytokines produced by Th1 cells, including IL-2 and IL-12, and an increase in cytokines produced by Th2 cells, which are mostly B-cell growth, differentiation, and stimulatory factors (e.g. IL-6, IL-10). However, this picture of cytokine imbalance is not entirely black and white. For instance, the ratio of IFN-gamma/IL-4-producing $CD4^+$ T cells correlates with SLE disease activity indexes and is significantly higher

among patients with lupus nephritis. Therefore, cytokines from each group are involved in the development of autoimmunity in lupus.

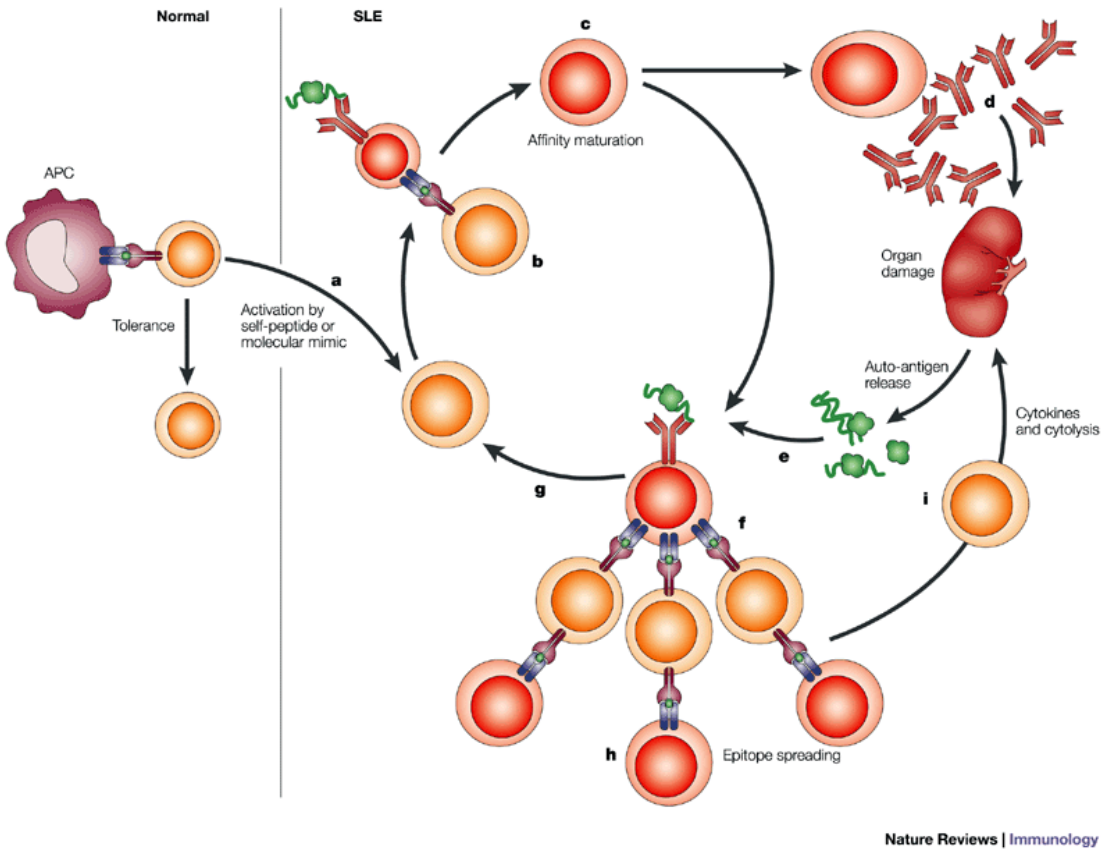


Figure 1 Model of autoimmune development in lupus

In normal situation, autoreactive T cells become tolerized to self antigens. In lupus, a) these T cells are activated by self-peptide; b) activated T cells interact with autoreactive B cells, which bind to self-antigen, and further activate B cells; c,d) these B cells undergo somatic mutation and affinity maturation, and differentiate into autoantibody-secreting cells; autoantibodies cause target organ damage directly or through immune complex deposition; e,g) autoantigens released from damaged tissue are captured by autoreactive B cells and presented to autoreactive T cell, which forms a positive-feedback loop; f,h) the interaction between autoreactive B cells and T cells facilitate epitope spreading and multiple clones activation; i) after activation, autoreactive T cells can migrate to target organ and cause tissue damage by releasing cytokines or cytotoxicity. Figure adapted from (123)

1.1.4. Animal models of SLE

Several murine models of spontaneous lupus-like disease have been established and widely used in research. The most well characterized are: the F1 hybrid of New Zealand Black and New Zealand White (NZB/W F1); the MRL/lpr mouse, an admixture of LG/J, AKR/J, and C3H/Di backgrounds; and the BXSB/MpJ (BXSB) mouse, a recombinant inbred of C57BL/6J (B6) and SB/Le strains. As in human SLE, all these mice develop high levels of IgG autoantibodies to nuclear antigens, including dsDNA. These autoantibodies mediate nephritis, probably as a result of *in situ* immune complex formation in the glomeruli. NZB/W F1 mice are more akin to human patients in that they show earlier and more severe disease in females, most likely related to the influence of sex hormones. Serologic and immunologic abnormalities in B/W mice also parallel that of humans, including the presence of LE cells, hypergammaglobulinemia, high antinuclear antibody titers, anti-dsDNA antibodies, hypocomplementemia, and severe immune complex GN (124, 125). Nephritis is evident in female mice by 5 to 6 months of age, and death from lupus nephritis follows relatively soon. Median life expectancy is 8 months for B/W female mice and 15 months for male mice. Based on these characteristics and similarities to human SLE, B/W mouse is the most commonly used murine model of lupus in research.

Even though the defining molecular and cellular events leading to SLE remain unclear, studies of murine models of lupus have provided important insight into its immunopathogenesis. Loss of self-tolerance and disease pathogenesis in SLE are thought to emerge from mutation or polymorphism in genes that influence lymphocyte signaling, the clearance of immune complexes, and apoptosis (126, 127). The MRL/lpr and BXSB mice carry single gene mutations that accelerate lupus-like disease. MRL/lpr mice are homozygous for the lymphoproliferation (lpr) mutation in Fas gene, which results in the acceleration of lupus-like autoimmunity, as well

as massive accumulation of $CD4^-CD8^-$ T cells (128). The hypothesis is that self-reactive T and B cells arise when they fail to undergo normal apoptosis. There is no counterpart to the lpr phenotype in human SLE. Male BXSB mice carry the Y chromosome-linked Yaa gene, resulting in a more rapid and severe lupus-like disease in male versus female BXSB mice (129). Being used extensively to map the genes responsible for SLE, NZM2410 mice were generated by breeding NZW and NZB/W mice (130), and thus represent an inbred strain that has acquired specific disease susceptibility loci from the NZB and NZW genomes. Three recessive loci, each comprised of approximately 100 genes and strongly associated with SLE susceptibility have been identified in the NZM2410 strain. Sle 1 on chromosome 1 initiates loss of self-tolerance and mediates antinuclear antibody development. Sle 2 on chromosome 4 lowers the activation threshold of B cells and sle 3 on chromosome 7 provides T cell hyperactivity (131, 132).

1.2. Immunotherapy

The survival of SLE patients has improved remarkably over the past decades because of the use of corticosteroids and cytotoxic agents, such as cyclophosphamide. While such medications have powerful anti-inflammatory and immunomodulatory effects, their use is severely limited by numerous side effects, such as immunosuppression and myelosuppression. A safe and efficient mode of immunomodulatory therapy for this disease is still lacking. Based on the rapid progress in understanding the molecular biology of the immune system, new strategies are available to regulate the immune response and inflammation by targeted molecular therapy. Methods under investigation, which may be beneficial, include manipulation of costimulatory pathways (133), manipulation of distinct cell-surface molecules that are required for T cell recognition of antigen (134), induction of tolerance through the administration of peptides derived from autoantibody (24), and blocking distinct cytokines that promote autoimmunity (98, 113).

1.2.1. B cell-directed

B cells have been found to be critical to the development of SLE. Besides autoantibodies secretion, they take up and present autoantigens, via specific surface immunoglobulins, to T cells, and they help regulate and organize inflammatory responses (78, 135). Therefore, several novel therapeutic schemes targeting B cells have been developed. CD20, a pan-B cell surface marker, is believed to function in B cell cycle initiation and differentiation (136). CD20 is first expressed in the early pre-B cell stage and remains present until terminal differentiation into plasma cells. Rituximab is a chimeric mouse/human anti-CD20 mAb and has been shown to be safe and effective in the treatment of B cell lymphoma (137). Phase I/II study of Rituximab on twelve SLE patients demonstrated variable effectiveness of B cell depletion (138, 139). The overall serum levels of anti-dsDNA antibodies did not significantly change, possibly reflecting a lack of effect on the plasma cell lineage where CD20 is not expressed. Higher doses of rituximab and additional immunosuppressive therapy may be necessary to achieve additional depletion of B cells, induce a serologic response and enhance clinical efficacy.

B lymphocyte stimulator (BLyS) is among the novel TNF ligands and receptor superfamily members recently described. Its receptors, TACI, BCMA and BAFFR, are largely restricted on B cells. The BLyS protein can promote B cell survival, expansion, and differentiation (140). Constitutive overexpression of BLyS protein results in SLE-like disease in mice, and treatment with a BLyS antagonist ameliorates disease in lupus-prone mice (141). Moreover, studies also show elevated serum BLyS levels in a subset of human SLE patients. LymphoStat-B is a fully human IgG1λ mAb that neutralizes human BLyS. The administration of LymphoStat-B to cynomolgus monkeys selectively reduces B cells in blood and tissue with no overt toxicity (142). Treatment of SLE mouse models with TACI-Ig fusion protein ameliorates

disease progression and improves survival (143). Recently, there is a study delineating 1,4-benzodiazepine (Bz-423) specifically induces apoptosis of activated B cells, casting light on lupus treatment (144).

Beside B cell depletion, there is another strategy to induce tolerance in B cells. LJP394, a B cell toleragen, consists of four double-stranded oligonucleotides attached to a nonimmunogenic polyethylene glycol platform. This molecule induces tolerance in B cells directed against dsDNA by cross-linking surface antibodies (145). It inhibits binding of DNA to anti-dsDNA Abs in an immunized mouse model (146). In a phase I trial, a single intravenous injection of LJP394 was followed by an immediate reduction in anti-dsDNA titers, which persisted for 4 weeks in some subjects (147). A double-blind, placebo-controlled study of LJP394 in 58 patients with clinically stable SLE demonstrates a 58% reduction in anti-dsDNA antibody at 8 weeks with the highest drug dose. This effect persists for 8 weeks after the drug is stopped (148).

1.2.2. T cell-directed

Numerous evidences suggest that autoantibody production is a T cell dependent process in lupus. Therefore, $CD4^+$ T helper cells become another target for inhibiting autoimmunity. Treatment with anti-L3T4 to delete T helper cells decreases the diverse histopathologic manifestations of murine lupus, including glomerular immunoglobulin and complement deposition, lymphocytic infiltration and vasculitis in the kidneys (34). A recent study attempts to induce immune tolerance by treatment with a consensus peptide based on amino acid sequences from the VH regions of murine anti-DNA IgG, which is likely to stimulate T cells in B/W mice. The data show that T cell help for IgG anti-DNA production is impaired, resulting in prolonged survival, delayed onset of nephritis, and decreased levels of IgG antibodies to nucleosomes (25).

1.2.3. Costimulatory blockade of T-B cell interaction

The interaction between the pathogenic T helper and B cells is critical for the autoantibody production in lupus. Two distinct signals are required for the interaction between T and B cells. The first signal originates from the engagement of the T cell receptor with the B cell receptor/peptide complex. The second signal operates through costimulatory pathways, including the CD28/B7 and CD40/CD40L receptor/ligand system (133, 149) (Fig.2). The interaction between CD28, which is constitutively expressed on T cells, and B7 on B cells is essential for T cell activation, Th1/Th2 differentiation, chemokine production and homeostasis of $CD4^+CD25^+$ regulatory T cells (133). CD40L is mainly and transiently expressed on activated T helper cells, and binds to CD40 on antigen-specific B cells. This costimulatory pathway induces B cell proliferation, Ig isotype switching, somatic mutation and terminal differentiation into memory cells and antibody-forming plasma cells (150, 151). CD40L is overexpressed on T cells from SLE patients (152, 153). Surprisingly, not only T cells, but also B cells of lupus patients expressed abnormally high levels of CD40L without any deliberate stimulation (57). Thus, in addition to being activated by CD40L on T helper cells, lupus B cells could amplify and sustain the pathogenic autoimmune response by interacting with CD40 on other autoimmune B cells in the vicinity, as well as follicular dendritic cells in the germinal center (154). Therefore, costimulatory blockade could work by affecting both the B and T cell responses, as well as amplifying the anti-inflammatory stimuli.

Preliminary evidence suggests that selective tolerance can be achieved by blocking interaction between costimulatory ligand pairs such as CD40/CD40L and B7/CD28 (155, 156). Considerable attention is focused on the use of agents that block T cell costimulation. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is only expressed on activated T cells. It has a

higher affinity to B7 and negatively regulates T cell function through B7/CTLA-4 pathway (157). Over the past few years, CTLA4Ig, a soluble protein of the CTLA4 extracellular domain fused to an immunoglobulin tail, has emerged as a particularly promising immunomodulator. It has been used as an inducer of peripheral tolerance in transplantation and autoimmune disease (158-160). It has also been shown to inhibit autoantibody production and prolong life span of lupus mice (161, 162). The observed beneficial effect of blocking B7/CD28 interaction by CTLA4Ig in murine lupus could also be due to its indirect effect on CD40-CD40L interaction (163, 164). This strategy has the advantage of not necessarily requiring knowledge of the autoantigen. In one study, treatment of murine lupus with a combination of CTLA4Ig and cyclophosphamide is more effective than either agent alone in blocking autoantibody production, reducing renal disease, and prolonging survival of mice with advanced nephritis (165).

Another way to block costimulatory pathway is using anti-CD40L mAb. Studies show that anti-CD40L mAb inhibits lymphocytes from lupus patients to produce antinuclear autoantibodies *in vitro* (57). Studies on lupus mice also show that anti-CD40L mAb is able to reduce anti-DNA autoantibody production and suppress disease progress (166-168), and even on nephritic mice, it is able to prolong survival, diminish vasculitis and fibrosis (169, 170). Because CD40L is expressed mainly and transiently by activated T cells, and most of the activated T cells in lupus are involved in driving autoantibody production, the anti-CD40L mAb therapy could preferentially affect the autoreactive T cells in lupus. Studies of anti-CD40L antibodies in SLE patients show decreased serum levels of anti-dsDNA antibodies, a reduction in the number of anti-dsDNA secreting cells in peripheral blood (171), and even improvement in hematuria in patients with advanced nephritis (172). Phase I trials demonstrate that anti-CD40L mAb (IDEC-131) is safe and well tolerated in SLE patients (173, 174).

Combination therapies of costimulatory blockade have been examined. In one study, CTLA4Ig, anti-CD40L mAb, or the combination of the two is given to B/W mice for 2 weeks early in life. Ten months later the survival is 0% in the CTLA4Ig group, 18% in the anti-CD40L mAb group, and 70% in the combination group (162). These data suggest that short-term treatment with combination therapy might result in a durable clinical response, a finding confirmed by a more recent study (175). These strategies are the subject of clinical trials that are designed to determine whether the promising results observed in lupus mice will herald a new era of improved treatments for human with SLE.

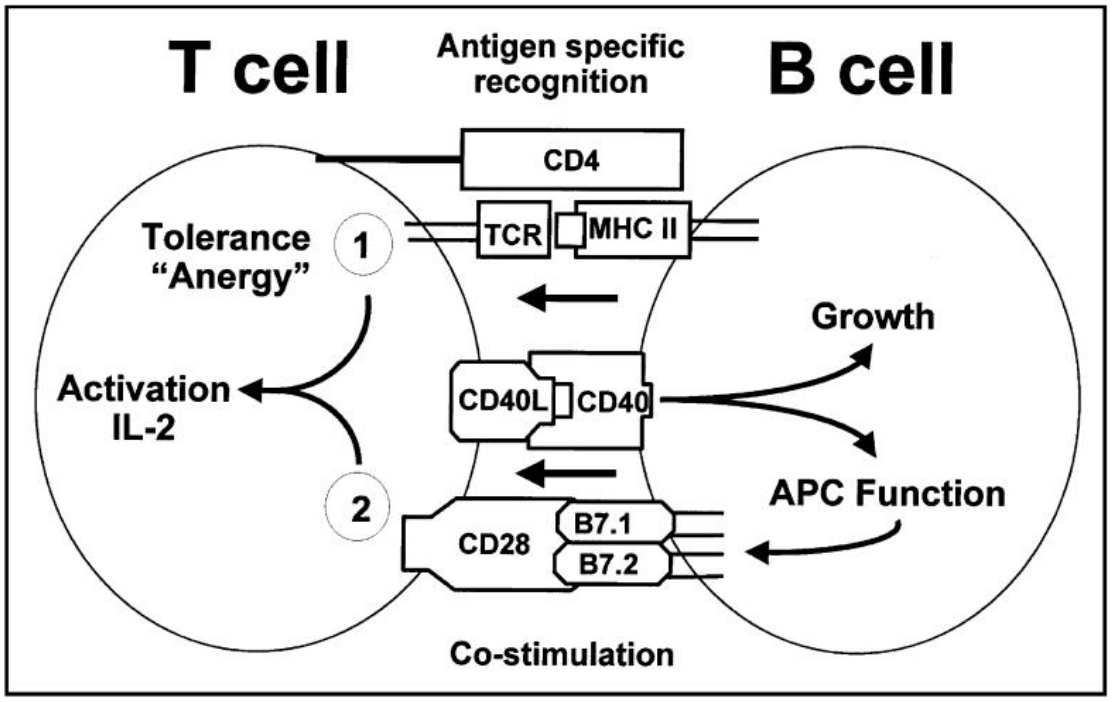


Figure 2 Costimulatory pathway

There are two signals required for T cell activation. Signal 1 is the binding of T cell receptor (TCR) to the MHC II-peptide complex on B cells. Signal 2 is costimulatory pathway, including the binding of CD28 to B7 and CD40L to CD40. When only signal 1 exists, T cells become anergy to the antigen. The binding of CD40L to CD40 stimullates B cell growth and differentiation. Figure adapted from (176)

1.2.4. Cytokine inhibitors

Excessive IL-10 plays an important role in autoantibody production in lupus. Thus, the administration of anti-murine IL-10 mAbs to B/W mice significantly inhibits serum anti-DNA antibody levels, while the onset of proteinuria and GN is delayed in the treated group (98). In human SLE, addition of neutralizing anti-IL-10 mAb to *in vitro* PBMCs culture inhibits total Ig and IgG production (177). Finally, in an open trial, six SLE patients with active lupus are injected with murine anti-IL-10 mAb. The treatment is safe and well tolerated, with 5 of the 6 patients achieving complete clinical remission and their corticosteroid treatment is significantly tapered (178). Even though elevated TNFα level is found in lupus, data collected from animal models of SLE strongly support the defective production of TNFα by monocytes as playing a facilitating role in the disease. Early administration of high doses of recombinant TNFα to B/W mice delays the onset of GN and improves survival (179-181).

1.2.5. Other strategies

By blocking the late membrane attack complex of complement (C5b-9), administration of anti-C5 mAb delays the onset of proteinuria, improves renal histology, and prolongs survival in B/W mice (182). Several case series demonstrate the efficacy of immunoablation and subsequent autologous hematopoietic stem cell transplantation in inducing remission in SLE patients (183-185). Although long-term follow-up data are not available, many of these patients have re-emergence of antinuclear antibodies within 2 to 3 years. Sex hormones may play a role in the etiopathogenesis of SLE. Use of the adrenal steroid hormone dihydroepiandrosterone (DHEA) in the treatment of SLE has been investigated in multiple clinical trials and controversy exists regarding the efficacy of this strategy (186, 187).

1.3. Gene therapy in autoimmune disease

The chronic nature, combined with the complex and insufficiently understood etiopathogenesis of SLE make it a special challenge for clinical therapy. Protein-based therapies with cytokines or antibodies have been available for some time, but production of these proteins in large quantities is expensive and parental delivery is problematic. Most cytokines and antibodies have a short half-life in body fluids, and bolus injections can lead to transient and dangerous toxic levels of these reagents. Moreover, limiting delivery to a specific diseased organ is either difficult or not feasible. Over the last decade, gene therapy has been developed as a particularly interesting and promising approach for treatment of genetic disease. For autoimmune disease, it offers an efficient and advantageous way of delivering immunomodulators and anti-inflammatory mediators, which can antagonize the chronic inflammatory process. After gene transfer, the immune mediators are produced at relatively constant, non-toxic levels and sometimes in a tissue-specific manner, obviating limitations of protein administration. Localized delivery can also be achieved in some tissues, such as joints and the central nervous system. Furthermore, genes can be transduced into T lymphocytes, which can pinpoint target organs in an antigen-specific manner. The key issues to consider in gene therapy include the therapeutic gene, the vector, and the delivery route.

1.3.1. Therapeutic genes

In several autoimmune/inflammatory disorders, protective effects have been observed by transfer of cDNA encoding IL-1 receptor antagonist (IL-1Ra), TNFα receptor, or TGFβ1 (188). For instance, intramuscular (i.m.) injection of naked plasmid DNA encoding TGFβ1 (pCMV-TGFβ1) protects against autoimmune lesions in NOD mice (189). The plasmid encoding IFNγR/IgG1 is protective in both spontaneous and drug-induced models of autoimmune diabetes (190, 191).

Lupus is associated with a wide range of immunological abnormalities. Attempts at developing specific biological therapies have targeted a number of these defects by using recombinant cytokines, blocking antibodies, and soluble receptors. With the recent advance in establishing protocols for gene therapy for a number of animal models of autoimmune diseases, gene therapy has become an obvious strategic approach to treat lupus. Monthly injections of plasmid cDNA encoding TGFβ1 into MRL/lpr mice between 6 and 26 weeks of age increase survival to 70% in the treated mice compared to 40% in control groups, decrease anti-chromatin and rheumatoid factor antibodies, and decrease total IgG production by 50% (192). However, other studies using similar strategies show no improvement in pathology in MRL/lpr mice (193, 194). These contradictory results highlight the risks inherent in using cytokines as therapeutic molecules.

Most cytokines may have stimulatory or inhibitory effects depending on their concentration, target tissue or cell, as well as interaction with other cytokines in the extracellular milieu. It becomes a prominent problem in the context of gene therapy because transgene products are produced long term. Compared with cytokines, cytokine inhibitors (such as antibodies or soluble receptors) are advantageously nontoxic and are often long-lived in body fluids. I.m. injection of IFNγR/Fc plasmid protects against lupus in MRL/lpr mice, particularly when enhanced by *in vivo* electroporation (195). The strikingly elevated serum IFNγ levels of these mice are depressed by this kind of therapy, and remarkably, amelioration of glomerular and other lupus-related lesions is noted even when the treatment is begun at an advanced stage of the disease. The fusion proteins used in these studies consist of segments of endogenous murine proteins, and no antibodies are reactive to these proteins in the treated mice (190). This strategy poses a major advantage over anti-IFNγ antibodies from other species, which can be neutralized

by the host's immune response. In addition, fusion proteins with immunoglobulin Fc segments often offer advantages over native proteins, particularly if the latter have low molecular weights. The increase in size can prolong the half-life of these proteins in body fluids (196). These fusion proteins are usually secreted as homodimers, which have a higher avidity for their ligand (191, 197, 198). As mentioned above, costimulatory blockade using CTLA4Ig also inhibit autoimmune responses. Gene therapy using this approach has also been shown to be beneficial in the MRL/lpr lupus mice. With a single intravenous injection of AdCTLA4/IgG into MRL/lpr mice, the autoantibody production and lupus nephritis are completely suppressed (199).

1.3.2. Vectors

There are basically two kinds of vector, namely viral and non-viral, that are used in gene therapy. They have been shown to be effective in several animal models of autoimmunity, including type 1 diabetes mellitus (DM), experimental allergic encephalomyelitis (EAE), SLE, colitis, thyroiditis and various forms of arthritis. When choosing a vector for gene therapy, there are several essential factors to consider, such as the capacity of transgene cassette, duration of transgene expression, immunogenicity, ability to target quiescent or dividing cells, and extra-chromosomal or genomic integration of a transgene, not to mention overall safety.

Viral and non-viral vectors each have advantages and disadvantages. Plasmid DNA is simple and cheap to construct, but unlike viruses, plasmids do not have the ability to enter cells, localize to the nucleus, or incorporate into the genome. Furthermore, with some exceptions, such as skeletal muscle and skin, plasmid DNA is not efficiently taken up and expressed by mammalian cells. Transduction has often been achieved by complexing DNA to cationic lipids/liposomes. These agents can enhance uptake of DNA by endocytosis, maintain its stability within the endosome and facilitate DNA release through their buffering and detergent properties

(200). For instance, intraperitoneal (i.p.) delivery of an IL-10-encoding plasmid in complex with cationic liposomes achieves therapeutic effects in the mouse collagen-induced arthritis (CIA) model (201). Other methods to improve plasmid transduction efficiency include gene gun delivery, jet injection, intravascular injection under pressure, or low-voltage *in vivo* electroporation (202-204). Since plasmids persist as extrachromosomal DNA, subsequent expression is transient, continuing for up to a week due to DNA loss during cell division or DNA degradation, except in skeletal muscle, where the transgene expression continues for at least 19 months (205). Another disadvantage of plasmid DNA is that they carry unmethylated CpG-containing immunostimulatory sequences (ISS), which promote secretion of inflammatory cytokines (206). However, this type of stimulation is not antigen specific, and vectors can be re-administered without the risk of a neutralizing immune response. Moreover, ISS motifs can often be deleted without abrogating the activity of the vectors.

Most vectors presently in use for gene therapy are viral. Retrovirus, adenovirus, adeno-associated virus (AAV) and herpes simplex virus (HSV) are most frequently used. As a group they are difficult and expensive to produce in large amounts, and there are lingering concerns about safety and immune response to the vectors. To circumvent the problem of viral immunogenicity, recent efforts have been focused on highly defective vectors deleted of viral proteins or *ex vivo* gene delivery.

Retroviruses are a group of RNA viruses characterized by the employment of the unique reverse transcription mechanism. They exist as lipid-enveloped nucleoprotein particles containing a single-stranded linear RNA molecule of 7-11kb. The limitations of retrovirus vectors are that transduction can occur only in actively dividing cells, and the possibility of insertional mutagenesis and possible carcinogenesis in infected cells. These limitations

significantly narrow the applicability of retroviruses for gene therapy, especially *in vivo* strategies. However, the ability of retroviruses to integrate into the host genome and their relatively low immunogenicity are advantageous features for gene therapy applications. Greatest success with retroviral vector gene therapy has been achieved following *ex vivo* transduction of various cell types. For example, dendritic cells (DCs) transduced with retrovirus to express bone morphogenetic protein 4 (BMP4) improve healing of a bone defect in rats (207). Similarly, retroviral transduction of lymphocytes with IL-4 protects BioBreeding rats from type I diabetes (208). Additionally, lentiviruses are complex retroviruses with a more developed genetic structure. Besides low immunogenicity and genome integration, they can infect nondividing cells. This applies particularly to gene therapy of autoimmune disease, as many potential target cells in the immune system are nondividing.

Adenoviruses (Ad) have been powerful tools in the development of experimental gene therapy for autoimmunity. Their ability to infect dividing and non-dividing cells with high efficiency, and their capacity to produce high-titer viral stocks have greatly aided their application. There are numerous examples of the successful application of Ad in gene therapy of animal models of various autoimmune disease including diabetes, rheumatoid arthritis, multiple sclerosis and SLE (199, 209-211). There are, however, significant drawbacks associated with the use of Ad. Ad particles elicit inflammatory and toxic reactions in the host, which leads to depletion of Ad-transduced cells. Most severely, a fatal immune response could occur (212).

Adeno-associated virus (AAV) is a small single-stranded DNA nonenveloped parvovirus. Recombinant AAV (rAAV) is able to transduce both dividing and nondividing cells. *In vivo* studies demonstrate efficient transduction of neurons, muscles, liver and airways (213-216). AAV vectors have been applied in gene therapy of experimental autoimmune models of arthritis

and diabetes. Delivery of rAAV vector encoding IL-10 i.m. in NOD mice effectively abrogates pancreatic insulitis and prevents diabetes development (217, 218). Constitutive expression of IL-4 through rAAV-mediated gene delivery prevents disease development in CIA mouse (219).

Herpes simplex virus (HSV) is a neurotrophic DNA virus. The huge 150kb-transgene capacity of HSV permits the introduction of multiple genes along with components for transcription regulation. LATP2 can be used as an enhancer to endure that transgene expression persists throughout the latency phase. HSV replication-deficient vectors encoding IL-4 have demonstrated therapeutic effect after onset of disease in a murine model of EAE (220).

1.3.3. AAV

AAV is a single-stranded DNA virus with a 5kb genome. Each end of the genome forms short inverted terminal repeat (ITR), which can fold into a hairpin structure and serve as the origin of viral DNA replication. The ITRs are the only cis elements required for replication and packaging (221, 222). The genome is packaged within a nonenveloped, icosahedral capsid of approximately 20nm diameter (223). The AAV genome allows for transfer of gene expression cassettes of up to approximately 4.5kb. AAV serotype 2 (AAV2) is most commonly used for the application of gene therapy, and its antibodies are detected in up to 96% of the population. To date, at least eight serologically distinct AAVs have been identified and isolated from human and primates (224, 225). AAV1 and AAV6 are muscle tropic, while AAV8 are kin to liver (225). Whereas the different serotypes differ in terms of tissue tropism, the genomes can be cross-packaged into heterologous capsids in order to take full advantage of different serytypes (226, 227).

The following features have made AAV an attractive vector for gene therapy. Firstly, AAV vector particles are nonpathogenic, noninflammatory, heat stable and resistant to solvents and changes in pH and temperature. This distinguishes recombinant AAV vectors from many

other viral vector systems such as retro/lentiviral and adenoviral vectors (228). Secondly, AAV vectors are devoid of all viral genes and retain only about 300 nucleotides of viral sequence in the nontranscribed ITRs. This greatly improves the safety of AAV vectors for human clinical applications by reducing the risk of recombination with wild type virus. Furthermore, the lack of viral coding sequences extends the duration of gene expression as no viral gene products are expressed in target cells, which reduces the risk of eliciting a cellular immune response. Thirdly, AAV vectors possess a broad host range and transduce both dividing and nondividing cells *in vitro* and *in vivo*. Lastly, the most promising and distinguishing feature of AAV vectors is the fact that they maintain (over several years) high levels of transgene expression in vivo in the absence of a significant immune response to the transgene product.

There are, however, a few drawbacks in using AAV vectors for gene therapy applications. First is the limited packaging capacity; the AAV genome allows for transfer of gene expression cassettes of up to approximately 4.5kb. Even though the strategy of viral DNA heterodimerization can double the cargo space, there is still many difficulties for the ultimate application (229). Secondly, the onset of gene expression after transduction is slow, especially when compared to Ad. This is because the single-stranded AAV genome has to be converted into double-stranded DNA before gene expression can be initiated (230). To overcome this limitation, double-stranded AAV (ds-AAV) vectors have been developed and shown to achieve faster and higher transgene expression (231). Thirdly, a study showed rAAV2 vectors preferentially integrate into transcriptionally active chromatin regions in hepatocytes, however, the overall integration frequency of rAAV2 vectors is extremely low (232). Despite the above described limitations and concerns, gene transfer vectors based on rAAVs still represent one of the most promising gene therapy systems and are gaining increasing popularity. So far, clinical

applications of rAAV2 vectors have focused on cystic fibrosis and hemophilia B, in which rAAV2 have been well tolerated (233-236).

1.3.4. Delivery methods

Regardless of the vector system that is used, gene transfer to the target tissues may be accomplished by *in vivo* or *ex vivo* strategies. In the former, vectors are injected directly into the body. In the latter, cells are removed, genetically altered outside the body, and then returned to the individual. In the *in vivo* gene transfer, local delivery produces fewer side effects and is suitable for autoimmune diseases, which primarily affect a discrete anatomical location, such as Grave's disease. Tissue-localized delivery can also be achieved, such as in joints or the CNS. An example is the injection of an IL-4 encoding retroviral vector directly into the joints of rats in the adjuvant arthritis model that results in a significant reduction of paw swelling and bone destruction (237). Adenoviral transfer of the viral IL-10 gene periarticularly to mouse paws suppresses development of CIA (238). For i.m. delivery, vector administration is simple and inexpensive, and transgene expression is usually more persistent than elsewhere. Therefore, it is also used in gene therapy of genetic disease. In a clinical trial of hemophilia B, rAAV encoding factor IX is injected into the muscle and factor IX is detected in the circulation (235). Systemic delivery seems better suited to the treatment of disseminated autoimmune diseases like lupus. Somatic gene therapy makes long-term, constant systemic delivery of many protein mediators feasible.

Ex vivo delivery is clearly more tedious, expensive, and cumbersome, but it permits selection and scrutiny of the genetically altered cells before reimplantation. It has been successfully applied to joint therapy and islet transplantation (239). It has become evident that T cells and their cytokines play a central role in the initiation, as well as the perpetuation, of organ-

specific autoimmune diseases (240, 241). Because of their importance as mediators in the pathogenesis of autoimmune disease, $CD4^+$ T cells have been considered to be ideal candidates for cell-based gene therapy. Moreover, after adoptive transfer, these autoantigen-specific T cells can home into the target organs. Effective prevention of EAE and arthritis has been achieved by adoptive cellular gene therapy using autoantigen-specific T cells and T cell hybridomas transduced by retroviruses to express the IL-12 receptor blocker IL-12p40 (242, 243), the regulatory cytokine IL-4 (244), and the TNF-antagonizing anti-TNF scFv (245). Moreover, adoptively transferred islet-specific Th1 clones transduced with IL-10 prevent diabetes in NOD mice (246). Despite these encouraging results, it has been difficult to transduce murine T cells efficiently. Another caveat is that T cell clones frequently produce several cytokines, and there is a risk of unexpected adverse effects following adoptive transfer. In addition, dendritic cells (DCs) not only provide a common set of signals for initial clonal expansion of T cells, but also provide T cells with selective signals that lead to either Th1 or Th2 immunity. This makes DCs optimal targets of cellular gene therapy. It has been demonstrated that transfer of TNF-treated, incompletely matured DCs induces peptide-specific IL-10 producing T cells *in vivo* and prevents EAE (247). Injection of bone marrow-derived DCs retrovirally transduced to express IL-4 before disease onset also reduce CIA incidence and severity (248).

1.4. Specific aims

SLE is a systemic autoimmune disease, which affects mostly young women. This disease is characterized by polyclonal B cell activation, autoantibody production and immune complex-mediated glomerulonephritis. In 60-70% of cases, renal involvement complicates the symptoms and its severity largely determines the prognosis. During long-term treatment using immunosuppressant and cytotoxic agents, lupus patients frequently suffer from life-threatening complications such as infection, malignant tumor growth and renal failure. A number of murine

models, such as NZB/W F1, MRL/lpr and BXSB mice, are available for the study of genetics, immunopathology and treatment of lupus. Even though the etiology of autoimmunity in both human and murine lupus is not clearly understood, mounting evidence has indicated the involvement of autoreactive B cells and T cells. Thus costimulatory blockade of T cell and B cell activation using mouse CTLA4Ig fusion protein and anti-CD40L mAb has been shown to suppress autoantibody production and prevent nephritis in lupus mice. Moreover, cytokine antagonist, such as anti-IL-10 mAb, also delays lupus onset. However, the application of these protein-based therapies is limited by inconvenient delivery, short half-life of protein and toxic effects after bolus administration. By obviating these drawbacks, gene therapy has become an obvious strategic approach to treating lupus. Furthermore, recombinant adeno-associated virus (rAAV) is a promising vector with regard to its nonpathogenicity and nonimmunogenicity.

To test the hypothesis that rAAV8-mediated delivery of immunomodulator transgene can prevent and suppress disease development in murine model of lupus, I used rAAV serotype 8 vectors encoding mouse CTLA4Ig fusion protein (mCTLA4IgG2a), mouse CD40IgG fusion protein (mCD40IgG1), or soluble receptor of mouse IL-10 (mIL-10RIgG2a). The findings in this study will broaden our understanding of rAAV-mediated gene therapy in autoimmune disease and facilitate its clinical trial in the future.

There are three specific aims in this study:

1. To test the transgene product secretion level *in vitro*, and to investigate the kinetics of transgene expression through different *in vivo* vector delivery routes.
2. To investigate whether rAAV8-mediated gene transfer was able to prevent lupus development by comparing different immune intervention strategies and to study the immunological mechanisms involved.

3. To determine whether rAAV8-mediated gene transfer was able to reverse SLE progress after the disease was already developed, and if an effect was observed, was it dose related.

2. RAAV-MEDIATED LONG-TERM TRANSGENE EXPRESSION IN NZB/W F1 MICE

2.1. Introduction

SLE is a prototypic autoimmune disease with the autoantibody production and IC-mediated target organ damage. The interaction between autoreactive T cells and B cells is believed to constitute a positive feedback loop which diversifies and amplifies the autoimmune response (123). There are two signals required for T and B cell interaction. One is the engagement of the T cell receptor with the B cell receptor-peptide complex, the other involves costimulatory pathways through the B7/CD28 and CD40/CD40L systems (249, 250). The major strategies adopted in this project were targeted at costimulation of lymphocyte activation, which takes place in the secondary lymphoid organs, such as spleen and lymph nodes. Another strategy was to use soluble IL-10 receptor to neutralize excessive serum IL-10, which is proven to be a growth and differentiation factor for B cells (99). In either case, the transgene products have to be secretory so that they can either get to the loci of lymphocyte activation or bind to their counterparts in the circulation. In this chapter, I firstly constructed plasmid cDNA encoding the extracellular domain of each immunomodulator followed by the Fc tail from mouse IgG. Then I determined whether the transgene products, namely mCTLA4Ig, mCD40Ig and mIL-10RIg, could be secreted following *in vitro* tranfection of 293 cells. Secondly, all transgenes were subcloned into rAAV expression cassettes, and rAAVs were produced using a three-plasmid cotransfection method of 293 cells. When choosing the rAAV serotype, the tissue tropism was the major concern. Liver is the largest secretory organ in the body, from which optimal secretion could be expected. AAV8 was reported to be 10- to 100- fold powerful than other serotypes in terms of liver transduction (225). Therefore, all transgenes were packaged into AAV8 capsid. In

gene therapy, the transgene expression level directly affects the intervention outcome, especially in a chronic autoimmune disease, where long-term intervention is necessary. After *in vivo* gene delivery, I measured serum mCTLA4Ig levels at different time points by ELISA. I also compared the transgene expression levels in different delivery routes. For systemic gene delivery, the vector distribution is also an interest of research. To address this issue, I isolated total protein from each tissue and organ after systemic delivery and measured transgene expression levels by ELISA to determine which organ or tissue has the highest transduction efficiency. The results from this chapter will help us to understand the expression pattern of rAAV8-delivered transgenes, and set the base for the study of gene therapy outcome.

2.2. Material and methods

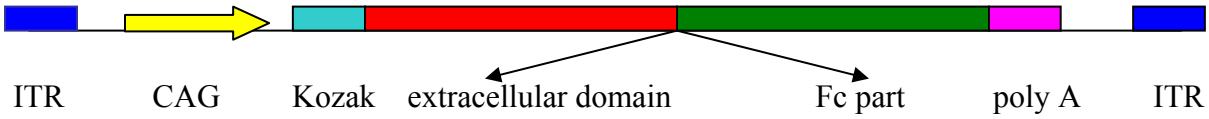
2.2.1. Plasmid construction

The 1269bp cDNA encoding mCD40IgG1 was obtained from the plasmid pAdlox-mCD40IgG1 (provided by Dr. Andrea Gambotto) by Hind III/BamH I digestion. It consisted of a 579bp extracellular domain of murine CD40 and a 684bp Fc part of IgG1, with a 6bp linker in between. The CD40 sequence corresponded to exon 1 through exon 6 of mouse CD40 type V isoform in GenBank, while the Fc part included the hinge, CH2 and CH3 sections of mouse IgG1. The 1194bp cDNA encoding mCTLA4IgG2a was excised from plasmid pXX-mCTLA4IgG2a (from our lab) by BamH I/Not I digestion It included a 483bp extracellular domain of murine CTLA4 and a 702bp Fc part of IgG2a, with a 9bp linker in between. The 5'end of the mCTLA4IgG2a cDNA sequence was further modified by introducing a Kozak sequence (GCCACC) to enhance transgene expression. In the mIL-10RIgG2a cDNA, the 714bp mIL-10 receptor extracellular domain was amplified by reverse transcription polymerase chain reaction (RT-PCR) with SuperScript reverse transcriptase using total RNA extracted from murine peripheral blood cells.

The forward primer was **5'-** TGTGCGGCCACCATGTTGTCGCGTTTGCTC **-3'**. The reverse primer was **5-** GTTGGTCACAGTGAAATA **-3'**. The fragment was then ligated to the 702bp Fc part of murine IgG2a, with a 9bp linker in between. In addition, a Kozak sequence was cloned to the 5' end of the mIL-10RIgG2a cDNA. These cDNAs were subcloned separately into the AAV plasmid backbone by blunt-end ligation. Since mCTLA4IgG2a was only 1194bp, it was further cloned into the double-stranded AAV plasmid backbone.

To construct a single-stranded AAV plasmid backbone, plasmid pXX-CAG-A1AT was used, which contained the 145bp AAV2 inverted terminal repeats (ITRs), flanking a cytomegalovirus immediate-early enhancer/chicken beta-actin promoter/human globin intron (CAG promoter) driving the alpha-1 anti-trypsin (A1AT) transgene. The A1AT cDNA was excised by Hind III/Not I digestion. To construct a double-stranded AAV plasmid backbone, plasmid pAAV-D(+)-CB-IL-18 was used, in which the D-sequence of the 5' ITR was deleted, while the 3' ITR was kept intact. Thus the modified plasmid generated double-stranded viral DNA (231, 251). The IL-18 cDNA, driven by a cytomegalovirus immediate-early enhancer/chicken beta-actin promoter (CB promoter), was then excised by Not I/Sal I digestion. Subsequently, the backbone was filled in using Klenow enzyme.

Single-stranded rAAV plasmid construct



Double-stranded rAAV plasmid construct

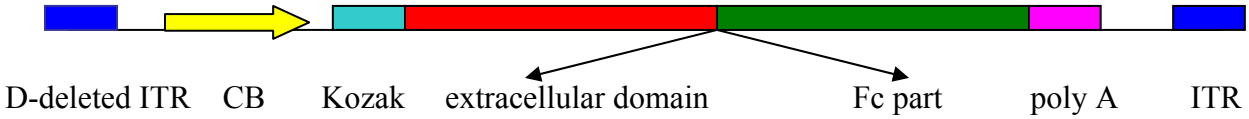


Figure 3 Adeno-associated viral vector constructs

The transgene cDNA was subcloned into the rAAV expression cassette, which contained ITR at each end. In the single-stranded rAAV plasmid, the transgene was driven by CAG promoter. For mCTLA4IgG2a with size of 1194bp, it was also subcloned into the double-stranded rAAV backbone, in which the D sequence of the 5' ITR was deleted.

2.2.2. AAV vector production

A standard triple-plasmid cotransfection method was used to produce rAAV vector. Briefly, 293 cell lines were propagated in 20 15-cm dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum at 37ºC in 10% CO_2/90% air. When the cells reached 90% confluency, 50µg of plasmid DNA, including 25µg of helper plasmid, 6.25µg of the AAV serotype 8 packaging plasmid and 18.75µg of the rAAV-transgene vector plasmid, was mixed in 2 ml of 0.25M $CaCl_2$ and then quickly mixed with 2 ml of HBS buffer (50mM HEPES, 280mM NaCl and 1.5mM Na_2HPO_4, pH 7.12) and added to the cells. Eight to 12 hours later, the medium was replaced with fresh DMEM. Forty-eight hours after cotransfection, the cells were harvested and resuspended in virus suspension buffer (50mM HEPES, 150mM NaCl, 50mM NaH_2PO_4, 2mM $MgCl_2$, 2.5mM KCl, pH 8.0), frozen and thawed three times. Cell lysate was further digested with 50 units/ml Benzonase at 37ºC for 2 hours and subsequently with 1% deoxycholate at room temperature for 30 minutes. Debris was removed by centrifugation.

For virus purification, CsCl salt was added to the supernatant at the concentration of 0.45g/ml to the density of 1.38g/ml. The mixture was then centrifuged in a SW41 rotor at 41,000 rpm for 24 hours with a 1.0ml underlying CsCl-PBS cushion (density 1.5g/ml). The rAAV band was collected and centrifuged as described above for 48 hours. Finally, the rAAV band was collected and dialyzed against virus dialysis buffer (2% mannitol, 1mM $MgCl_2$, 1xPBS). The particle titer of viral preparation was determined by quantitative dot-blot hybridization. Briefly, the rAAV stock was treated with 50 units/ml DNase I for 1 hour at 37ºC to degrade any unencapsidated DNA, then treated with 0.5mg/ml proteinase K for 30 minutes at 37ºC in the presence of 0.5% SDS and 10mM EDTA to liberate the rAAV genomes, which were then

phenol-extracted, ethanol-precipitated, denatured in alkali buffer and applied to a nylon membrane. Serial dilutions of the corresponding vector plasmid were used as standards to determine rAAV virion copy number. A biotin-labeled DNA probe specific for the CB promoter was hybridized and detected using North2South Chemiluminescent Nucleic Acid Hybridization and Detection Kit (Pierce, IL). The rAAV titers were approximately 10^{12}-10^{13} physical particles per milliliter. The virus preparations were stored in -80ºC for animal studies.

2.2.3. *In vitro* transfection and secretory protein detection

After clones were confirmed by restrictive enzyme digestion, the transgene expression was tested on the human 293 cell lines in 6-well plates by transfection using calcium phosphate precipitation method described above. Six hours after transfection, the medium was replaced with protein-free OPTI-MEM. Twenty-four hours later, medium and cells were collected separately. To evaluate the level of transgene expression and fusion protein secretion, 15μl each of medium and crude cell lysate were electrophoresed on a 10% SDS-PAGE gel and transferred for 30 minutes at 15V onto a nitrocellulose membrane. The membrane was then blocked in 10% non-fat milk and probed with horseradish peroxide (HRP)-conjugated anti-mouse IgG antibodies at a 1:5000 dilution. Blots were further developed using the Western Blot Chemiluminescense Reagent Plus Kit (NEN Life Science Products, MA). The clones displaying optimal fusion protein secretion were selected for rAAV production.

2.2.4. New Zealand Black/White F1 female mice

New Zealand Black/White F1 (NZB/W F1) mice were bred in our colony using NZB males and NZW females purchased from The Jackson Laboratory. The gender was identified using tail DNA in PCR assays after birth. Only female F1 mice were used in this study because of their more rapid onset of autoimmunity. All mice were maintained at an AAALAC-approved animal

housing facility at the University of Pittsburgh, with a 14 hour light-10 hour dark cycle and were provided water and food ad libitum throughout the experiment. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

2.2.5. Experimental protocol

In this study, there were early and late prevention groups, with 10 NZB/W F1 female mice in each group. In the early prevention group, NZB/W F1 female pups were given an intraperitoneal (i.p.) injection of $5x10^{11}$ viral genome (vg) of ds-rAAV8-CB-mCTLA4IgG2a in 0.15 to 0.30ml PBS within the first week after birth. At this age, i.p. injection was the only feasible route for systemic gene delivery. While in the late prevention group, 6-week old NZB/W F1 female mice were given $3.5x10^{11}$ vg of ds-rAAV8-CB-mCTLA4IgG2a, $1x10^{12}$ vg of rAAV8-CAG-mCD40IgG1 and $4x10^{11}$ vg of rAAV8-CAG-mIL-10RIgG2a mixed in 0.3ml PBS and injected into the spleen. In early prevention groups, mice were bled from the tail at 2, 4, 6 and 8 weeks of age and monthly thereafter. In the late prevention group, mice were bled biweekly after gene delivery for 2 months and then monthly thereafter. Sera were obtained by centrifugation of blood at 4000rpm for 10min at 4ºC and stored at -80ºC.

2.2.6. mCTLA4Ig measurement

Serum mCTLA4IgG2a levels were measured by ELISA using the mouse CTLA4/Fc ELISA kit (Chimerigen, MA) according to the manufacturer's instructions. To evaluate mCTLA4Ig expression in each tissue and organ after systemic delivery, total protein was extracted from each organ. Briefly, tissues were homogenized in RIPA buffer containing 50mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1mM PMSF, 1mM Na_3VO_4, 1mM NaF and a protease inhibitor cocktail tablet (Roche Diagnostic). Then the homogenate was centrifuged at 13,200 rpm in Centrifuge 5415 R (Eppendorf) for 5 minutes to eliminate cell

debris, and the supernatant was recovered. Total protein concentrations of tissue extracts were determined using the Bradford method. mCTLA4IgG2a was measured using the mouse CTLA4/Fc ELISA kit (Chimerigen, MA), which was then normalized in 100ng total protein from tissue extracts.

2.2.7. Statistical analysis

All data were analyzed using Excel software. The student t test was used to compare the mCTLA4Ig levels. The p value was two-tailed and considered statistically significant when $p < 0.05$.

2.3. Results

2.3.1. Transgene expression and protein secretion *in vitro*

After the plasmid constructs were confirmed by restrictive enzyme digestion, the transgene expression of the clones was tested on 293 cells by transfection, and fusion protein secretion was evaluated by Western blot. For both clone #4 and clone #6 of pXX-CAG-mCTLA4IgG2a, the transgene was highly expressed within the cells and the 47.64kDa mCTLA4Ig was secreted into the medium at a level correlating to that of the intracellular transgene expression. For clone #9 of pXX-CAG-mIL-10RIgG2a, the transgene expression level was lower compared to the two clones of pXX-CAG-mCTLA4IgG2a. The secretion of 56.88kDa soluble mIL-10 receptor was also detected, however, at an even lower level than those of mCTLA4Ig. For clone #15 and clone #21 of pXX-CAG-mCD40IgG1, the transgene expression within the cells was comparable to that of mCTLA4IgG2a and mIL-10RIgG2a. Nevertheless, the secretion of 50.64kDa mCD40IgG1 was much weaker, but still detectable by Western blot (Fig.4).

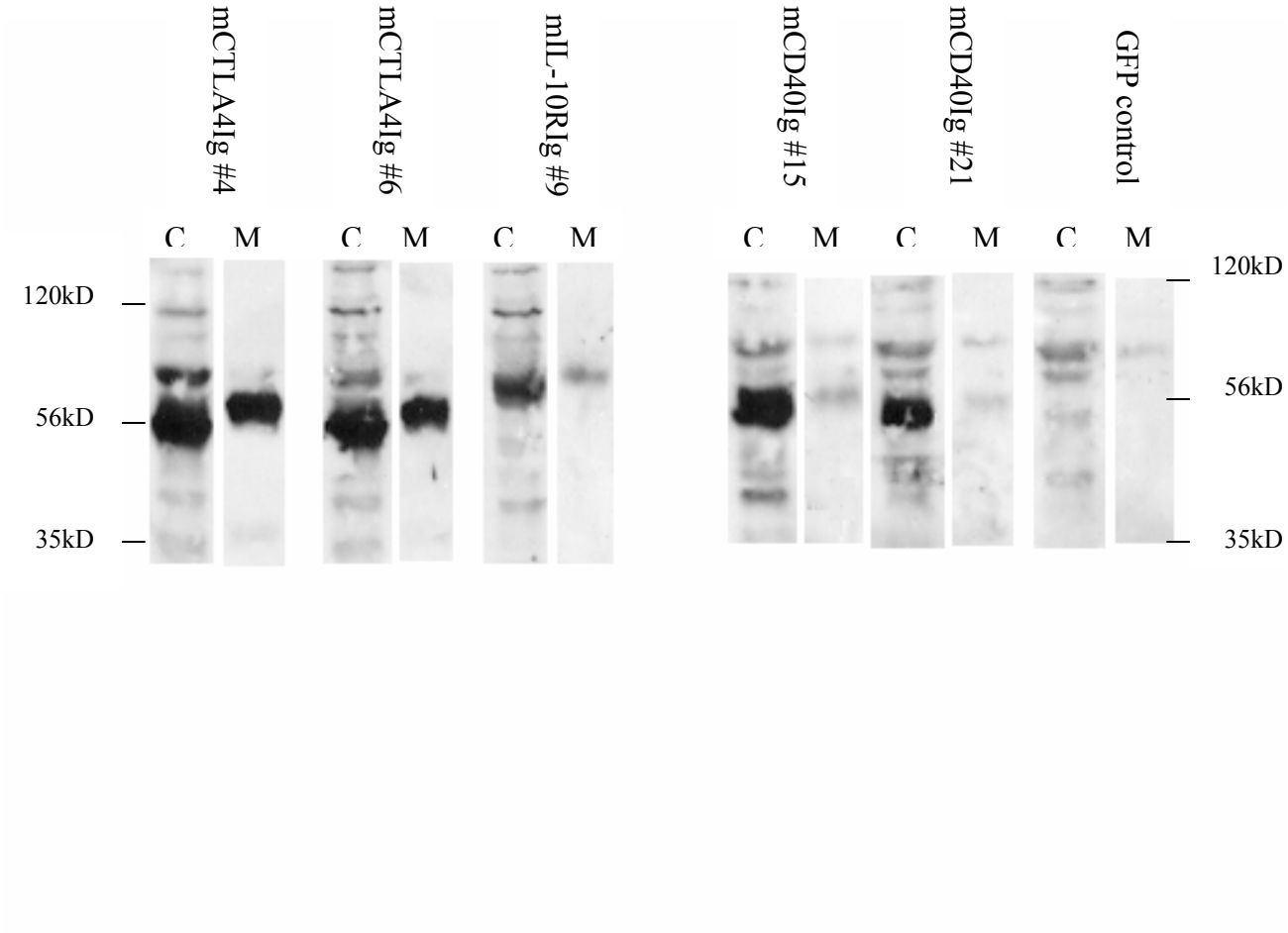


Figure 4 Western blot

Plasmid was transfected into 293 cells in a 6-well plate. Six hours later, the medium was changed to protein-free OPTI-MEM. Twenty-four hours after transfection, medium and cells were collected separately and loaded on SDS-PAGE gel for western blot. Protein was detected by HRP conjugated anti-mouse IgG Ab. C: crude cell lysade; M:medium.

2.3.2. Serum mCTLA4Ig level after i.p. injection of rAAV into B/W F1 newborn

In B/W newborn pups, the only feasible way for systemic gene delivery was by intraperitoneal injection (i.p.). It was important to follow up transgene expression levels, because they directly affected gene therapy outcome. After i.p. injection of 5 x 10^{11} vg ds-AAV8-CB-mCTLA4IgG2a, serum mCTLA4Ig levels reached 455.00±156.10μg/ml at 2 weeks and increased to 522.72±93.80μg/ml at 1 month. At 6 weeks the levels decreased to 483.13±51.62μg/ml and further decreased to 400.33±76.32μg/ml at 2 months. However, at 10 weeks of age, the levels again peaked at 543.52±146.83μg/ml and quickly decreased to 462.98±103.17μg/ml at 3 months. From 4 months to 9 months of age, serum mCTLA4Ig levels stabilized at 300μg/ml with only slight fluctuation (Fig.5). Even at 11 months after gene delivery, serum mCTLA4Ig levels were still detected at 210.20±89.06μg/ml, which was not significant lower than the peak values of 522.72μg/ml and 543.52μg/ml ($p>0.05$). Interestingly, when studying serum mCTLA4Ig levels in individual mice, only one peak in serum mCTLA4Ig levels was observed. Obviously, these results demonstrated two patterns. In some treated mice, mCTLA4Ig levels peaked at 1 month of age; while in the others, they peaked at 10 weeks of age. Moreover, in some long-surviving treated mice, serum mCTLA4Ig levels maintained at a consistent, extremely low level.

In addition, two other serotypes, rAAV2 and rAAV1, were tested. In the rAAV2 group, 9 x 10^{10} vg AAV2-CAG-mCTLA4IgG2a were injected i.p. into newborn B/W mice. Serum mCTLA4Ig was detected as 40.73±16.56μg/ml at 1 month after gene delivery and the levels increased to 92.00±17.49μg/ml after 2 weeks. While in rAAV1 group, 4.5 x 10^{10} vg ds-AAV1-CMV-mCTLA4IgG2a were delivered systemically into the newborn pups. Serum mCTLA4Ig level peaked at 44.97±11.46μg/ml after 6 weeks.

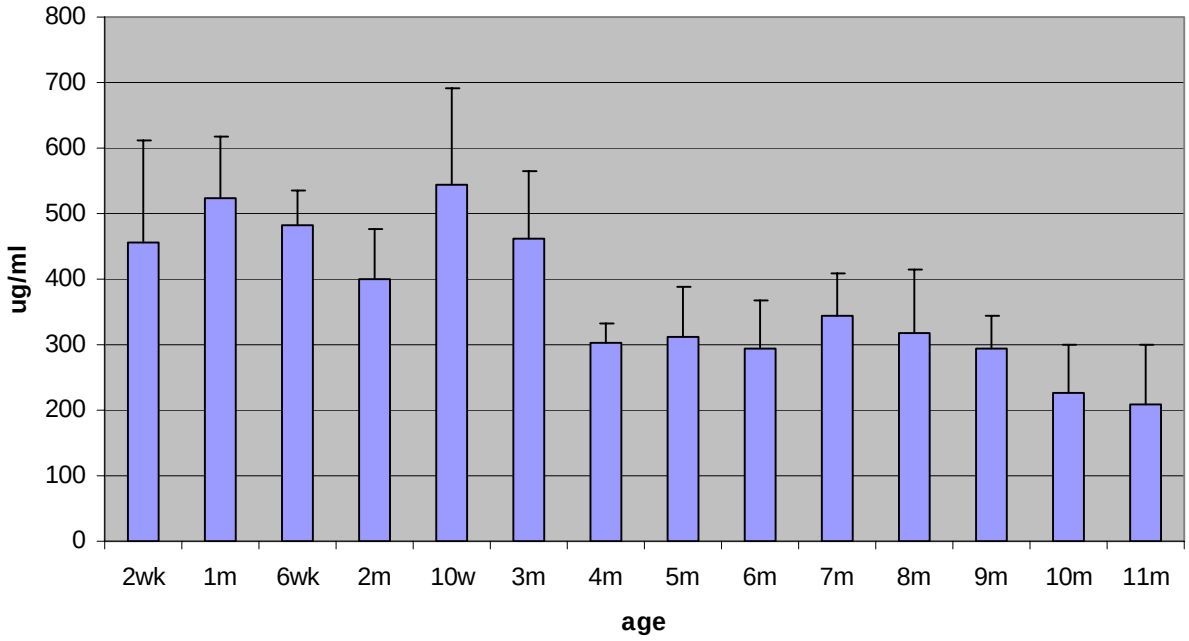


Figure 5 Serum mCTLA4IgG level after I.P.injection into B/W newborns

Following i.p. injection of 5 x 10^{11} vg ds-AAV8-CB-CTLA4IgG2a into NZB/W F1 female mice within one week after birth, serum was collected at different time points and mCTLA4Ig levels were measured by ELISA and presented as μg/ml (mean±SE). n=8.

2.3.3. mCTLA4Ig expression in tissue after systemic delivery of rAAV8 into B/W F1 newborns

After a single i.p. delivery of rAAV8 into newborn mice, transgene products were detected in the circulation for more than 11 months. What was the transduction efficiency in each organ and which organ did contribute the most to sustaining the high levels of mCTLA4Ig in the circulation? To address these issues, total protein was extracted from the homogenate of major organs and skeletal muscles from 8 month to 13 month old treated mice. Then mCTLA4Ig was measured by ELISA and normalized to 100ng total tissue protein. The results showed that heart, with 305.32±93.03pg mCTLA4Ig per 100ng total tissue protein, had a significantly higher transgene expression level than all other inner organs ($p<0.05$). When compared to skeletal muscles, mCTLA4Ig levels in the heart was 3.09 fold higher than biceps femoris, 5.68 fold higher than diaphragm, 5.72 fold higher than gastrocnemius(GAS) and tibialis anterior(TA), and 6.4 fold higher than quadriceps (Fig.6). Moreover, heart had significantly higher levels of mCTLA4Ig than abdominal and intercostal muscles ($p<0.05$). Among skeletal muscles, biceps femoris, with 98.80±27.89pg mCTLA4Ig per 100 total tissue protein, had the highest transgene expression level, followed by the diaphragm, GAS and TA, quadriceps and abdominal muscles. Intercostal muscles demonstrated only one-third the transgene expression of the biceps. All inner organs, including kidney, spleen, liver, brain and intestine, had expression levels only slightly above the baseline levels of mCTLA4Ig, except lung and pancreas, whose levels of transgene expression were comparable to that of intercostal muscle.

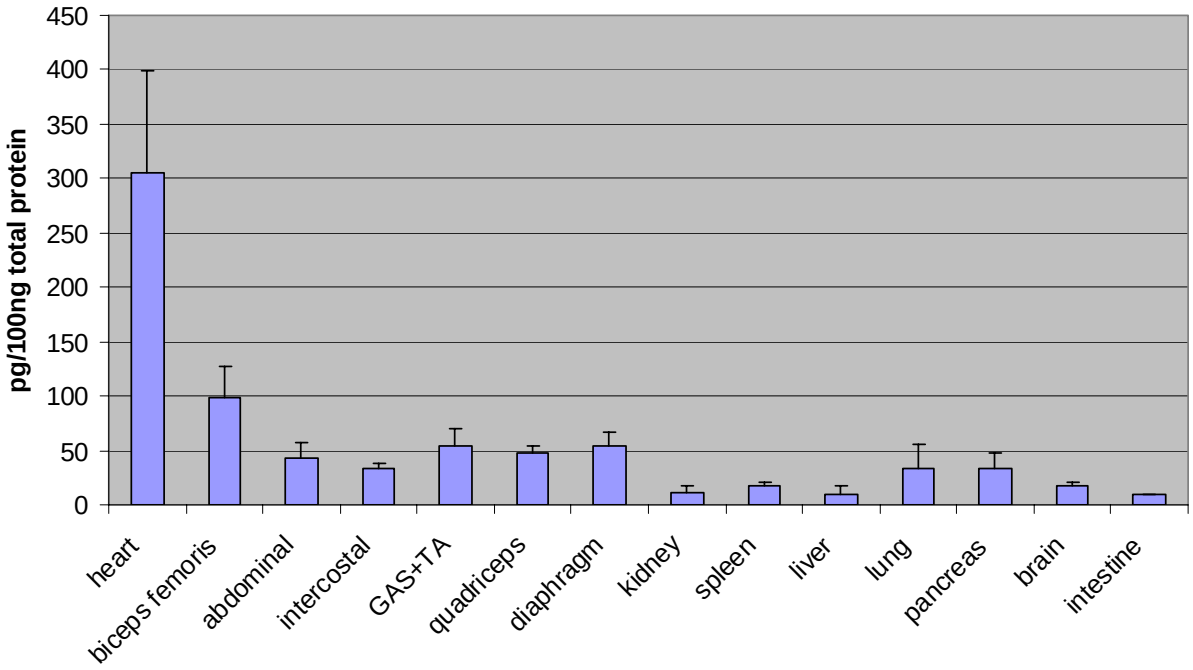


Figure 6 mCTLA4IgG level in tissue after I.P. injection into B/W newborns

Total protein was extracted from each tissue and organ from 8 months to 13 months old NZB/W F1 mice, which were i.p. injected with AAV8-mCTLA4IgG2a as newborns. Tissue mCTLA4Ig levels were measured by ELISA, normalized to 100ng total tissue protein and presented as mean±SE. n=5.

2.3.4. Serum mCTLA4Ig levels after intrasplenic injection of rAAV8 into B/W F1 young adult mice

In the late prevention group, rAAV8 vectors were delivered into 6 week old B/W mice, just prior to the typical age of onset of autoantibody production. Based on studies of rAAV8 biodistribution by our lab, we knew that most AAV8 ended up in liver after intrasplenic injection (unpublished data). Other groups have also showed that AAV8 has a natural tropism to liver (225), which is the largest secretory organ in the body. To achieve the maximal transgene expression and secretion in the young adult lupus-prone mice, three vectors, including 3.5×10^{11} vg of ds-AAV8-CB-mCTLA4IgG2a were coinjected into spleen. After gene delivery, serum was collected at different time points and mCTLA4Ig levels were determined by ELISA. The results showed that serum mCTLA4Ig levels were 248.00±16.72μg/ml at 2 weeks post gene delivery and steadily increased thereafter. At 6 weeks, the levels doubled and peaked at 571.60±42.27μg/ml. The levels then slowly decreased over time, and by 22 weeks after gene delivery, there was still 345.11±38.53μg/ml of mCTLA4Ig in circulation (Fig.7). Compared to the early prevention group, mice in this group showed a smaller standard deviation of serum mCTLA4Ig levels. Interestingly, when studying long-surviving mice in this group, serum mCTLA4IgG2a levels were about 16.77±8.52μg/ml at 16 months of age, which was significantly lower than that at 22 weeks of age ($p<0.01$).

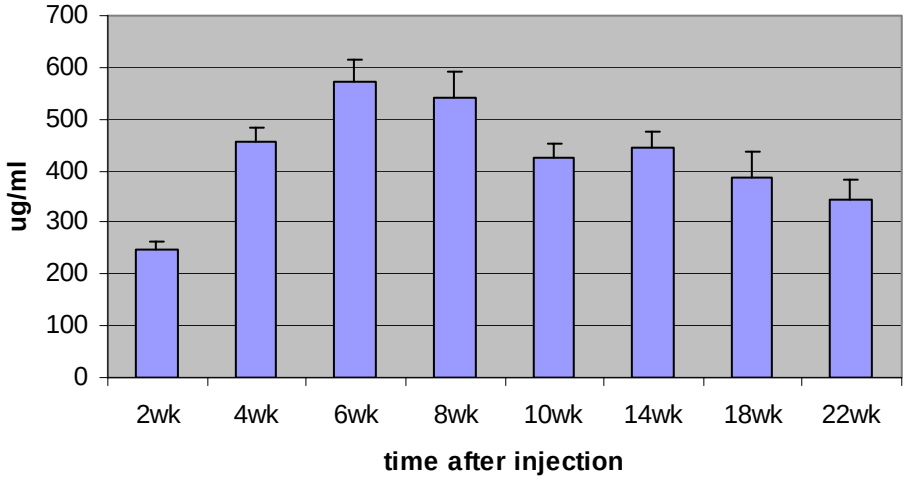


Figure 7 Serum mCTLA4IgG levels after intrasplenic delivery into B/W young adult mice

In the late prevention group, 3.5 x 10^{11} vg of ds-AAV8-CB-mCTLA4IgG2a, 1 x 10^{12} vg of AAV8-CAG-mCD40IgG1 and 4 x 10^{11} vg of AAV8-CAG-mIL-10RIgG2a were coinjected into the spleens of 6 week old NZB/W F1 female mice. Serum was collected at different time points and mCTLA4Ig levels were determined by ELISA and presented as μg/ml (mean±SE). n=10.

2.4. Conclusions

SLE is a chronic systemic autoimmune disease, in which long term intervention is necessary. rAAV has been proven to be a prominent vector for gene therapy of genetic diseases by successfully introducing transgenes to correct genetic defects (234, 236). However, it is not known whether rAAV can be applied to the treatment of autoimmune disease like lupus by delivering immunomodulator transgenes. When studying rAAV-mediated gene therapy for lupus, the first issue addressed here was the transduction efficiency. In this chapter, rAAV8 vectors encoding mCTLA4IgG2a, mCD40IgG1 or mIL-10RIgG2a were constructed and transgene products were confirmed to be secreted *in vitro*. For the *in vivo* transgene expression, serum mCTLA4Ig levels were evaluated. After a single i.p. delivery of rAAV8 into the B/W newborns, high levels of transgene expression were achieved. Serum mCTLA4Ig levels reached a peak of 522.72μg/ml at 1 month after injection and maintained at a level of 300μg/ml for at least 9 months. When studying systemic transduction efficiency, the results showed that the heart had significantly higher transgene expression levels than skeletal muscles and all other organs. mCTLA4Ig levels in heart were 3 to 6 fold higher than those in skeletal muscles, and all other organs had only baseline levels of transgene expression. Therefore, the heart represented the major source of the high mCTLA4Ig levels observed in the circulation.

For gene therapy in young adult B/W mice, rAAV8 vectors were delivered into the spleen, through which maximal liver transduction was expected. Serum mCTLA4Ig levels peaked at 571.60μg/ml after 6 weeks and maintained a level of 345.11μg/ml for at least 22 weeks. In conclusion, these results indicate that rAAV8-mediated gene transfer, delivered either systemically or directly into liver, is able to achieve high levels and long-term transgene expression in B/W mice. These data set the base for the application of rAAV8 vectors in this murine model of lupus.

2.5. Discussion

With genetic predisposition, the onset of autoimmune responses can appear as early as two months of age in NZB/W F1 mice (252). Protein- or mAb-based therapies, such as costimulatory blockade and cytokine antagonists, have been shown to be effective in the prevention of lupus (98, 166, 199). However, in the case of gene therapy, it will take time for transgene expression and transgene products to reach stable levels following gene transfer. Therefore, when to deliver the vectors and how the transgene will be expressed, are two critical issues concerning the intervention outcome. In this study, rAAV vectors were delivered either into newborn pups or into young adult lupus-prone mice in order to compare the intervention time points. It should be noted that the transgene expression pattern of rAAV-mediated gene delivery in the newborn pups was different from that of the adult mice. Moreover, there are at least 8 known different serotypes of AAV, each having a unique tissue tropism. Which serotype to choose was also a point of study. These questions will be discussed in detail below, but in general, the study of transgene expression and transduction efficiency will help us to better interpret the intervention outcomes in the following chapters.

2.5.1. rAAV8 is the serotype of choice

In this study, the purpose of gene delivery was to achieve long-lasting high levels of transgene products in the circulation, which depended on transgene expression in the target organs. Skeletal muscle and liver are two common targets for gene delivery of secreted transgene products. From a study of gene therapy of hemophilia B, we knew that even though rAAV-mediated factor IX gene delivery showed high transduction efficiency in the skeletal muscle, its circulation level was not >1%, indicating poor secretion of the transgene products (253). Moreover, liver transduction through the vascular system guaranteed more target cells to be infected than the limited intramuscular injection. Not to mention, liver is the largest secretory

organ in the body, which is also efficient at posttranscriptional modification. Therefore, liver was the target organ in this study of rAAV-mediated gene transfer.

For all known AAV serotypes, each has a unique tissue tropism. For example, AAV1, 6 and 7 show higher transduction efficiency in skeletal muscle, AAV5 targets airway epithelia and AAV2 is less efficient at tranducing liver and muscle (225, 254, 255). AAV8 was recently isolated from rhesus monkeys and its unique feature of liver tropism soon gained popularity in liver-directed gene therapy (225). Studies have shown that regardless of whether delivery is through the portal or tail vein, rAAV8 shows superior liver transduction efficiency to that of rAAV2, 5 and 7 (256). The molecular mechanism is proven to be the rapid uncoating of the vector genome in the liver cell nucleus, which ensures efficient transduction (257). Furthermore, a recent study from our lab shows that AAV8 is more efficient in systemic gene transfer than AAV1 and AAV2 after i.p. delivery into neonatal mice. The mechanism lies in the capability of crossing blood vessel barrier (258).

In this study, a single i.p. injection of $5x10^{11}$vg ds-AAV8-CB-CTLA4IgG2a achieved more than 10 fold higher serum CTLA4Ig level than that of $9x10^{11}$vg AAV2-CAG-CTLA4IgG2a. Even though double-stranded AAV contributed to the higher and faster transgene expression, the dramatic difference in transgene expression level did suggest superior transduction efficiency of AAV8 to that of AAV2, even when a lower vector dose and a weaker promoter was used for AAV8 vector. Another supporting evidence lied in the two vector combination early prevention group, where $2x10^{11}$vg AAV8-CAG-CTLA4IgG2a were injected into the newborn mice. Serum mCTLA4Ig level reached 916.50μg/ml after 1 month (data not shown), while only 40.73μg/ml mCTLA4Ig level was detected in the $9x10^{11}$vg AAV2-CAG-CTLA4IgG2a injection group. It showed that even with a much lower vector dose, AAV8

archieved significantly higher transgene expression level than AAV2 after systemic delivery. These data were consistent with those from other groups (259, 260).

2.5.2. The advantage of double-stranded AAV-mediated gene transfer

AAV-mediated gene transfer has a delayed onset of transgene expression and lower transduction efficiency when compared to adenoviral vector, for instance in gene transfer to rabbit vein segments (261). The rate-limiting step of transgene expression in rAAV-mediated gene delivery is believed to be the conversion of the single-stranded (ss) AAV genome into a double-stranded (ds) expressible form through either second strand synthesis, or the plus and minus strands annealing (262). To overcome this limitation, researchers have recently developed a novel strategy in which the terminal resolution site at one terminal repeat was deleted, so that a rAAV genome less than half the size of the wild type formed self-complementary (sc) double-stranded DNA. Study using scAAV carrying mouse erythropoietin (mEpo) shows faster onset of transgene expression and higher transduction efficiency after intramuscular delivery (251). One study from our lab, using dsAAV carrying the enhanced green fluorescent protein (GFP) as the reporter gene for intravenous delivery, demonstrates that 10% hepatocytes are GFP-positive at 1 week after delivery and it increases to 90% hepatocytes by 2 month (231). In this project, mCTLA4Ig was only 1194bp and was further subcloned into the ds AAV plasmid backbone. In the ds-AAV8-CB-mCTLA4IgG2a-treated group, serum mCTLA4Ig levels rapidly increased 2 weeks after gene delivery and peaked at 1 month. There was no lag period of transgene expression commonly seen in the ssAAV-mediated gene transfer. These results, cosistant with the above studies, also suggest higher and faster transgene expression delivered by the double-stranded AAV vector.

2.5.3. The gene delivery route and tissue transduction at different ages

In the newborn pups, the easiest method of systemic delivery was intraperitoneal injection. High levels and long-lasting transgene expression were observed, however, the biodistribution of rAAV8 and systemic transduction efficiency was still not clear in this route of vector delivery. To answer that question, mCTLA4Ig in the homogenate of each organ/tissue was evaluated by ELISA in the treated mice older than 8 months. Surprisingly, the heart, instead of the liver, was the organ with the highest transgene expression. Interestingly, another group has also observed high transgene copy number and transgene product activity in the heart 5 months after rAAV2 was injected into newborn pups (263). Moreover, a study from our lab also shows higer transgene expression in heart than other organs 2 months after AAV8 i.p. delivery into neonatal mice (258). The tissue distribution after systemic gene delivery into newborns can not be explained by AAV8 liver tropism. An alternative explanation could be the dilution of viral genome during hepatocyte clonal expansion in normal liver growth. There is evidence that most rAAV genomes exist episomally after transduction and that the integration rate is extremely low (264). Therefore, there is a great chance that rAAV genomes are lost when the host cell replicates. After birth, hepatocytes actively divide with the organ growth, which then ceases in the adult mice. In contrast, cardiac myocytes and skeletal myofiber are terminally differentiated cells. Once transduced, the rAAV genome persists within these cells for a long time. As supporting evidence, serum mCTLA4Ig levels in mice treated as newborns became strikingly stable after 4 month of age. Furthermore, for the young adult B/W mice in the late prevention group, all vectors were injected into the spleen. Based on the biodistribution study of rAAV8 by our lab, most vectors administered via this delivery route ended up in the liver. As a matter of fact, the change of serum mCTLA4Ig levels over time was not as dramatic as that in the early

prevention group, indicating stable transgene expression in the adult liver, where hepatocyte proliferation was negligible.

3. LUPUS PREVENTION

3.1. Introduction

SLE is a complex autoimmune disease with a wide range of clinical manifestations. Because of the pleiomorphic nature of this disease, the mechanisms underlying the autoimmunity are still not totally understood, and this hinders the search for de novo treatments. With genetic predisposition, NZB/W F1 mice spontaneously develop autoantibodies and immune complex (IC)-mediated glomerulonephritis (GN), and have been widely used as a murine model of lupus. Through decades of research using this murine model, some progress has been achieved in lupus treatment with therapeutic schemes, including costimulatory blockade, cytokine antagonists, and immunoablation. Gene therapy introduces a promising way to conquer chronic autoimmune diseases like lupus, by achieving long-term and stable transgene expression, with the added benefits of convenient delivery, and possible gene expression regulation. In this chapter, the feasibility of the rAAV8-mediated gene delivery for lupus prevention was tested on B/W mice. Although there are many mechanisms involved in the development of autoimmunity, the strategies adopted in this study targeted the most critical ones, namely the costimulatory pathways of autoreactive T and B cell activation and cytokine imbalance. These strategies were applied separately or combined in an attempt to investigate possible synergistic effect. Moreover, in the setting of gene therapy for lupus prevention, the optimal time for gene delivery is as yet unknown. Therefore, early and late prevention groups were set up. The prevention efficacy in each group was evaluated in terms of the autoantibody titer, the onset of proteinuria, and the change of life span. Besides that, the kidneys, the most commonly damaged organs in lupus, were also assessed for pathological changes and IC deposits. In addition, splenic T cell subpopulations in the prevention groups were also analyzed, in order to dissect the intertwining

immune mechanisms responsible for the prevention outcome. When applying costimulatory blockades, one major concern was the resulting side effects, such as immunosuppression. To address this issue, treated mice were challenged with foreign antigen and the antigen specific-humoral responses were evaluated. Taken together, all these studies in lupus prevention will not only broaden our understanding of the pathogenesis of autoimmune disease, but also facilitate further application of gene therapy in lupus treatment.

3.2. Material and methods

3.2.1. Experimental protocol

To determine the best time for gene delivery of immunomodulators to prevent lupus, early and late prevention groups were set up. In the early prevention groups, 6 x 10^{11} vg of AAV8-CAG-mCD40IgG1 or 5 x 10^{11} vg of ds-AAV8-CB-mCTLA4IgG2a in 0.1 to 0.3 ml PBS was injected into the peritoneum of newborn mice separately. Furthermore, to test whether there is a synergistic effect between the two costimulatory blockades, 4x10^{11} vg of AAV8-CAG-mCD40IgG1 and 2x10^{11} vg of AAV8-CAG-mCTLA4IgG2a were coinjected into the peritoneum of newborn mice. While in the late prevention group, 1x10^{12} vg of AAV8-CAG-mCD40IgG1, 3.5x10^{11} vg of ds-AAV8-CB-mCTLA4IgG2a and 4x10^{11} vg of AAV8-CAG-mIL-10RIgG2a mixed in 0.3ml PBS were injected into the spleen of 6 week old lupus-prone B/W mice. After gene delivery, mice were bled via the tail vein every two weeks for 3 months, and then monthly. Serum was collected and stored at -80ºC. Mice were weighted monthly. All mice, which were long-surviving in the prevention groups, had to be sacrificed when the animal facility in Plum Boro site closed.

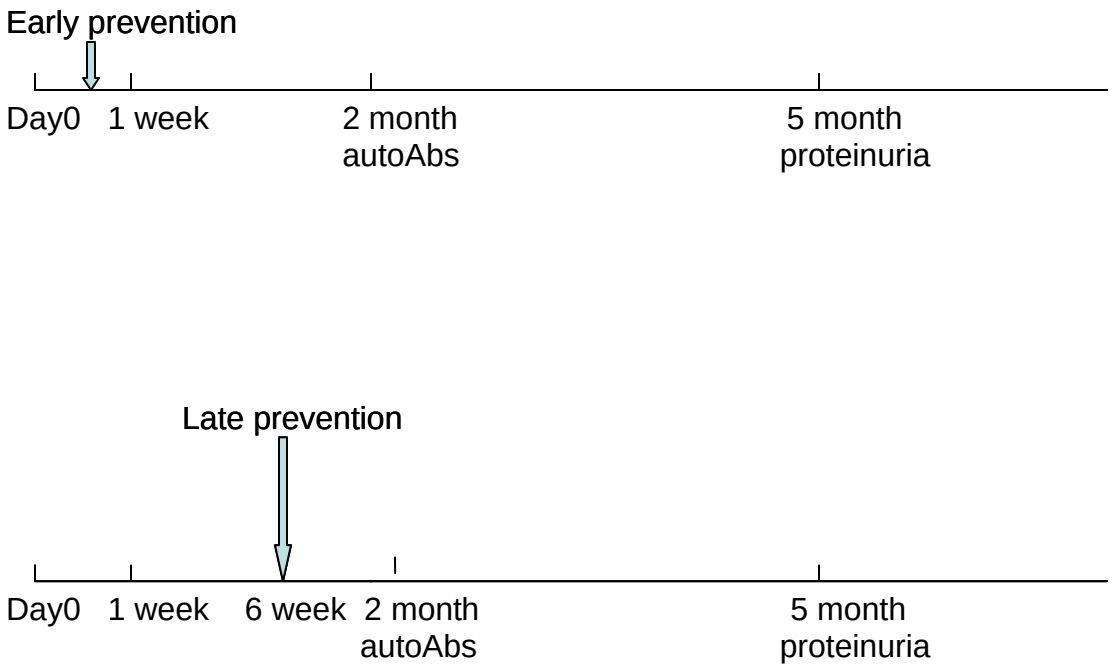


Figure 8 Experimental time-line in the murine SLE model

In the early prevention group, rAAV8 vectors were injected i.p. into B/W newborns. To study a possible synergistic effect, two vectors of costimulatory blockades were coinjected into newborn mice. In the late prevention group, rAAV vectors were delivered into the spleen of 6 week old lupus-prone B/W mice.

3.2.2. Serum anti-dsDNA IgG by ELISA

Among all autoantibodies found in lupus, anti-dsDNA IgG is considered pathogenic and its titer closely correlates with disease progress and prognosis (265, 266). Serum anti-dsDNA IgG levels were measured by ELISA using an anti-dsDNA antibody ELISA kit (Alpha Diagnostic, TX) following the manufacturer's instructions. Titers were presented as OD readings at 450nm.

3.2.3. Proteinuria

Proteinuria is a criterion that directly reflects kidney dysfunction and is widely used for lupus assessment in both clinical and research settings. Urine from the mice was monitored monthly starting at 2 months of age. To assess proteinuria, urine pressed from the bladder was tested colorimetrically using Albustix (Bayer corp. IN). Proteinuria levels were scored as followed: 0 (none), 1+ (30-99mg/dl), 2+ (100-299mg/dl), 3+ (300-1999mg/dl) or 4+ (2000mg/dl and more). Because there was fluctuation, only 3+ and above at two independent measurements was considered to represent severe proteinuria.

3.2.4. Kidney pathology and immunofluorescence staining

To evaluate renal histopathology, kidneys were longitudinally dissected into two parts. One was snap-frozen in liquid nitrogen for cryostat section; the other was fixed in 4% paraformaldehyde and embedded in paraffin. Five μm thick sections of paraffin-embedded tissue were stained with periodic acid-Schiff (PAS) according to conventional procedures. The glomerular, tubular, and interstitial pathologies were evaluated morphometrically under light microscopy by two pathologists (SB and TM) in a blind manner in accordance with clinical standards. The histopathological changes were expressed as an Activity Index (AI) and Chronicity Index (CI), as described by Austin et al (267). The activity index was graded on a scale of 0 to 24 by calculating the sum of individual scores (0 to 3+) for each of the six histological parameters:

glomerular endocapillary proliferation, glomerular neutrophil infiltration, wire-loop deposits and hyaline thrombi, glomerular karyorrhexis and fibrinoid necrosis, cellular crescents, and interstitial inflammation. Glomerular features (including endocapillary proliferation, wire-loop deposits, necrosis, and cellular crescents) were graded as follows: 0, absent; 1+, <25% of glomeruli affected; 2+, 25% to 50% of glomeruli affected; 3+, >50% of glomeruli affected. Neutrophil exudation was defined as more than 2 neutrophils per glomerulus and scored as mild (1+), moderate (2+), and severe (3+). Fibrinoid necrosis and cellular crescents were weighted double because of their more ominous prognostic importance. For interstitial inflammation, the scoring was as follows: 0, absent; 1, mild; 2, moderate; and 3, severe. A similar score for chronicity was computed by summing the individual scores (0 to 3+) for each of the following parameters: glomerular sclerosis, fibrous crescents, tubular atrophy, and interstitial fibrosis. For glomerular sclerosis, segmental or global glomerulosclerosis in <25% of glomeruli was regarded as 1+, in 25% to 50% of glomeruli as 2+, and >50% as 3+. Interstitial fibrosis and tubular atrophy were graded as mild (1+), moderate (2+), or severe (3+).

To examine IgG and C3 deposits within renal glomeruli, 6 μm thick kidney cryostat sections were stained with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories; West Grove, PA) or FITC-conjugated goat anti-mouse C3 (Cappel Laboratories; Aurora, Ohio), both at a 1:200 dilution. Immune complex deposition was scored by the percentage of glomeruli stained per field and by the brightness of fluorescence: 1+, dim and not uniform; 2+, bright, uniform, and observable in >50% of the glomeruli; 3+, very bright, uniform, and observable in >75% of glomeruli; 4+, very bright, uniform, and observable in all glomeruli. At least 100 glomeruli per section were analyzed.

Table 1 Assessment of kidney histopathology by activity index and chronicity index

Activity Index	Chronicity Index
glomerular endocapillary proliferation/hypercellularity	glomerular sclerosis
glomerular neutrophil infiltration	fibrous crescents
wire-loop deposits and hyaline thrombi	tubular atrophy
glomerular karyorrhexis and fibrinoid necrosis	interstitial fibrosis
cellular crescents	
interstitial inflammation	

Adapted from (267, 268)

3.2.5. KLH challenge and anti-KLH antibody detection

To investigate whether costimulatory blockades suppress host humoral immune responses to foreign antigen, 3 to 5 mice from the prevention groups, 2 mice from the control group and 1 wild type ICR mouse were challenged with keyhole limpet haemocyanin (KLH; Calbiochem, CA), a typical T-dependent foreign antigen. Before immunization, mice were bled via the tail vein. Each mouse was immunized i.p. with 100μg of KLH in 0.2ml PBS, emulsified in Complete Freud's Adjuvant (CFA; Sigma, St. Louis, MO) at a 1:1 ratio. After 2 weeks, the mouse was boosted i.p. with 50μg of KLH in 0.05ml PBS. Sera were collected weekly. Anti-KLH IgG titers were quantitated by ELISA. Briefly, microtiter plates were coated with antigen by incubation with 100 μl of 10μg/ml KLH in sodium bicarbonate buffer (pH 9.6) at 37ºC for 2 hours, washed with PBS containing 0.05% Tween 20 (PBS-T; pH 7.4), then blocked with 5% nonfat dry milk in PBS-T for 2 hours at room temperature to reduce nonspecific binding, and washed again with PBS-T. Serum samples were serially diluted with PBS-T to 1:50, 1:300 and 1:1800 ratios. One hundred μl of each serum dilution was added to duplicate wells of the KLH-coated plates. Positive control samples (sera from KLH-hyperimmuned Balb/C mouse) and negative control samples (sera from KLH-naïve mouse) were also added in duplicate to each plate; plates were sealed, incubated overnight at 4ºC, then washed with PBS-T. To detect mouse anti-KLH IgG, 100μl of HRP conjugated goat anti-mouse IgG, diluted 1:100 with sample diluent, was added to

each well, and the plates were sealed and incubated at room temperature for 30min. Plates were washed again with PBS-T, and 100µl TMB substrate was added to each well. Plates were protected from light during the enzyme-substrate reaction, which was terminated after 15 min by adding 100µl of stop solution to each well. The optical density (OD) was determined using a VERSAmax microplate reader (Molecular Devices; Sunnyvale, CA) equipped with a 450nm wavelength filter, and the mean OD for each set of duplicate wells was calculated. HRP conjugated goat anti-mouse IgG, sample diluent, TMB substrate and stop solution were all from the anti-dsDNA antibody ELISA kit (Alpha diagnostic; San Antonio, TX).

3.2.6. Splenic T cell subpopulations by flow cytometry

Single cell suspensions were made by mashing spleens through a cell strainer with 70µm pore size (BD Falcon; Bedford MA) and then washed with RPMI medium 1640 (Invitrogen, Grand Island, NY). Red blood cells (RBCs) were removed using RBC lysing buffer (Sigma). Cell numbers were counted with a cell counter and $1x10^6$ cells per aliquot were dispensed into 12x75mm FALCON 5ml tubes. Before staining the cells, Fcγ receptors were blocked by incubation with 20µg/ml rat anti-mouse CD16/CD32 monoclonal antibody clone 2.4G2 (BD Pharmingen) at room temperature for 15min. Samples were then stained with various combinations of fluorchrome conjugated mAbs in 2% fetal calf serum, 0.1% sodium azide in PBS (PBS-azide) at 4ºC for 30min, avoiding light. Finally, cells were washed twice with 2% fetal calf serum, PBS-azide and fixed with 1% paraformaldehyde in 0.5ml PBS. Flow cytometry of the stained cells was performed using Beckman Coulter EPICS XL 4-color cytometer (flow cytometry facility in University of Pittsburgh Cancer Institute). Spleen cells were analyzed for T cell markers using fluorescein isothiocyanate (FITC)-anti-CD4(H129.19) and phycoerythrin (PE)-anti-CD8a(53-6.7). The presence of naïve and activated/memory CD4 cells was determined

by triple staining with FITC-anti-CD4, PE-anti-CD62L(MEL-14), and PE-Cy5-anti-CD44(IM7). The presence of early activated T cells was determined by double staining with FITC-anti-CD4 and PE-anti-CD69(H1.2F3). The presence of regulatory T cells was determined by double staining with FITC-anti-CD4 and PE-anti-CD25(3C7). Negative controls were set by using isotype-matched Ig directly conjugated to fluorchromes. All antibodies were purchased from BD Pharmingen. Corresponding isotype controls were kindly provided by Dr. Yukai He. Collected events were gated for total lymphocytes defined by forward and side scatter characteristics using WinMDI 2.8 software. A second gate was applied for all CD4+ cells. A logarithmic scale was used for the axis of the dot-plots, which gave the relative fluorescence intensities of events.

3.2.7. Statistical analysis

The student's t-test was used to determine the statistical significance of serum anti-dsDNA IgG titers, anti-KLH IgG titers, T cell subpopulations, and weight changes among the groups. Proteinuria and survival data were analyzed by the log-rank test. The survival curve was drawn using SigmaPlot software. Kidney pathological scores were analyzed using the nonparametric Mann-Whitney U test. The p value was two-tailed and considered statistically significant when $p < 0.05$.

3.3. Results

3.3.1. The change of serum anti-dsDNA IgG titer in prevention groups

The criteria directly reflecting autoimmunity status were the autoantibody titers, in which anti-dsDNA IgG was the most important one. Serum anti-dsDNA IgG was measured by ELISA, and the titers were presented as OD readings at 450nm. In the untreated control B/W mice, the autoantibodies started to slowly increase at a very early age and the increase dramatically accelerated at 5 months of age. Within another 2 months the anti-dsDNA IgG reached the

saturation level of the ELISA assay and was sustained at this level thereafter (Fig.9). In comparison, mice in the mCTLA4Ig early prevention group had significantly lower levels of autoantibodies at all time points ($p<0.01$), except at 6 months of age when a spike of autoantibody titers was observed. In the mCD40Ig early prevention group, the autoantibody levels were close to those of the control group, except at 6 months of age when an obvious delay of the increase occurred in the treated group. However, this delay didn't last long and the autoantibody levels soon caught up with those of the control group. In the mCTLA4Ig and mCD40Ig combination early prevention group, anti-dsDNA IgG titers fell into the same pattern as that of the mCTLA4Ig single vector prevention group and were significantly lower than that of the control group ($p<0.01$). Obviously, mice in the two vector combination prevention group had even lower autoantibody titers than those in the single vector prevention groups at all time points tested. Moreover, in the three vector combination late prevention group, the increase of serum anti-dsDNA IgG was significantly inhibited, and maintained a baseline level all the time ($p<0.01$, Fig.10). In conclusion, the three vector combination achieved the best results with regard to inhibition of autoantibody production, even though the vectors were delivered into young adult lupus-prone mice. In the early prevention groups, where the vectors were injected into newborn mice, the two vector combination strategy was the best, followed by the mCTLA4Ig single vector prevention. The mCD40Ig did not prevent autoantibody production.

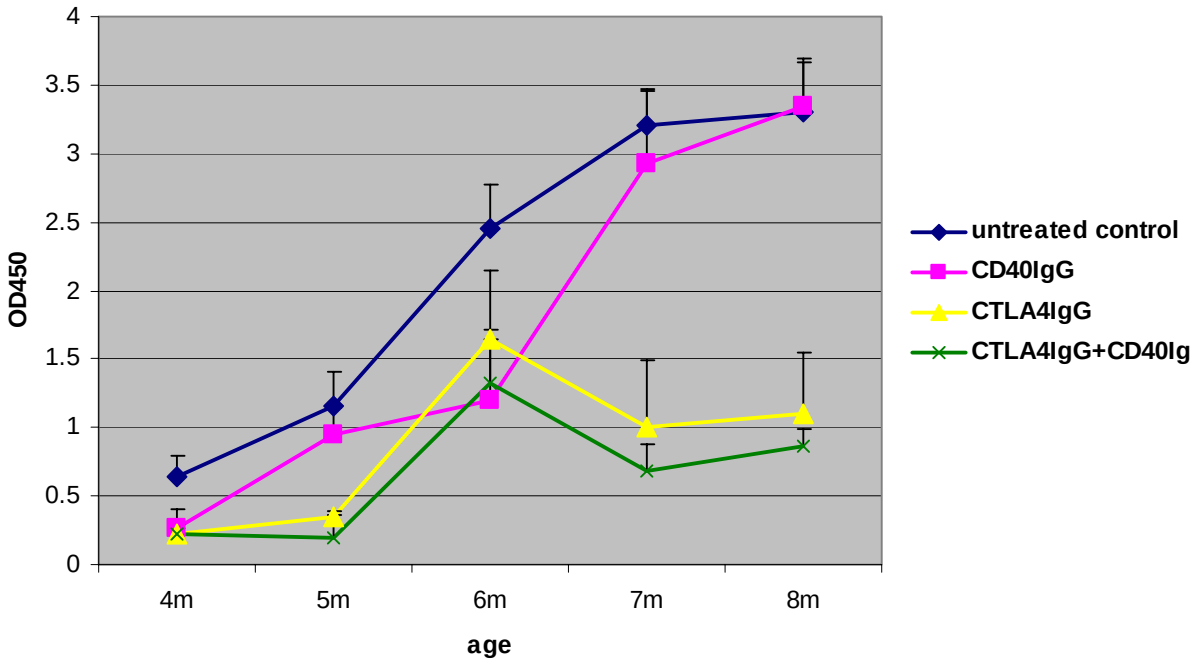


Figure 9 Serum anti-dsDNA IgG titer in the early prevention groups

Serum anti-dsDNA IgG as detected by ELISA. Levels are presented as OD readings at 450nm (mean±SE). Compared to the untreated control group, mice in all early prevention groups, except CD40Ig, had significantly lower autoantibody titer at the age of 8 month ($p<0.01$).

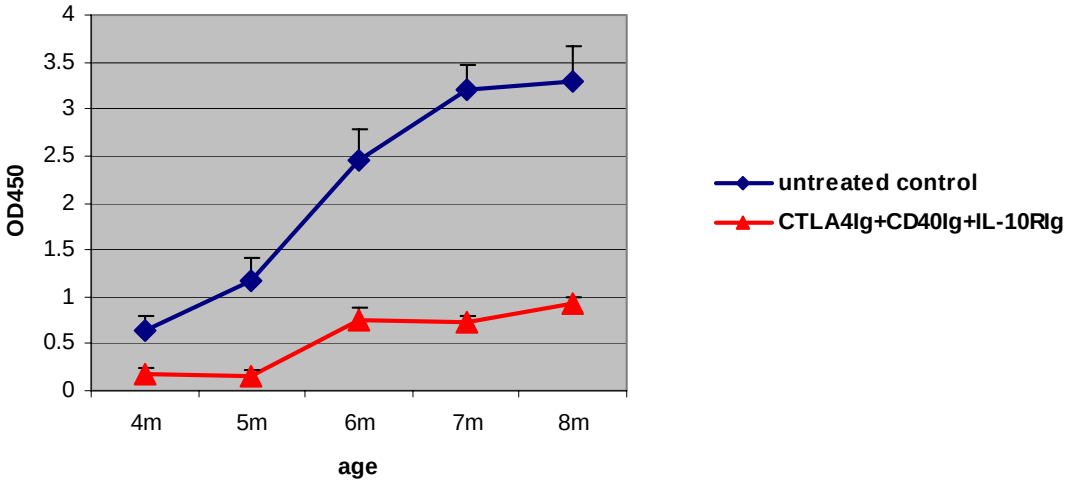


Figure 10 Serum anti-dsDNA IgG titer in AAV8-CAG-CD40IgG1, ds-AAV8-CB-CTLA4IgG2a and AAV8-CAG-IL-10RIgG2a combination late prevention group

Serum anti-dsDNA IgG as detected by ELISA. Levels are presented as OD readings at 450nm (mean±SE). Compared to the untreated control group, the autoantibody titers in the late prevention group were significantly lower at all time points tested ($p<0.01$).

3.3.2. The effect on the onset of proteinuria in prevention groups

Proteinuria is the most prominent and life-threatening symptom in lupus mice. It reflects the damage to the kidneys and closely correlates with disease outcome. In untreated control mice, severe proteinuria occurred as early as 5 months of age. Thereafter, more and more mice became symptomatic. By 10 months of age, all mice in the control group suffered from proteinuria (Fig.11). In comparison, the onset of proteinuria in the mCTLA4Ig single vector early prevention group was delayed by 2 months, and only 28.57% mice in this group developed fixed proteinuria by the age of 12 months ($p<0.001$). In the mCD40Ig single vector early prevention group, the onset of proteinuria was also delayed by 2 months and significantly different from that of the control group ($p<0.01$). However, more mice developed proteinuria over time than those in the mCTLA4Ig prevention group ($p<0.05$). Therefore, by 12 months of age, 90% of mice in this group developed proteinuria. Moreover, in the two vector combination early prevention group, not a single mouse developed proteinuria by the age of 12 months, which was significantly different from that of the mCD40Ig prevention and untreated control groups ($p<0.001$). These results strongly suggest a synergistic effect on proteinuria prevention between the two costimulatory blockades. Surprisingly, all mice in the three vector combination late prevention group were proteinuria negaive for more than 12 months, which was significantly different from the control group ($p<0.001$, Fig.12). To conclude, both the vector combination groups showed significant effects on preventing the development of proteinuria. In the single vector groups, mCTLA4Ig was better than mCD40Ig, in terms of reducing proteinuria occurrance.

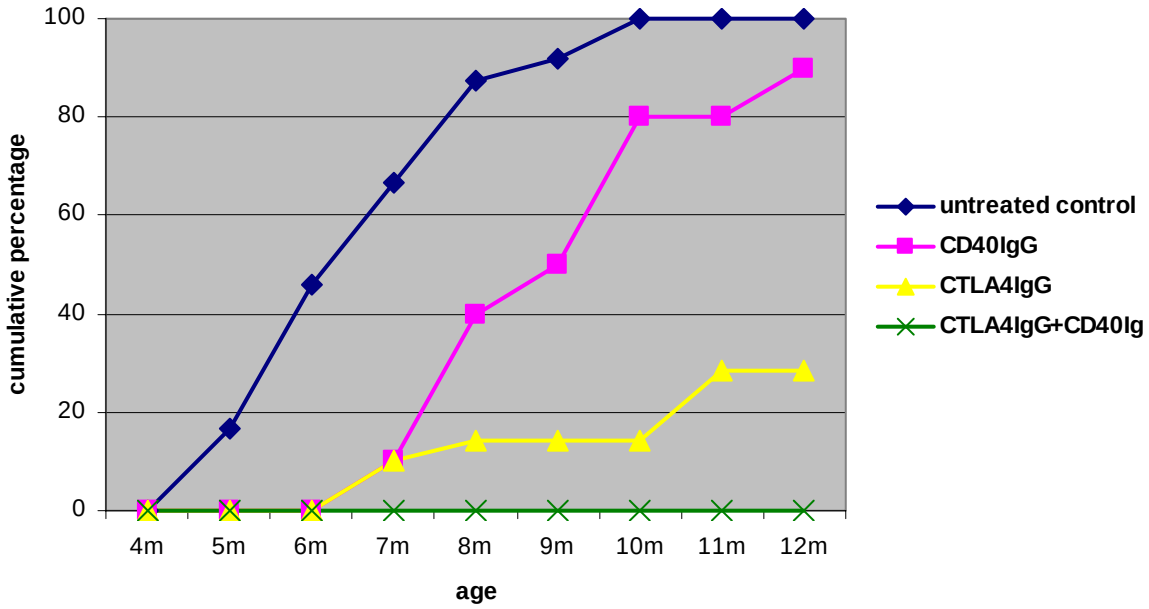


Figure 11 The occurrence of proteinuria in the early prevention groups

Proteinuria was evaluated with Albustix. Levels were scored as followed: 0 (none), 1+ (30-99mg/dl), 2+ (100-299mg/dl), 3+ (300-1999mg/dl) or 4+ (2000mg/dl and more). Severe proteinuria was defined as 3+ and above at two independent occasions. Shown is the cumulative percentage of mice that developed severe proteinuria over time. Compared to the untreated control group, mice in all prevention groups had significantly delayed onset of proteinuria (CD40Ig $p<0.01$, CTLA4Ig $p<0.001$, CTLA4Ig+CD40Ig $p<0.001$).

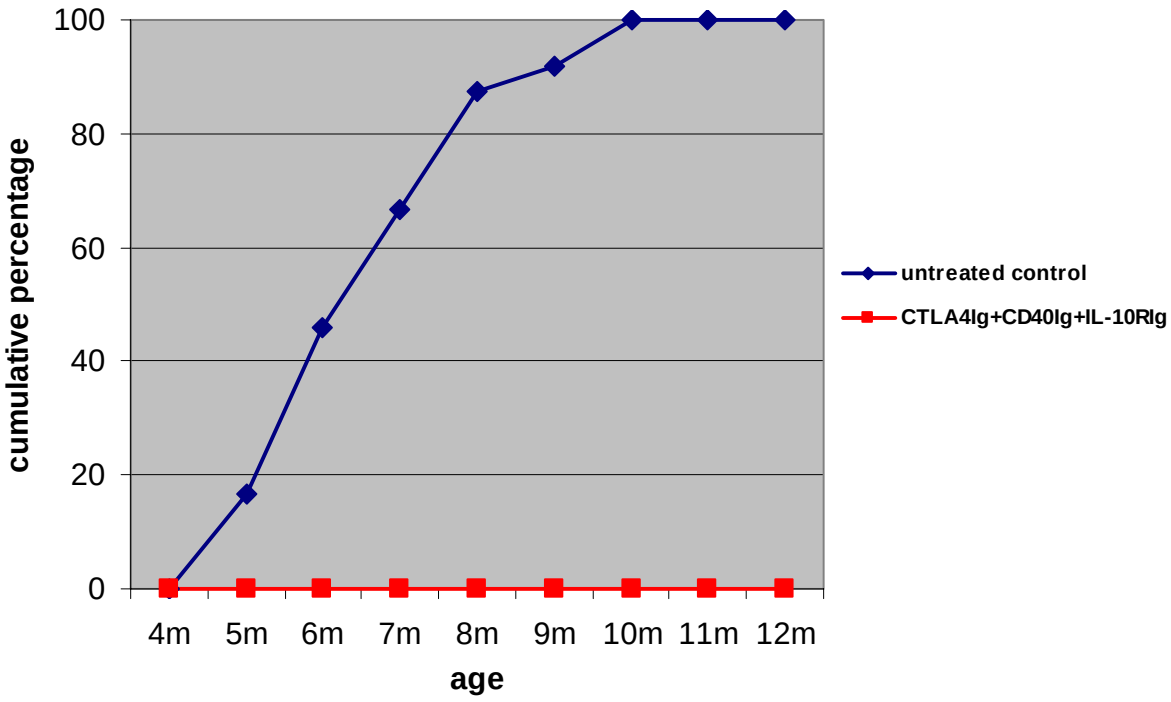


Figure 12 The occurrence of proteinuria in AAV8-CAG-CD40IgG1, ds-AAV8-CB-CTLA4IgG2a and AAV8-CAG-IL-10RIgG2a combination late prevention group

Proteinuria was evaluated with Albustix. Levels were scored as followed: 0 (none), 1+ (30-99mg/dl), 2+ (100-299mg/dl), 3+ (300-1999mg/dl) or 4+ (2000mg/dl and more). Severe proteinuria was defined as 3+ and above at two independent occasions. Shown is the cumulative percentage of mice that developed severe proteinuria over time. Compared to the untreated control group, mice in the late prevention group had significantly delayed onset of proteinuria ($p<0.001$).

3.3.3. The effect on survival rate in prevention groups

In lupus mice, nephritis dramatically influences life span, and the survival rate inversely correlates to the onset of proteinuria. The earlier the onset of proteinuria, the shorter the mouse lives. In the untreated control group, death was first observed at the age of 5 months. The death toll increased as more mice developed proteinuria over time. By 10 months of age, all mice in the control group had died (Fig.13). In comparison, there was not a single death in the mCTLA4Ig single vector early prevention group until 8 months of age. By 12 months of age, 50% of the mice were still alive ($p<0.0001$). However, in the mCD40Ig single vector early prevention group, the first death occurred at 7 months of age. By 12 months of age, 50% of the mice were alive, demonstrating significantly prolonged survival ($p<0.01$). When compared the survival rates of the mCTLA4Ig and the mCD40Ig single vector groups, no statistical difference was detected ($p>0.05$). In the two vector combination early prevention group, all mice survived for at least 10 months. By 12 months of age, 75% of the mice were still alive ($p<0.0001$). Moreover, the survival rate in the two vector combination group was significantly higher than those in single vector groups ($p<0.05$ compared to mCTLA4Ig; $p<0.01$ compared to mCD40Ig). Not surprisingly, the three vector combination significantly prolonged survival in the late prevention group ($p<0.0001$, Fig.14). 77.78% of the mice were alive by the age of 12 months. In general, mice in the vector combination prevention groups had prolonged survival. Single vectors also increased the survival rate in the prevention groups.

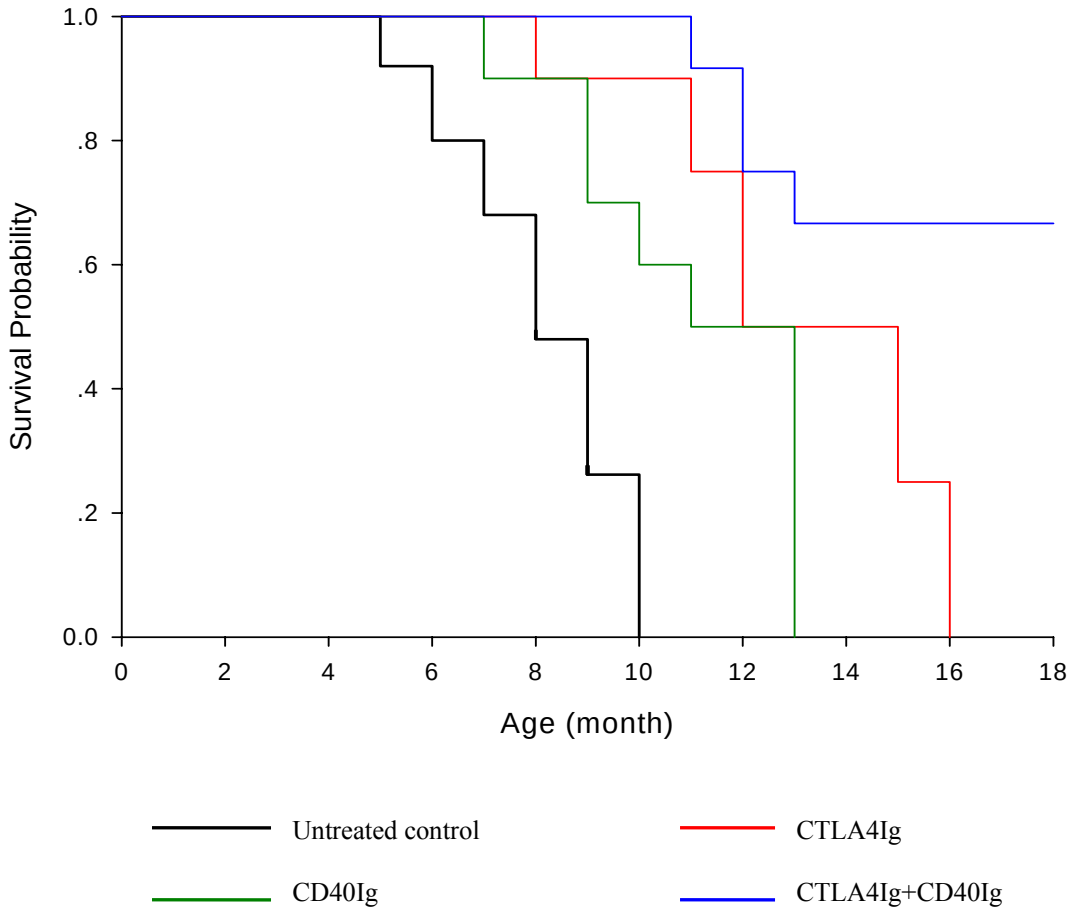


Figure 13 Survival curve in the early prevention groups

Shown is the cumulative percentage of mice that survived each time point. All mice were followed until death. Compared to the untreated control group, all mice in the prevention groups had prolonged survival (CD40Ig $p<0.01$, CTLA4Ig $p<0.0001$, CTLA4Ig+CD40Ig $p<0.0001$).

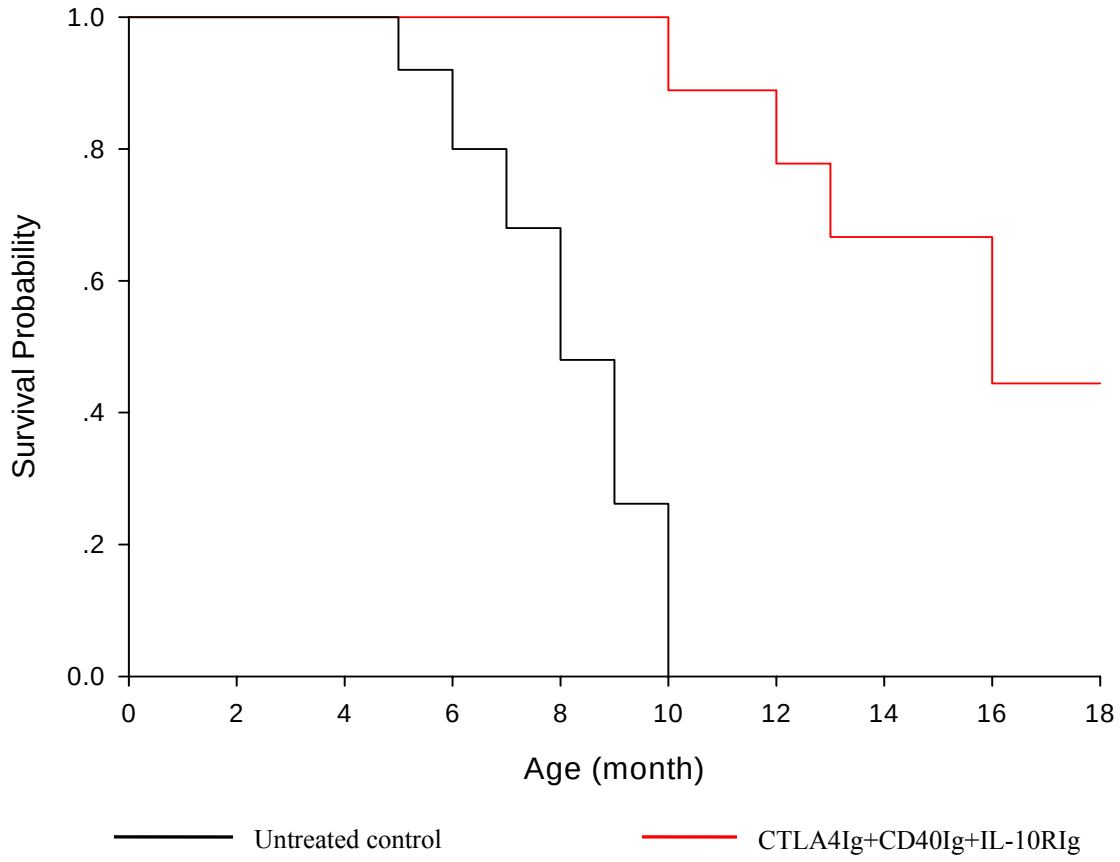


Figure 14 Survival curve in AAV8-CAG-CD40IgG1, ds-AAV8-CB-CTLA4IgG2a and AAV8-CAG-IL-10RIgG2a combination late prevention group

Shown is the cumulative percentage of mice that survived each time point. All mice were followed until death. Compared to the untreated control group, mice in the late prevention group had prolonged survival ($p<0.0001$).

3.3.4. Kidney study

The kidneys are the most important target organs in lupus. Even though proteinuria reflects the severity of renal dysfunction, pathological study directly reveals its tissue damage. Clinically, kidney biopsy is widely used for disease classification and making prognosis. Here I studied the kidney histopathology and also stained the tissue for two immune complex components--IgG and C3.

3.3.4.1. Kidney histopathology

The kidney histopathology was evaluated by two pathologists (S.B and T.M) in a blind manner. The glomerular, tubular and interstitial pathological changes were semi-quantitatively scored according to clinical criteria. These scores were also sorted out using the activity index (AI) and the chronicity index (CI), which are commonly used to evaluate disease activity and irreversible damage. Common characteristics of severe glomerulonephritis (GN) in lupus kidneys include proliferative changes in the mesangial and endothelial cells of the glomeruli, capillary basement membrane thickening, severe interstitial infiltrates in the interstitium, tubular atrophy and large protein casts. Fig.15 compares representative renal pathological findings in the prevention groups and control group. The 10 month old untreated control B/W mice exhibited typical GN changes, including glomerular hypercellularity, hyalinosis, segmental or global sclerosis, and cast formation in tubules (Fig.15A). In contrast, age-matched mice in both the mCTLA4Ig single vector prevention group and the two vector combination prevention group presented almost intact glomeruli, tubules and interstitium (Fig.15C, 15D), with significantly lower pathological scores ($p<0.05$, Fig.16a). Mice in the mCD40Ig single vector prevention group had milder glomerular, tubular, and interstitial damage than mice in the untreated control group (Fig.15B), however, with no statistically significant difference in the scores ($p>0.05$, Fig.16a). When

comparing AI and CI, similar results were obtained ($p<0.05$ in the mCTLA4Ig and the two vector prevention group; $p>0.05$ in the mCD40Ig group, Fig.16b). In conclusion, mCTLA4Ig alone or with mCD40Ig protected kidneys from autoantibody induced damage. These data were consistent with the observed improved symptoms and prolonged survival.

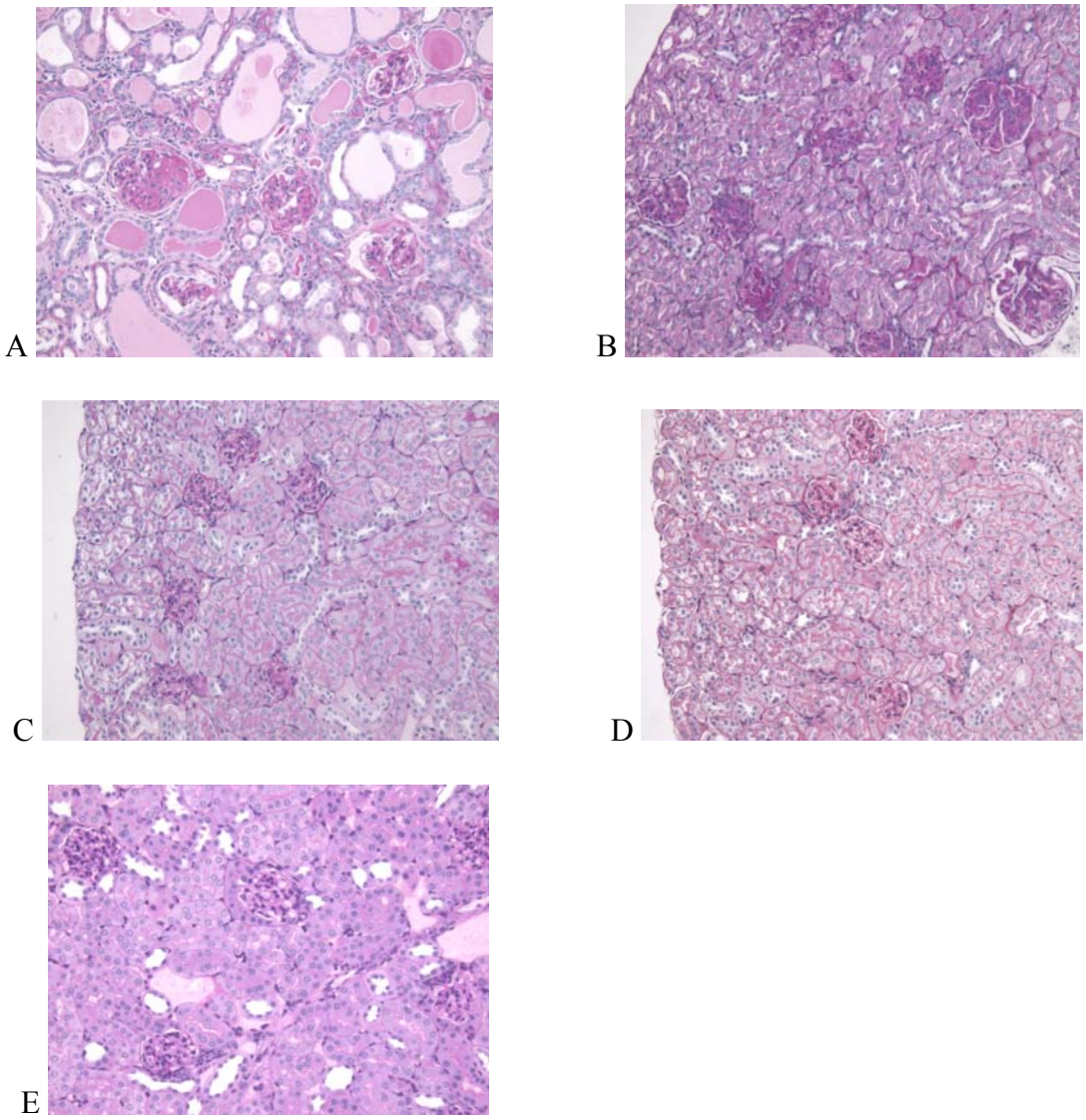
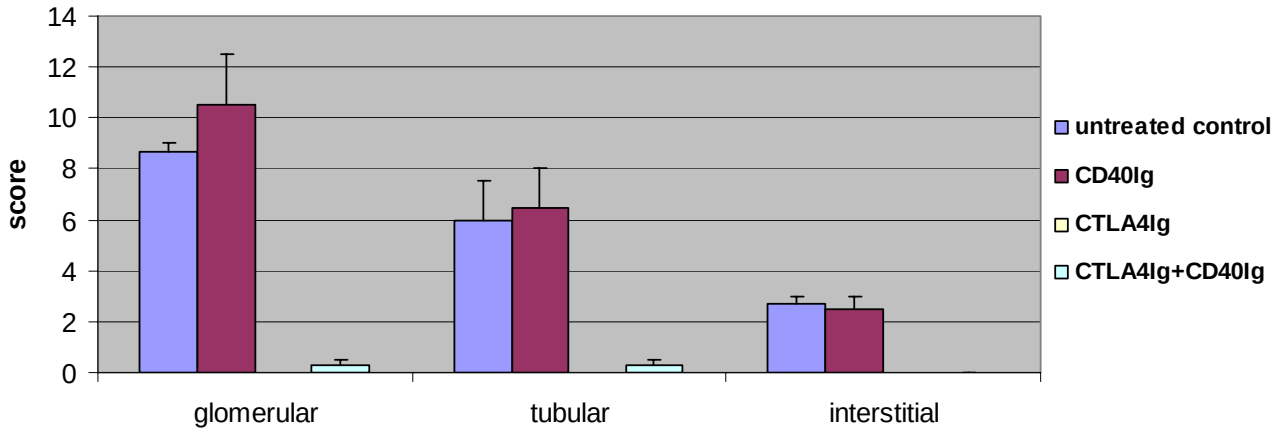


Figure 15 Kidney histopathological findings

Shown are PAS-stained, paraformaldehyde-fixed, paraffin-embedded renal sections. A, kidney of a untreated control B/W mouse at 10 months of age. Note some of the typical light microscopic features of GN, including glomerular hypercellularity, cellular crescent and tubular dilatation with casts. B, kidney of a mouse in the mCD40Ig prevention group at a comparable age, showing mild mesangial proliferative GN with obliteration of capsular lumina. C, kidney of a mouse in the mCTLA4Ig prevention group at a comparable age. D, kidney of a mouse in the two vector early prevention group at a comparable age. Both C and D show essentially normal renal histological findings. E, kidney of a wild type ICR mouse, showing normal histology. (Original magnification x 200)

(a)



(b)

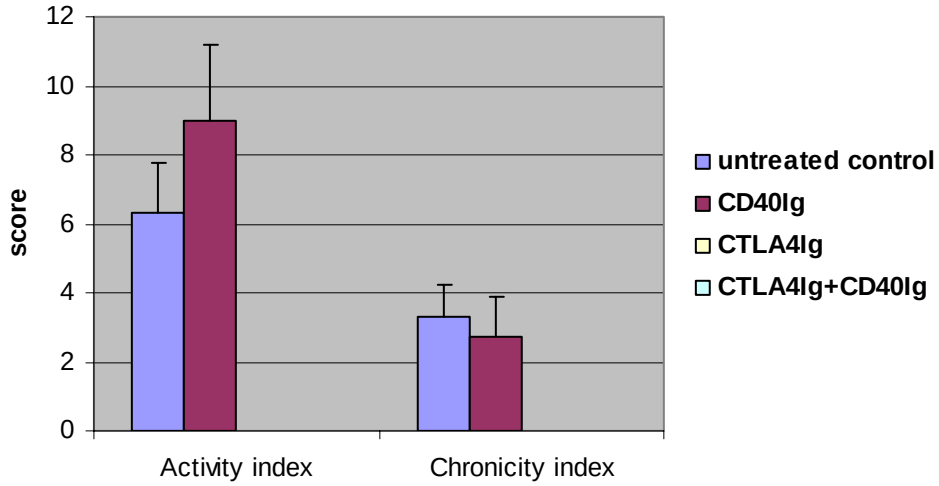


Figure 16 Differences in kidney histopathological scores

Kidney histopathological changes were evaluated by two pathologists in a blind manner on PAS staining of kidney paraffin sections from 8 month to 12 month old mice. Scores are presented as mean±SE. (a) scores of glomerular, tubular and interstitial damage. Compared to the control group, mice in the mCTLA4Ig and the two vector combination groups had lower scores ($p<0.05$). (b) activity and chronicity index of renal sections. Mice in the mCTLA4Ig and the two vector combination groups had lower scores than those in the control group ($p<0.05$).

3.3.4.2. Kidney immune complex (IC) deposit

In the study on the kinetics of autoantibody production, an age-dependent increase of serum autoantibody levels was observed in lupus mice. Serum autoantibodies are involved directly in the formation of IC, which may deposit in the glomeruli. While serum anti-dsDNA IgG titers represent the autoantibody titers in circulation, IC deposits in the kidneys provide direct evidence for the pathogenicity of the autoantibodies in the target organ. Moreover, IC deposits in the kidneys are also used as a criterion for treatment evaluation. Therefore, I stained renal cryosections for IgG and complement 3 (C3), two major components in IC. In the untreated control mice, there was pronounced IgG and C3 depositon in the glomeruli (Fig.17, Fig.18). In comparison, deposition of IgG and C3 in the glomeruli was markedly diminished in mice from the mCTLA4Ig single vector and the two vector combination early prevention groups ($p<0.05$, Fig.19). However, in the mCD40Ig single vector early prevention group, IgG and C3 deposits in the glomeruli were comparable to that of the control group ($p>0.05$). Again, the IC deposits were strikingly consistent with the kidney histopathological changes.

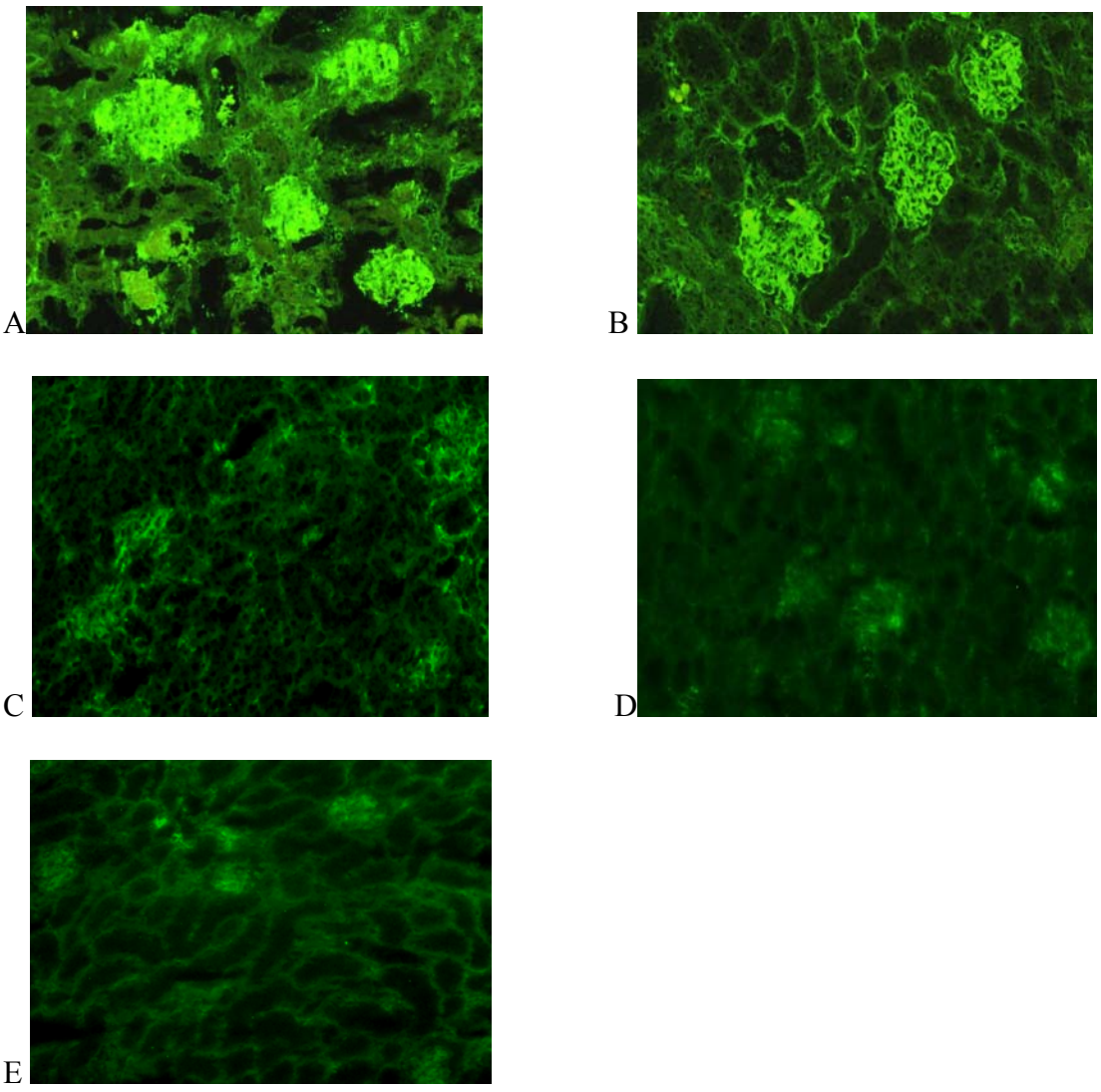


Figure 17 Kidney IgG staining

Shown are immunofluorescence stainings for mouse IgG in the kidney cryosections. A, untreated control; B, CD40Ig; C, CTLA4Ig; D, CTLA4Ig+CD40Ig; E, wild type ICR. (Original magnification x 200)

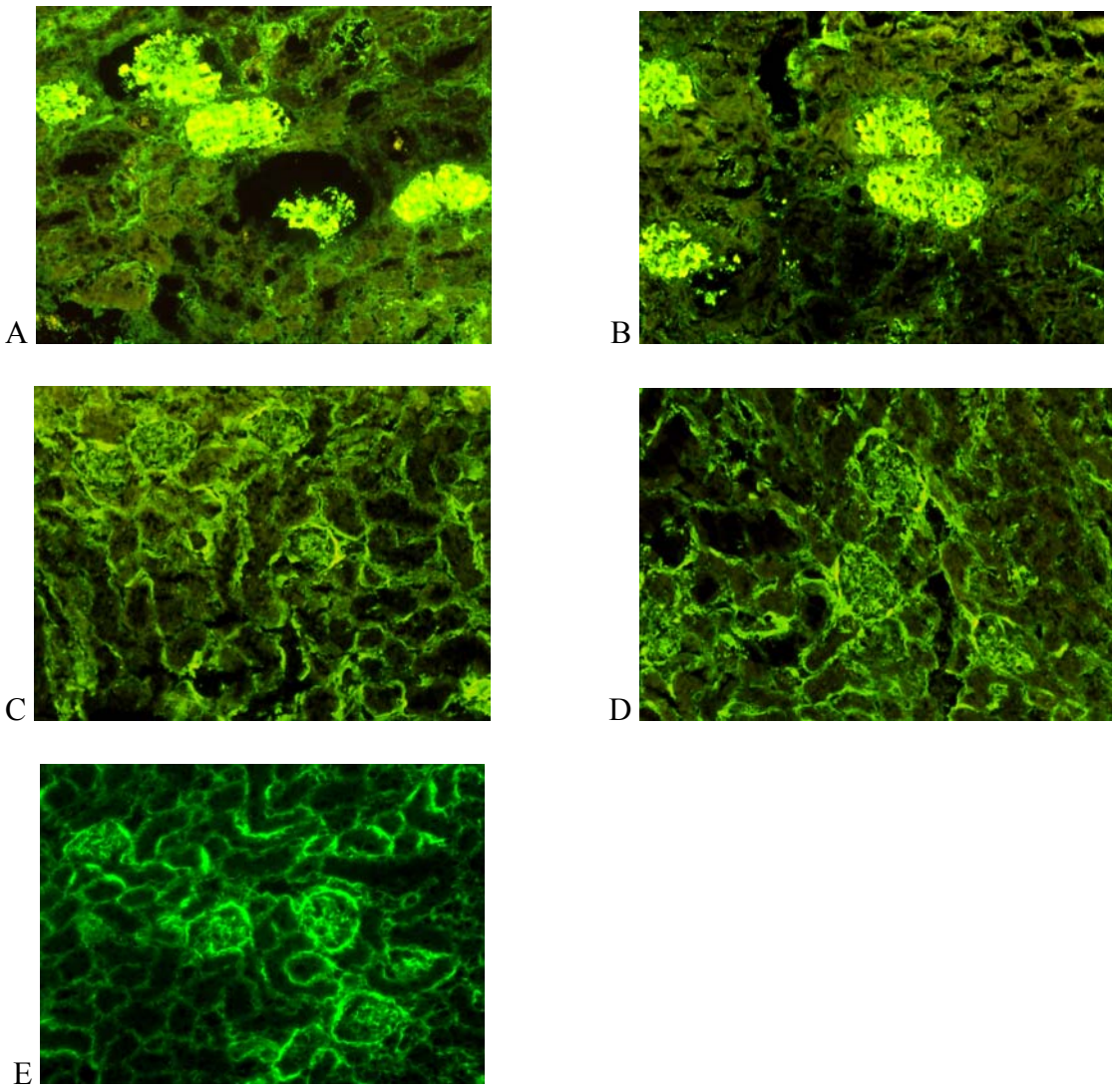


Figure 18 Kidney C3 staining

Shown are immunofluorescence stainings for mouse C3 in the kidney cryosections. A, untreated control; B, CD40Ig; C, CTLA4Ig; D, CTLA4Ig+CD40Ig; E, wild type ICR. (Original magnification x 200)

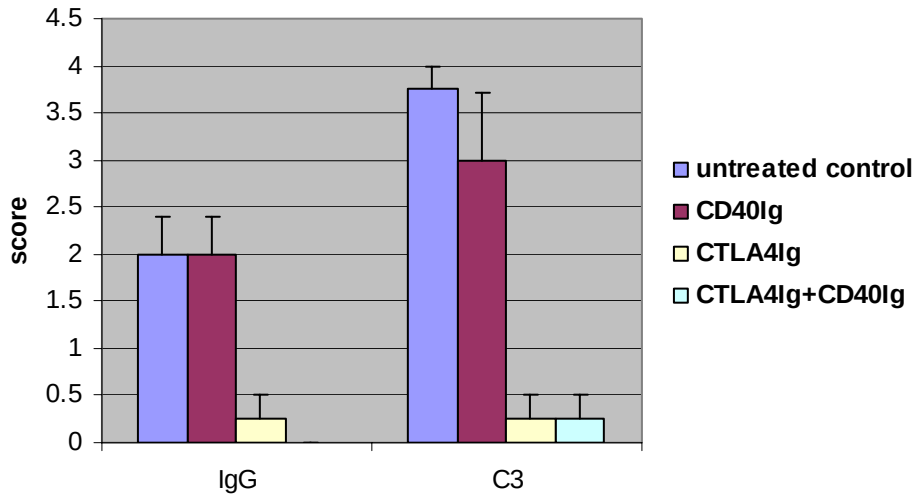


Figure 19 Score of immune complex deposit in kidney

The immune complex deposits in kidneys were evaluated by two pathologists in a blind manner on tissue cryosections from 8 month to 12 month old mice. The scores were determined by fluorescence intensity and the number of glomeruli affected, and presented as mean±SE. Mice in the mCTLA4Ig and the two vector combination groups had lower scores than those in the untreated control group ($p<0.05$).

3.3.5. Humoral response to foreign antigen

When using the costimulatory blockade strategy, one major concern is generalized immune suppression. To study whether costimulatory blockades inhibited host humoral immune responses to foreign antigens, mice were challenged with KLH, a T-cell dependent antigen, and then boosted two weeks later. Serum was collected pre-immunization and one week after boosting, and tested for anti-KLH IgG by ELISA. In the control group, mice were able to mount humoral immune responses to KLH at a level comparable to that of wild type ICR mouse. In comparison, mice in the mCTLA4Ig single vector early prevention group also produced anti-KLH IgG, but at a lower level. Its anti-KLH IgG titer was significantly lower than that of the control group at 1:1800 dilution ($p<0.01$). Mice in the two vector combination early prevention group also showed lower anti-KLH IgG titers, which became significant at 1:1800 dilution ($p<0.05$). Surprisingly, mice in the three vector combination late prevention group were able to mount anti-KLH humoral responses at a level comparable to that of the control group (Fig.20). In conclusion, costimulatory blockades did not completely inhibit host humoral responses to T-dependent antigen.

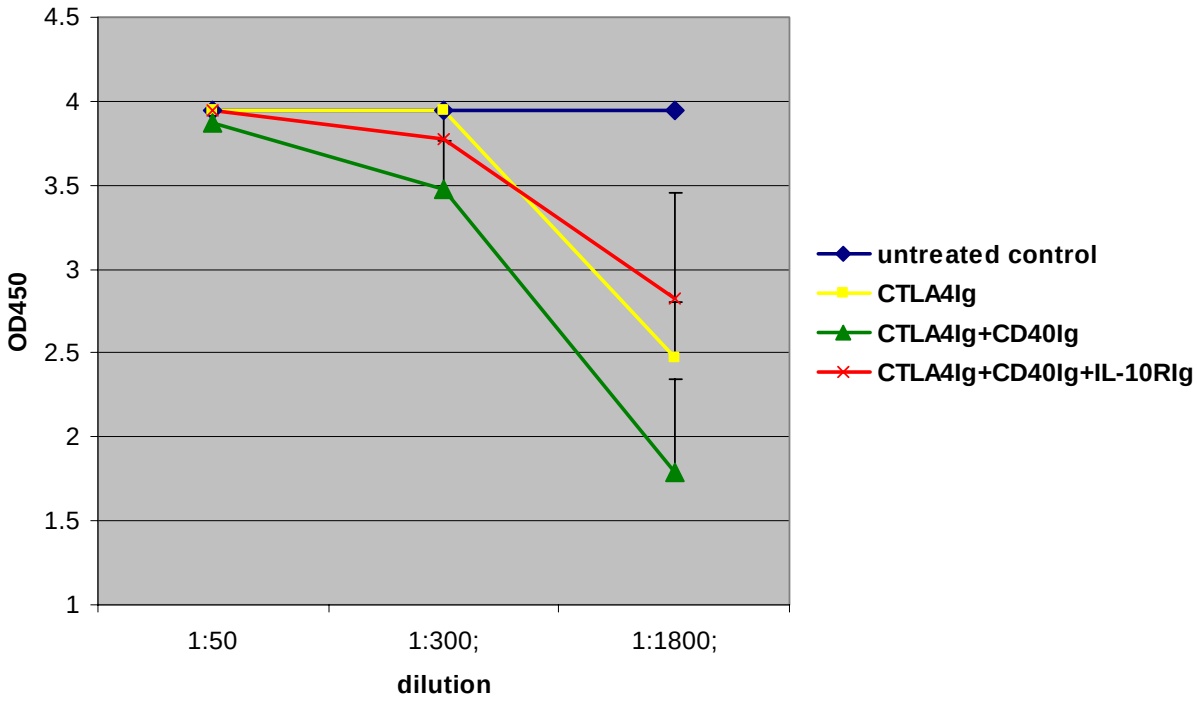


Figure 20 Humoral response to T-dependent foreign antigen

Mice were challenged i.p. with KLH in CFA and boosted two weeks later. After one week, serum anti-KLH IgG titers were determined by ELISA at different dilutions, and presented at OD readings at 450nm (mean±SE). Only at 1:1800 dilution, mice from CTLA4Ig single vector and the two vector combination prevention groups had lower anti-KLH IgG than those from the control group ($p<0.01$ mCTLA4Ig, $p<0.05$ the two vector combination).

3.3.6. Side effects

To investigate whether rAAV8 mediated gene therapy had side effects on the treated mice, weights were followed up monthly. In the control group, there was a significant weight loss when mice became moribund. The same pattern of weight change was also seen in all single vector early prevention groups. In the two vector combination early prevention group, only a few mice showed significant weight loss at death. In the three vector combination late prevention group, all except one mouse maintained a stable weight at death or when sacrificed (Fig.21). In the mCTLA4Ig single vector prevention group, one mouse had an eye infection at the age of 13 months. No such symptom was observed in other groups. In the two vector combination early prevention group, one mouse died at the age of 12 months and was found to have a colon obstruction by autopsy. One mouse was sacrificed at the age of 16 months and found to have an enlarged spleen and liver tumor. Two mice were sacrificed at the age of 17 months and also found to have enlarged spleens, and one of them had a neck tumor. Interestingly, all three of the mice with enlarged spleens were challenged with KLH. Moreover, one mouse in the control group that was challenged with KLH also had an enlarged spleen, it further implicates KLH injection involvement. For all other 10 mice in the two vector combination group, no obvious abnormalities were observed at autopsy. In the three vector combination late prevention group, no organ abnormalities were observed. In conclusion, the prevention strategies had no impact on weight, and there was no direct evidence indicating the relation between malignancy and costimulatory blockades.

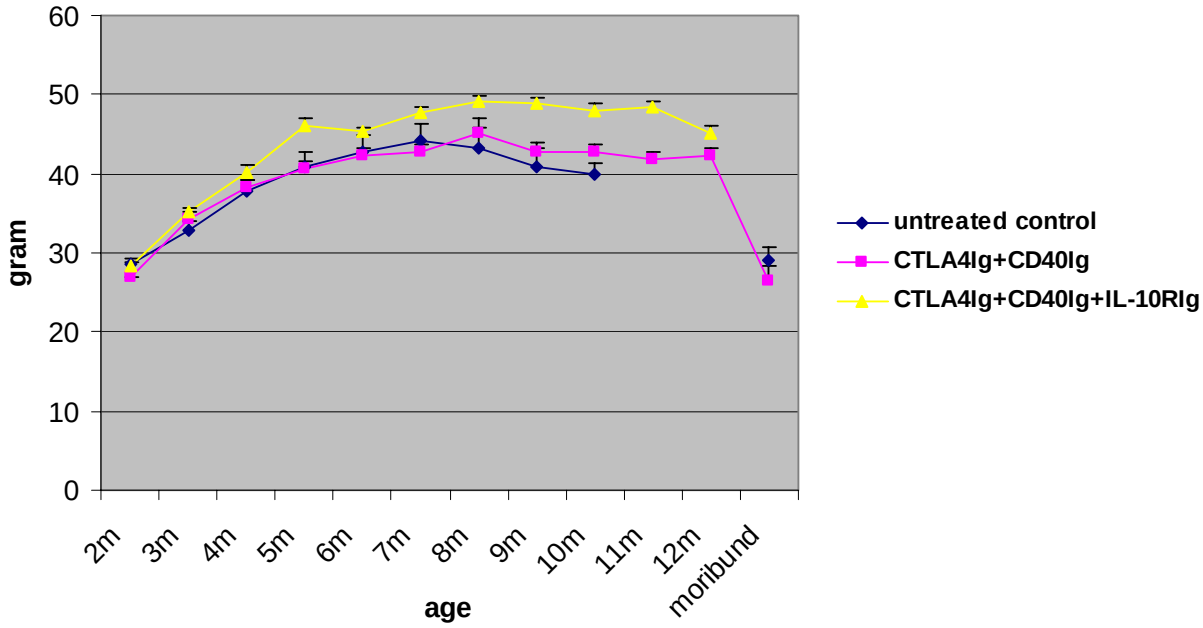


Figure 21 Weight change in the vector combination prevention groups

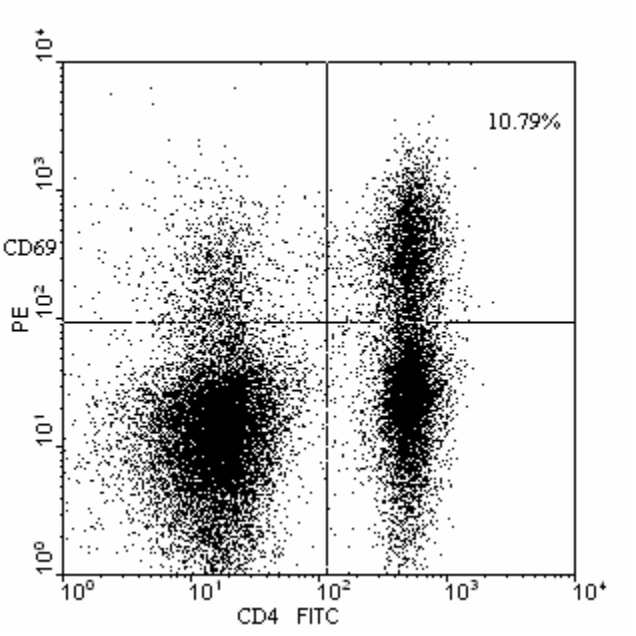
Mice were weighted monthly.The weight is presented as mean±SE. There was a significant weight loss when mice became moribund.

3.3.7. Splenic T cell subsets

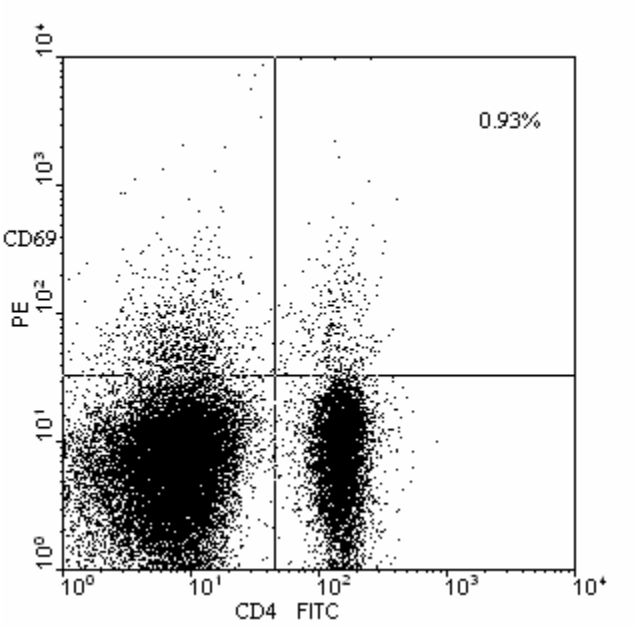
Since autoantibody production in lupus is T-cell dependent and costimulatory blockades are targeted at T cell-B cell interaction, it is important to study T cell changes after gene delivery. To determine whether the activated phenotype of splenic T cells could be altered by gene therapy, flow cytometry was performed on splenocytes by staining with different combinations of T cell surface markers. In this study, mice in the mCTLA4Ig single vector prevention group, the two vector combination early prevention group, and the untreated control group were compared. First, the total number of splenocytes in mice of the control group was 2.14-fold of that in the mCTLA4Ig prevention group and 1.4-fold of that in the two vector prevention group. Second, the percentage of $CD4^+$ and $CD8a^+$ cells in splenocytes were similar among all of these groups. Furthermore, there was no difference in the CD4:CD8a ratio. However, control mice showed a significantly higher percentage of $CD4^+CD69^+$ early activated T cells in all splenocytes than those from both prevention groups ($p<0.01$, Fig.22a,b,c). When evaluating $CD4^+CD69^+$ cells in $CD4^+$ cells, the percentage of early activated T cells was also higher in the control group ($p<0.01$ in the two vector prevention group, $p<0.05$ in the mCTLA4Ig prevention group). Interestingly, mice in the mCTLA4Ig single vector prevention group had a higher percentage of $CD4^+CD69^+$ T cells than mice in the two vector combination group ($p<0.01$). Finally, the frequency of naïve T cells ($CD62L^{high}CD44^{low}$) and memory T cells ($CD62L^{low}CD44^{high}$) in $CD4^+$ T cells were analyzed. Mice in the control group showed a larger subset of memory T cells and a smaller naïve T cell subpopulation than those in both prevention groups ($p<0.01$, Fig.22d). There was even less memory T cells in the two vector combination group than in the mCTLA4Ig single vector prevention group ($p<0.05$). In conclusion, both the mCTLA4Ig single vector and the two

vector combination prevented T cell activation and inhibited the transition from naïve T cells to memory T cells.

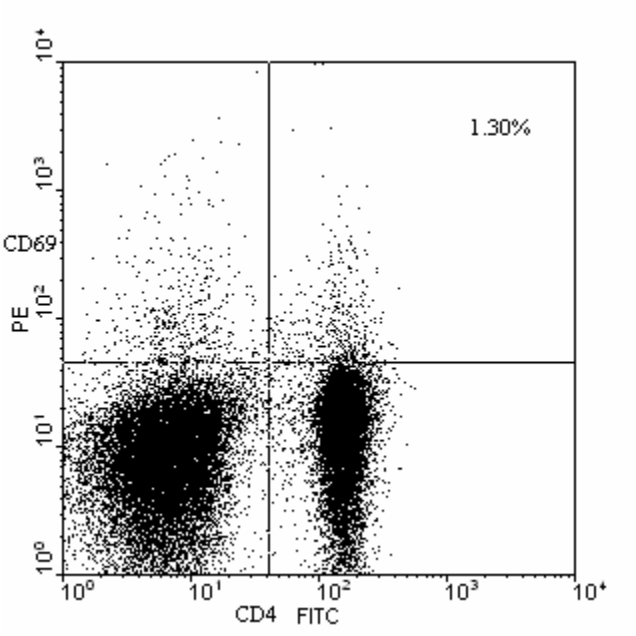
(a)


10.79%
CD69
PE
CD4 FITC
10^0
10^1
10^2
10^3
10^4

(b)


0.93%
CD69
PE
CD4 FITC
10^0
10^1
10^2
10^3
10^4

(c)



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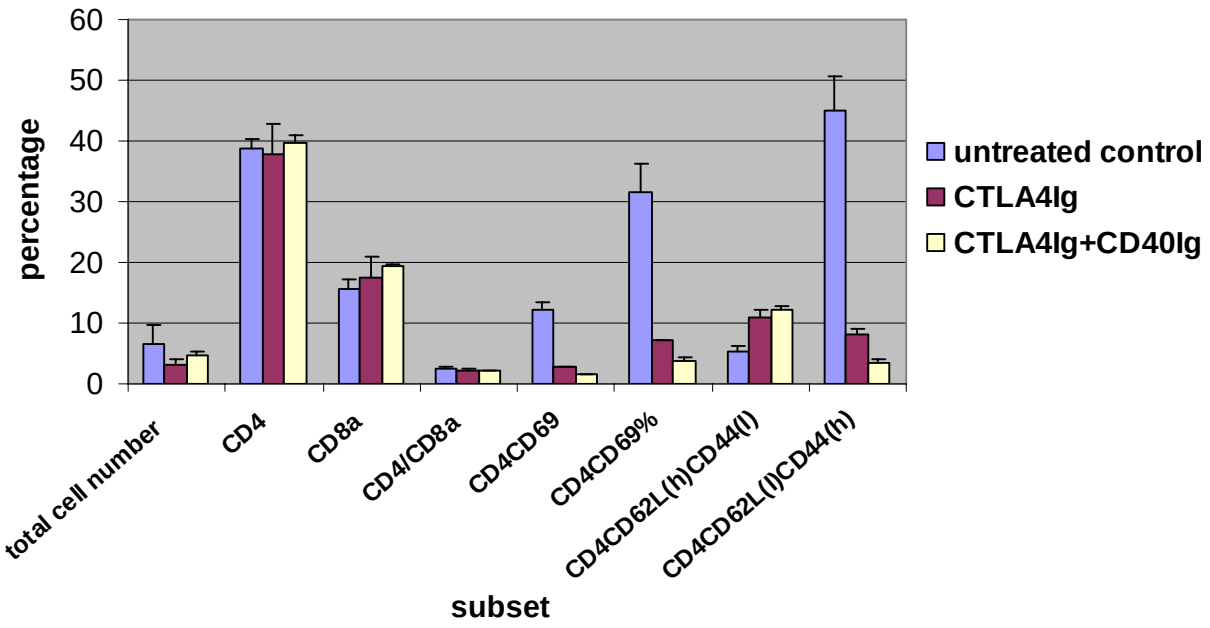

Figure 22 Flow cytometry analysis of spleen cells

Splenocytes were isolated and stained with different combinations of T cell surface markers. The immunophenotype was assayed by FACS. Shown are representative dot plots of $CD4^{+}CD69^{+}$ cells in the (a) the untreated control group, (b) CTLA4Ig+CD40Ig early prevention group, and (c) CTLA4Ig early prevention group. (d) A histogram depicting the total number of splenocytes ($x10^{7}$), the percentage of $CD4^{+}$, $CD8a^{+}$, early activated T cells ($CD4^{+}CD69^{+}$), naïve T cells ($CD62L^{high}CD44^{low}$) and memory T cells ($CD62L^{low}CD44^{high}$), presented as mean±SE. Compared to the untreated control, mice in both prevention groups had fewer early activated T cells and memory T cells, and more naïve T cells ($p<0.01$).

3.4. Conclusions

rAAV8-mediated gene delivery opens up a new field for lupus intervention. After achieving long-lasting and stable transgene expression, the next step was to investigate what kind of effect gene therapy will have on lupus mice, and how the transgene products will interact with the host immune system. In this chapter, the disease prevention efficiency of different immune modulation strategies was compared in B/W mice. In single vector early prevention groups, mCTLA4Ig was able to prevent autoantibody production, delay the onset of proteinuria, protect kidneys from IC-induced damage, and prolong survival. However, mCD40Ig was less effective in lupus prevention, resulting in a dramatic increase in autoantibody titers and typical GN pathological changes in the kidneys. The two vector combination strategy showed better preventive effects on lupus mice than each single vector. In this group, not a single mouse died at 10 months of age, when all the control mice had died of proteinuria. This result suggests a synergistic effect between mCTLA4Ig and mCD40Ig.

B/W mice spontaneously produce anti-dsDNA antibodies as young adults, and it is not known whether gene delivery at a late time point can still prevent lupus. Interestingly, when three vectors were coinjected into 6 week old B/W mice, they prevented lupus with efficiency comparable to that of the two vector combination early prevention. This result indicates that late intervention for lupus prevention is feasible in a gene therapy setting. As to the side effects from long-term transgene expression, no significant weight change was observed, and two long-term survival mice in the two vector combination group developed neck and liver tumors. With respect to immunosuppression from long-term costimulatory blockades, when challenged with T-dependent antigen KLH, treated mice were able to mount foreign antigen-specific humoral responses at a level comparable to that of control mice. This result indicates that costimulatory blockades did not cause immunosuppression.

Finally, in an attempt to elucidate the mechanism of lupus prevention by rAAV-mediated gene delivery, splenic T cell subsets were studied. Treated mice showed a significantly larger population of naïve T cells compared to control mice, which had more early activated T cells and memory T cells. This result suggests that costimulatory blockades inhibited $CD4^+$ T cell activation and transition from naïve T cells to memory T cells. In conclusion, rAAV8-mediated gene delivery was able to prevent lupus development in lupus prone B/W mice by inhibiting T and B cell activation.

3.5. Discussion

In the application of gene therapy for disease prevention, the first issue to study is transgene expression in the disease setting, and has been covered in the first chapter. The next step is to study the preventive effects of transgene expression. In this chapter, the following issues were discussed 1) whether rAAV8-mediated gene delivery was able to prevent lupus development in B/W mice, 2) what was the best time for gene transfer, 3) which one was the best prevention strategy, single vector or vector combinations, 4) how will these immunomodulators affect the host immune system, 5) whether there were any side effects. Clarifying these questions will help us to understand the mechanism of rAAV-mediated gene delivery in lupus prevention and facilitate gene therapy for lupus treatment.

3.5.1. The mechanism of lupus prevention and effects on lupus development in rAAV-delivered costimulatory blockade

It is well known that the autoantibody production in lupus is a T-dependent process. Because of loss of peripheral tolerance, autoreactive T cells activate autoreactive B cells, which in turn, present autoantigens to T cells and drive the epitope spreading and perpetuation of autoimmunity (123). The generation of autoantibodies and immune complexes, along with autoreactive T cells, cause damage to several target organs, including skin, blood vessels, lung and kidney. The aim of costimulatory blockades was to interrupt autoreactive T and B cell interaction in order to break up the positive feedback loop. The binding of CD28 constitutively expressed on T cells with the B7 molecule on B cells is critical for T helper cell differentiation and activation. With higher affinity, mCTLA4Ig competes with CD28 for the binding to the B7 molecule in order to block their interaction (158). On the other hand, mCD40Ig binds to CD40L expressed on T cells and blocks the activation signal essential for isotype switching, B cell growth and differentiation into memory B cells (175).

In this chapter, the effect of gene therapy on T cells was studied by evaluating the splenic T cell subset composition. Therers are three different T helper cell subsets, including naïve, activated and memory T cells. Their relative population size indicates the activation status of T cell pool. In control mice, early activated $CD4^+$ T cells took up 12.15% of the splenocytes, and in all $CD4^+$ T cells 5.47% were naïve T and 44.85% were memory T, showing activated phenotype of T cells (Fig.22). In comparison, for mice in the mCTLA4Ig early prevention group, only 2.72% of the splenocytes were early activated T cells, and $CD4^+$ T cells showed more naïve phenotype (10.94%) and less memory phenotype (8.07%). In the two vector combination prevention group, there were even more naïve T cells (12.30%) and fewer activated and memory T cells (1.49%, 3.56% respectively). These data suggest that costimulatory blockades inhibited the activation of $CD4^+$ T cells and also their differentiation into memory T cells. These results are also supported by another group the used CTLA4Ig and anti-CD40L mAb on B/W mice (175).

Autoreactive B cells are the main source of autoantibodies (56). Anti-dsDNA IgG titer not only directly reflect autoimmunity status but also indicate autoreactive B cell activity (269). The study of serum anti-dsDNA IgG shows that rAAV8-mCTLA4Ig alone, or combined with rAAV8-mCD40Ig, significantly prevents anti-dsDNA IgG production in lupus mice. Interestingly there was a noticeable increase in the autoantibody titer at the age of 6 months, which was soon suppressed and the autoantibody level kept low thereafter. This observation seemed not to be related to serum mCTLA4Ig levels, which were stable around that time. Consistent with the low autoantibody titers, the onset of proteinuria was significantly delayed in mice from these two groups. However, when the relationship between serum mCTLA4Ig levels, anti-dsDNA IgG titers and the onset of proteinuria was evaluated in individual mice, no certain

correlation was found. Instead, different patterns were presented. In one mouse, serum mCTLA4Ig levels were far below the average after gene delivery, its autoantibody titer did not increase until 7 months of age, and proteinuria was detected 4 months later. While in another mouse, serum mCTLA4Ig level dropped significantly at 6 months of age, soon autoantibody titers became elevated, and proteinuria developed only 1 month later. From the view point of pathogenesis, these data indicate a complex process of lupus development. From the autoantibody production to the target organ damage, there are multiple factors involved, such as proinflammatory cytokines, chemokines, complement and leukocytes (270, 271). Numerous evidence has shown that autoantibodies induce target organ damage directly, or indirectly by causing inflammation through immune complex deposits (272).

In this study, kidneys were the most prominent target organs and presented pathological changes typical of lupus. Kidney histopathology studies on control mice showed typical GN changes, such as glomerular endocapillary proliferation, hyaline thrombi, tubular atrophy and interstitial fibrosis. Nevertheless, judged with a scoring criterion, mice from the mCTLA4Ig single vector or the two vector combination early prevention groups had significantly less GN damage. In some cases, kidneys from these prevention groups showed almost normal histology. It is believed that immune complex deposits forming from autoantibodies is the major reason for kidney damage in lupus. In this study, kidney sections were stained for IgG and C3, the major components of immune complexes. Mice in the treated groups had significantly less IgG and C3 deposits in the glomeruli compared to those in the control group. Moreover, the treated mice survived much longer than the control mice.

Taking all these evidence together, it suggests the mechanism of rAAV-delivered costimulatory blockades in lupus prevention. Long-term mCTLA4Ig expression inhibited $CD4^+$

T cell activation, prevented spontaneous autoantibody production from autoreactive B cells, inhibited IC deposits in the target organ of kidney, further ameliorated the lupus symptoms, and eventually prolonged survival. On the other hand, these data also confirm the paradigm of lupus development proposed by other researchers, in which autoantibodies produced by autoreactive B cells form immune complexes which further cause tissue damage by IC deposition in the target organs.

To further investigate the effect of AAV-mediated costimulatory blockade on T and B cells, I will focus on the following studies. Firstly, analysis of B cell activity in vitro will provide direct evidence on whether the intervention affects B cell antibody production. I will isolate splenic B cells using CD19-conjugated microbeads and evaluate the spontaneous IgG secretion in vitro. Secondly, when B cells get $CD4^+$ T cell help and undergo proliferation and somatic mutation, germinal center (GC) is formed in white pulp of spleen. CD40-CD40L interaction is essential for GC formation and also the development of B cell memory (150). Costimulatory blockade is expected to inhibit GC generation by blocking T-B cell interaction. Therefore I will stain splenic cryosection with GC marker (PNA) and B cell marker (B220) to compare the number of double-positive GC in the treated and control groups. Finally, there has been great interest in the possibility that a skewing toward Th2 type response plays a major role in murine lupus. Some evidence from cytokine profile of $CD4^+$ T lymphocytes in aged lupus supports this opinion (109, 273). Therefore, it is necessary to study serum cytokine profile. The change in serum level of Th1 cytokine (IFNγ and IL-2) and Th2 cytokine (IL-4, IL-6 and IL-10) indicates Th1/Th2 skewing in treated and untreated control groups.

3.5.2. The best time point of gene delivery for lupus prevention

For protein-based therapy, as soon as molecules are injected, they are ready to take effect. However, under the setting of gene therapy, it takes time for transgenes to be expressed and for the transgene products to reach an effective level after gene delivery. In the previous chapter, rAAV-mediated gene expression in lupus mice was elucidated. Here, the focus was on the relationship between time points of gene delivery and prevention outcome. It is known that lupus mice spontaneously produce anti-ssDNA IgM at a very early age, and the anti-dsDNA IgM takes over (274). At the age of 3 to 4 months, with the autoreactive B cells proliferating, anti-dsDNA IgM is class-switched to anti-dsDNA IgG, one of the major pathogenic autoantibodies (275).

For the purpose of lupus prevention, two groups were set up. In the early prevention group, rAAV8 was delivered i.p. into the B/W newborns; while in the late prevention group, rAAV8 was injected into the spleen of 6-week old B/W mice. In either case, there were no autoantibodies in the circulation at the time of gene delivery. Moreover, data in the previous chapter suggests that for both groups, serum mCTLA4Ig levels peaked before the massive production of autoantibodies from B cells. However, unlike mice in the two vector combination early prevention group, which had an increase of autoantibody at 6 months of age, mice in the late prevention group showed baseline levels of anti-dsDNA IgG at all time points tested. One possible explanation is that the combination with mIL-10RIg maximizes the preventive effects by neutralizing excessive mIL-10 in the circulation. This possibility will be discussed in detail below. As to the onset of proteinuria, all mice in both the early and the late prevention groups were proteinuria-free for more than 12 months. Furthermore, late prevention increased the survival rate to a level similar to that of early prevention. Based on all these data, as far as the time point of gene delivery is concerned, late prevention is as effective as early prevention.

However, when considering immunosuppression, mice in the late prevention group had higher anti-KLH IgG titers than those in the two vector early prevention group. This could have resulted from an enormous interference within the host immune system as the trangenes were delivered into the newborn mice. Another supporting line of evidence is the tumor growth in the early prevention group, while mice in the late prevention group had no detectable malignancies. Therefore, considering the similar efficiencies on lupus prevention, late intervention is recommended based on fewer side effects.

3.5.3. Comparison of different prevention strategies

Since lupus is a complex disease with multiple steps in its pathogenesis and formation, many strategies have been developed to prevent or treat lupus. Recently, costimulatory blockade has drawn more and more attention in this field because of its efficiency, selectivity and fewer side effects. The feasibility of rAAV-delivered costimulatory blockade for lupus prevention was studied here. Both CTLA4Ig and anti-CD40L mAb have been shown to decrease autoantibody titers and inhibit lupus development (166, 199). In theory, CD40Ig should work in the same way as anti-CD40L mAb by blocking CD40-CD40L binding. However, CD40Ig has never been applied to lupus, even though it has been used for transplantation where it successfully induced tolerance to the allograft (276).

This is the first report using CD40Ig in the murine model of lupus. *In vitro* studies in the previous chapter showed that mCD40Ig was secreted from plasmid transduced 293 cells. For the *in vivo* studies, even though serum mCD40Ig levels were not measured, mice treated with rAAV8-mCD40Ig had delayed onset of proteinuria and increased survival rates, which suggest there was a certain level of mCD40Ig in the circulation. On the other hand, compared to the untreated controls, the increase of anti-dsDNA IgG was not inhibited in the mCD40Ig single

vector early prevention group. The *in vitro* data indicate low secretion levels of mCD40Ig, which would explain the lack of significant lupus prevention. In this chapter, mCTLA4Ig and mCD40Ig were also combined in the early prevention group and compared to the single vector groups. These studies showed that the two vector combination was much better than each single vector in terms of inhibiting autoantibody production, delaying disease onset and prolonging survival. On one hand, this suggests a synergistic effect on autoimmune development between these two costimulatory pathways, namely B7/CD28 and CD40/CD40L; on the other hand, it also indicates that rAAV8-mCD40Ig had some inhibitory effect on lupus development.

Another strategy evaluated in this chapter was the use of cytokine antagonist. It is well known that cytokine imbalance plays a critical role in lupus development. Abnormally high levels of IL-10, a B cell growth factor, has been observed in both SLE patients and lupus mice (91, 177). Anti-IL-10 mAb has been shown to inhibit the differentiation and proliferation of autoantibody-producing B cells and efficiently prevent lupus in the murine model (98). Here, rAAV8 was used to deliver the transgene of soluble IL-10 receptor for lupus prevention. mIL-10RIg showed little, if any, effect on the increase of autoantibody, and instead seemed to accelerate autoantibody production in B/W mice. At the age of 5 months, the autoantibody titers in this group were significantly higher than those of the control group, and the autoantibody levels were saturated in the ELISA by the age of 6 months (data not shown). Moreover, the onset of proteinuria followed the same pattern as that of the control group, and no difference was detected statistically ($p=0.47$). Consistent with proteinuria data, mice in the mIL-10RIg early prevention group had similar low survival rates when compared to the control untreated group ($p>0.05$). No mice lived longer than 12 months. Even though the *in vitro* data showed positive mIL-10RIg secretion, the *in vivo* study with the mIL-10RIg single vector failed to protect B/W

mice from lupus development. Serum mIL-10 levels were also measured by ELISA (data not shown). In the mIL-10RIg single vector early prevention group, the levels kept increasing, from 420.17pg/ml at 2 months, 848.33pg/ml at 6 months, to 1060.75pg/ml at 8 months of age. While in the control group, serum mIL-10 levels were 1391pg/ml at 10 months and 1149.5pg/ml at 11 months of age. These results suggest that mIL-10 was not neutralized by the soluble mIL-10 receptor in the mIL-10RIg treated mice. On one hand, the low level of secretion should be taken into account, which was supported by the *in vitro* secretion data; on the othe hand, it is necessary to measure the bioactivity of mIL-10R for neutralizing IL-10. Interestingly, when costimulatory blockades were combined with mIL-10RIg in the late prevention group, they showed the same preventive effect as that of the two vector combination early prevention group. Because rAAV8-mIL-10RIg did not have much effect on lupus prevention, it would be reasonable to speculate that the mCTLA4Ig and mCD40Ig combination will be enough to succeed in the late prevention of lupus. Furthermore, rAAV-mCD40Ig was much less efficient than rAAV-mCTLA4Ig in the early prevention of lupus. There is a great chance for rAAV-mCTLA4Ig single vector to prevent lupus in the young adult mice.

3.5.4. Transgene expression level and AAV tissue tropism in lupus prevention

For gene therapy, transgene expression level directly affects the intervention outcome. In the rAAV8-mediated lupus prevention, when studying individual mice, I observed that a decrease in serum CTLA4Ig level was followed by the onset of proteinuria. It suggests that sustained high level of CTLA4Ig is necessary to prevent the activation of naïve autoreactive T and B cells. Controversially, in the AAV1 and AAV2 groups, where serum CTLA4Ig level was significantly lower than that of AAV8 group, all mice were long-surviving and proteinuria free. Study shows that the development of regulatory T cells is CD28-dependent (277, 278). It is possible that

during the development and maturation of immune system, high level of CTLA4Ig in the newborn mice suppresses the development of host immune regulatory machinery, such as regulatory T cells, while it is less affected by the low level of CTLA4Ig. Therefore, in the AAV1 and AAV2 groups, self immune regulatory mechanism, instead of high level of CTLA4Ig, keep the autoreactive T and B cells in check. To clarify this issue, analysis of T cell compartment of the newborn mice with low level CTLA4Ig need to be performed.

Another theory is that for the prevention of autoimmune disease like lupus, immune cell-targeted transduction is more effective than systemic high level of immunomodulator transgene expression. T cells and B cells play the major role in lupus pathogenesis, with some contribution from dendritc cells (279). It has been reported that AAV is able to transduce lymphocyte-derived cell lines and B cells (280, 281). However, controversies still exist about whether AAV can transduce hematopoietic stem cells (HSCs) (282). Since AAV exist mostly episomal in the host cell, it is unlikely that the transgene will be carried on in the T and B cell lineage derived from HSCs. Even though the immune cells are transduced, the possibility of long-term effect from AAV-delivered transgene is very slim because of the short half life of these cells. Solid evidence supporting this theory is still missing.

3.5.5. Immunosuppression and side effects

When using costimulatory blockades, one major concern is immunosuppression, mostly to the humoral arm of the immune response. This is still a disputable issue. Some studies have shown that costimulatory blockades result in antigen-specific tolerance, while others insist it causes general immunosuppression (283, 284). The results varied depending on the testing system used. For example, gene transfer of CD40Ig into heart allografts induced donor-specific

hyporesponsiveness with intact humoral responses to cognate antigens, while intravenous injection of Ad-CTLA4Ig inhibited host humoral response to KLH (284-286).

To address this issue, mice were challenged with the T-dependent antigen KLH and the anti-KLH IgG titers were measured by ELISA. The results showed that before challenge, there were low titers of anti-KLH IgG in the control and treated mice, while no such antibody was detected in wild type ICR mouse (data not shown). This result could be explained by nonspecific binding from other IgGs, considering the autoantibody background in these mice, and also the low dilution factor used in the ELISA assay. Three weeks after boosting, mice in the control group mounted KLH-specific humoral responses at levels comparable to that of the wild type ICR mouse. Anti-KLH IgG titers in the mCTLA4Ig single vector early prevention group and the three vector combination late prevention group were not significantly different from those of the control group. For the two vector combination early prevention group, anti-KLH IgG titers were significantly lower only at the 1:1800 dilution. These results indicate that all treated mice were able to mount considerable KLH specific humoral responses. When studying the splenic T cell immunophenotype, we knew that the costimulatory blockade inhibited $CD4^+$ T cell activation. In this case, mice were challenged with KLH emulsified with CFA, which dramatically enhanced the immunogenicity of this antigen. For such a strong antigen, costimulatory pathways are not required for T cell activation (287, 288). KLH-CFA could circumvent the costimulatory blockade and directly activate T helper cells. In addition, the effect of costimulatory blockade on the cellular arm of host immune system was not studied. As far as $CD8^+$ T cell population size was concerned, there was no significant change in the treated mice.

As to the side effects in general, there was no observable deleterious effects on the treated mice. All mice in the prevention groups carried stable weight after gene delivery. The disease

was not accelerated in the prevention groups. Even though there was one mouse from the mCTLA4Ig single vector group that experienced an eye infection, the incident was too low and it could be considered a random event. Noticeably, there were two mice in the two vector combination early prevention group that had tumor growths. Since all these treated mice lived far beyond the normal longevity of B/W mice, it's hard to conclude whether the tumor growth was an age-dependent event or the result from immunosuppression.

4. LUPUS TREATMENT

4.1. Introduction

The ultimate goal of gene therapy is to treat the disease. Especially for chronic systemic diseases like lupus, early intervention is necessary to prevent multiple organ involvement. A recent study on SLE patients shows that autoantibodies are present in the circulation years before the occurrence of clinical manifestation (289). The present paradigm for the development of SLE is that autoimmunity develops in four stages: (1) genetic predisposition, (2) initiation, (3) perpetuation and progression (associated with epitope spreading), and finally, when autoimmunity has crossed a certain threshold, (4) clinical disease. Thus, there exists a window time for early intervention, when the serological abnormality already exists, but tissue damage in the target organs is mild and reversible. It is important to keep in mind that lupus is the result of a cascade of events that occur on the background of a genetic predisposition, and that environmental factors also influence the development of autoimmunity. As evidence, even inbred lupus-prone mice vary considerably in the time of disease onset, the severity and the pathology elicited by the autoimmune responses (290). The studies described in this chapter focused on whether rAAV8-mediated gene delivery was able to treat lupus after the autoimmunity had already developed in B/W mice. Another issue that was addressed was whether the treatment effect was dose-dependent. The results will help us to apply rAAV-mediated gene therapy to clinical trials for lupus.

4.2. Material and methods

4.2.1. Animal protocol

To study whether costimulatory blockades established through gene delivery are able to reverse lupus progression and inhibit proteinuria, rAAV was delivered into NZB/W F1 mice only after anti-dsDNA IgG titers were significantly elevated. In the single vector treatment group, 8×10^{11} vg of AAV8-CAG-mCTLA4IgG2a in 0.3 ml PBS were injected into the spleen. To evaluate the dose-effect relationship, two different vector doses were given in the two vector combination treatment groups. For the high dose treatment group, $2x10^{11}$ vg of AAV8-CAG-mCTLA4IgG2a and AAV8-CAG-CD40IgG1 in 0.3ml PBS were delivered into the spleen of lupus mice; in the low dose treatment group, $2x10^{10}$ vg of AAV8-CAG-mCTLA4IgG2a and AAV8-CAG-CD40IgG1 in 0.3ml PBS were delivered into the spleen of lupus mice. After gene delivery, mice were bled via the tail vein every two weeks for 2 months, and then monthly until death. Serum was collected and stored at -80ºC.

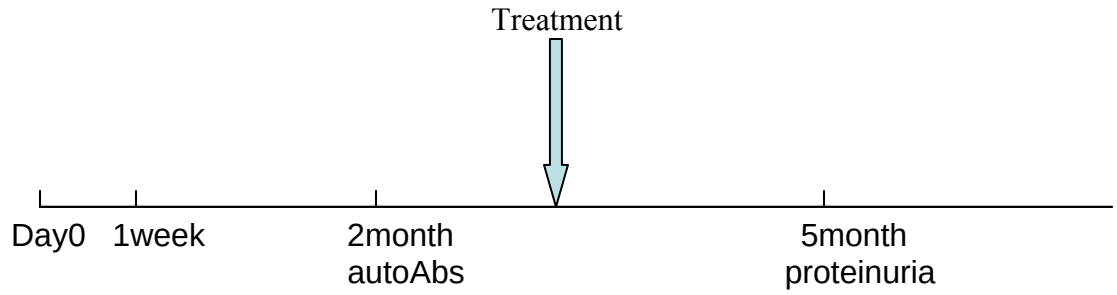

Figure 23 Experimental time-line of lupus treatment in B/W mice

Lupus mice were intrasplenically injected with AAV8-CAG-mCTLA4IgG2a alone or with AAV8-CAG-mCD40IgG1, after they had highly elevated serum anti-dsDNA IgG titers, but were still symptom-free.

4.2.2. Serum anti-dsDNA IgG

Among all autoantibodies in lupus mice, anti-dsDNA IgG most closely correlates with disease progression and prognosis. Serum anti-dsDNA IgG levels were measured by ELISA using an anti-dsDNA antibodies ELISA kit (Alpha Diagnostic, TX) following the manufacturer's instructions. Levels were presented as OD readings at 450nm.

4.2.3. Proteinuria

Urine was collected monthly by pressing the bladders of the mice beginning at 2 months of age. To assess proteinuria, urine was monitored colorimetrically with Albustix (Bayer corp. IN). Proteinuria levels were scored as followed: 0 (none), 1+ (30-99mg/dl), 2+ (100-299mg/dl), 3+ (300-1999mg/dl) or 4+ (2000mg/dl and more). Severe proteinuria was defined as 3+ and above at two independent measurements.

4.2.4. Statistical analysis

The student's t-test was used to determine the statistical significance of serum anti-dsDNA IgG titers among the groups. Proteinuria and survival data were analyzed by the log-rank test. The survival curve was drawn using SigmaPlot software. The p value was two-tailed and considered statistically significant when $p < 0.05$.

4.3. Results

4.3.1. The change of serum anti-dsDNA IgG titer in the treatment groups

In the treatment groups, mice were treated only after they had developed autoantibodies, as judged by ELISA OD450 readings higher than 1.0. It turned out that most mice were treated from age 4 months to 6 months. After gene delivery, the mice were bled every 2 weeks to monitor the changes in autoantibody titers. The results showed that in the AAV8-CAG-CTLA4IgG2a single vector treatment group, the autoantibody titers continuously dropped after

gene delivery, but started to increase 1 month after treatment. In the AAV8-CAG-CTLA4IgG2a and AAV8-CAG-CD40IgG1 two vector combination low dose treatment group, the decrease in autoantibody titers continued for 6 weeks after treatment, then went up at 2 months after treatment (Fig.24). Interestingly, in the two vector combination high dose treatment group, the autoantibody titers continuously went down and at 2 months after gene therapy, titers were significantly lower than that before treatment ($p<0.01$) and those of the single vector treatment group ($p<0.05$).

Autoantibody titers were also monitored long term. Compared to the untreated control group, mice in the treatment groups had significantly lower level of autoantibody titer at the age of 8 month ($p<0.05$ in the mCTLAIg group, $p<0.01$ in the two vector combination low dose group, $p<0.001$ in the two vector combination high dose group). From 9 months to 12 months of age, anti-dsDNA IgG titers slowly increased in the low dose group, while it kept at low levels in both the mCTLA4Ig single vector and the two vector combination high dose groups. By the age of 12 month, autoantibody titers in the low dose group were much higher than those of the single vector and the two vector high dose treatment groups (Fig.25). In conclusion, rAAV8-mediated gene therapy suppressed autoantibody production and decreased autoantibody titers. In the two vector combination treatment groups, a high dose was more efficient than a low dose in terms of decreasing autoantibody titers.

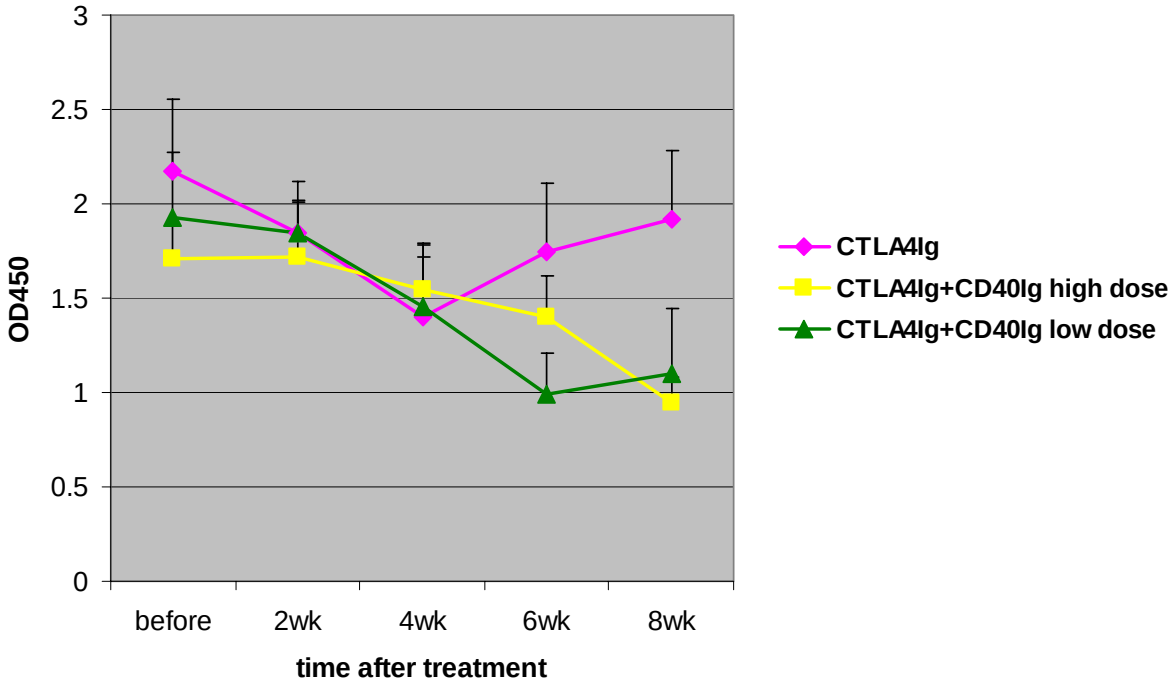

Figure 24 Serum anti-dsDNA IgG titer 2 months after treatment

Mice were treated only after serum anti-dsDNA IgG titer highly increased but were negative for proteinuria. Sera were collected every 2 weeks after gene delivery. Anti-dsDNA IgG titers were determined by ELISA and presented as mean±SE. Two months after gene delivery, the autoantibody titers in the high dose treatment group decreased dramatically ($p<0.01$), and were much lower than those of the single vector treatment group ($p<0.05$).

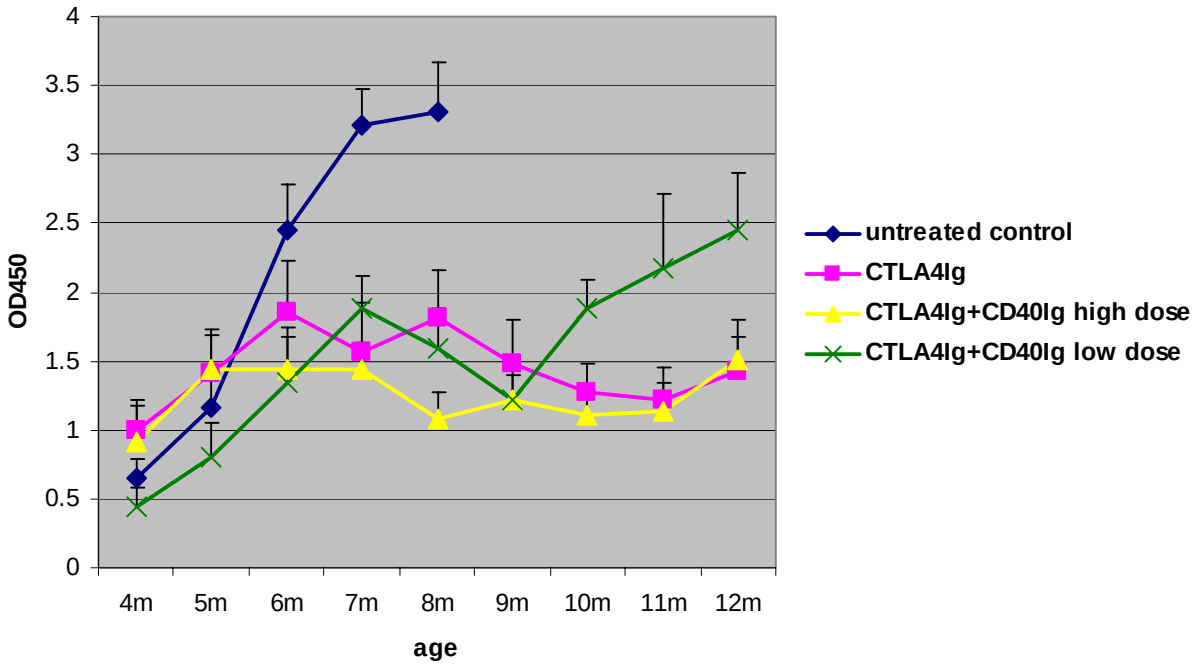

Figure 25 Serum anti-dsDNA IgG titer in the treatment groups

Mice were treated only after serum anti-dsDNA IgG titers highly increased but were negative for proteinuria. To monitor the autoantibody changes, mice were bled every month. Anti-dsDNA IgG titers were determined by ELISA and presented as mean±SE. Compared to the untreated control group, mice in the treatment groups had lower level of autoantibody titer at the age of 8 month (CTLA4Ig $p<0.05$, CTLA4Ig+CD40Ig low dose $p<0.01$, CTLA4Ig+CD40Ig high dose $p<0.001$).

4.3.2. The effects on the onset of proteinuria in the treatment groups

At the time of gene delivery, the mice were tested negative for proteinuria even though they had significantly elevated serum autoantibody titers. In the mCTLA4Ig single vector treatment group, the onset of proteinuria was delayed by 2 months and the development of proteinuria was significantly inhibited ($p<0.0001$). At the age of 12 months, only 50% of the mice in this group suffered from proteinuria (Fig.26). Mice in the two vector combination high dose treatment group also had delayed onset of proteinuria and suppressed proteinuria development ($p<0.0001$). By the age of 12 months, only 33.33% of these mice had developed proteinuria. However, for mice in the two vector combination low dose treatment group, the onset of proteinuria was as early as that of the control group. Also, the development of disease was faster than that observed in the other treatment groups, and by 12 months of age 80% of the mice in this group were proteinuria positive. Nevertheless, mice in the low dose treatment group still had inhibited proteinuria progression when compared to the control group ($p<0.001$). In addition, the efficiency of proteinuria inhibition was not statistically different among all treatment groups ($p>0.05$). To conclude, rAAV8-mediated costimulatory blockades delayed proteinuria onset and suppressed disease development in lupus mice. Again, a high dose was observed to yield better results than a low dose treatment in terms of proteinuria inhibition.

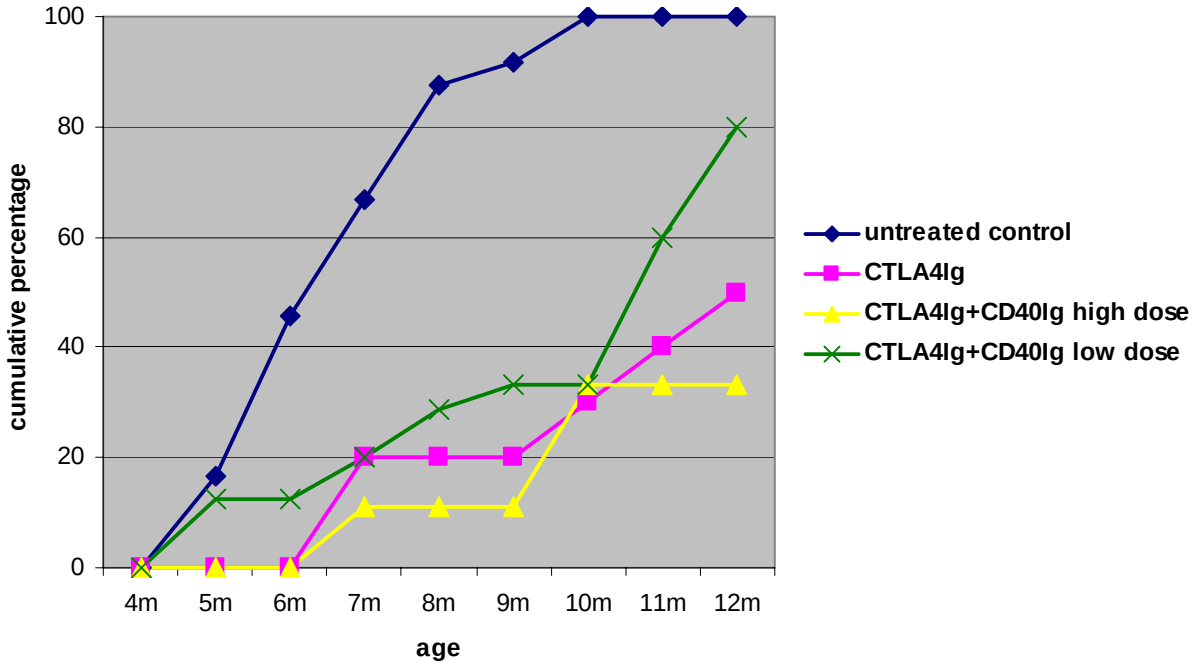

Figure 26 The onset of proteinuria in the treatment groups

Mice were treated only after serum anti-dsDNA IgG titers highly increased but were negative for proteinuria. Shown is the cumulative percentage of mice that developed severe proteinuria over time. Compared to the untreated control group, mice in all treatment groups had significantly delayed onset of proteinuria (CTLA4Ig $p<0.0001$, CTLA4Ig+CD40Ig high dose $p<0.0001$, CTLA4Ig+CD40Ig low dose $p<0.001$).

4.3.3. The effects on the survival rate in the treatment groups

After confirming that rAAV-mediated gene therapy suppressed lupus development, another aspect reflecting the therapeutic effects was the survival rate. In the mCTLA4Ig single vector treatment group, 80% of the mice were alive when all mice in the control group had died by the age of 10 months ($p<0.001$). Thereafter, the death toll increased slowly and 2 months later 60% of the mice still survived (Fig.27). In comparison, mice in the two vector combination high dose treatment group showed higher survival rates. By the age of 10 months, 88.89% of the mice were alive, and 2 months later the survival rate had dropped to 66.67%, which was still significantly higher than the control group ($p=0.0001$). Interestingly, mice in the two vector combination low dose treatment group had similar survival rates as that of the control group at the age of 5 months. However, as time went by, mice in the low dose treatment group showed significantly prolonged survival than that of the control group ($p<0.01$). By 12 months of age, 75% of the mice in this group were still alive. Moreover, when comparing mice in all the treatment groups, no significant difference in survival rates was observed ($p>0.05$). In conclusion, rAAV-mediated gene therapy extends the life span of lupus mice.

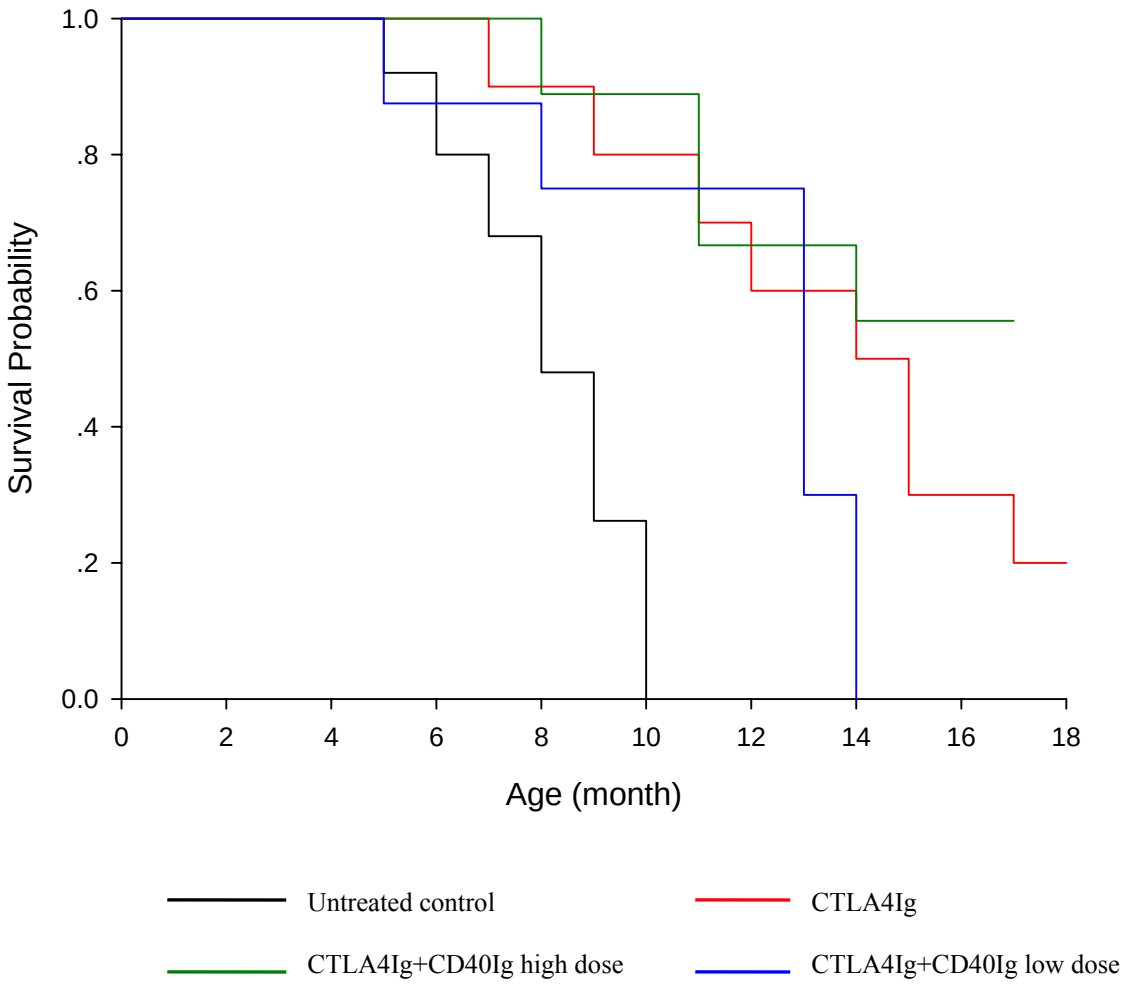

Figure 27 Survival rate in the treatment groups

Shown is the cumulative percentage of mice that remained alive at each time point. All mice were followed until death. Compared to the untreated control group, all mice in the treatment groups had significantly prolonged survival (CTLA4Ig $p<0.001$, CTLA4Ig+CD40Ig high dose $p=0.0001$, CTLA4Ig+CD40Ig low dose $p<0.01$).

4.4. Conclusions

In the previous chapter, it was demonstrated that rAAV8-mediated gene delivery was able to prevent lupus development in the murine model and the immunological mechanisms involved were studied. The ultimate goal of gene therapy is to treat disease. In this chapter, the therapeutic efficacy of rAAV8-mediated gene transfer was studied in lupus mice. Clinical studies have shown that SLE patients have autoantibodies in their circulation years before they experience any clinical symptoms (289). The murine model also presents a similar pattern of lupus development, providing a window time for early intervention. In this study all mice were injected with vectors only after they had developed high levels of autoantibody but remained symptom free. The rAAV8-mCTLA4Ig single vector was able to decrease serum autoantibody titers and keep them at low levels, delay the onset of proteinuria by 2 months and increase the survival rate. For the combination of rAAV-mCTLA4Ig and rAAV-mCD40Ig, which showed the maximum preventive effects in the previous chapter, the relationship between dose and the therapeutic efficiency was evaluated. A high dose of rAAV8 significantly decreased the autoantibody titers after gene delivery, delayed the onset of proteinuria by 2 months and prolonged survival. While a low dose did not show such a robust effect on autoantibody inhibition, it still suppressed proteinuria development and increased the survival rate. These data suggest a possible dose-effect relationship in gene therapy of lupus mice. In summary, rAAV8-mediated gene therapy was able to treat lupus, and the efficacy was evidenced to be dose-related.

4.5. Discussion

The purpose of gene therapy is to treat the disease after it is already established. In the case of lupus, the disease development is a long process that includes several stages. Gene therapy induces a new method for lupus treatment by achieving long-lasting transgene expression and prolonged therapeutic effects after gene delivery. So far, there are only a few studies done concerning gene therapy of lupus, and not a single one used AAV as the vector. In this study of rAAV8-mediated gene transfer in lupus prevention, we have described the transgene expression pattern of rAAV-mediated gene delivery and have come to better understand how it affects the host immune system. In this chapter, the feasibility of rAAV8-mediated gene therapy for treatment of lupus was tested. The topics to be discussed here include: 1) the best time for intervention, 2) whether there is a dose-effect relationship.

4.5.1. The best time for lupus treatment by rAAV8-mediated gene delivery

For lupus prevention, time is not a critical issue. Both early and late intervention can prevent lupus development. However, when it comes to lupus treatment, the time of intervention directly determines the outcome. In the early stage of lupus, autoreactive T and B cells proliferate and their clone size expands to some extent. As autoantibodies build up, they cause tissue damage of target organs directly or indirectly through the inflammation process initiated by IC deposits. When disease develops to an advanced stage, the dysfunction of target organs resulting from the tissue damage begins to appear. Patients start to have complaints and clinical symptoms. In lupus mice, the most prominent symptom is proteinuria. The kidney is an organ with great compensational capability. Only when there is a massive destruction of nephron will kidney function be affected, and the clinical presentations include elevated levels of BUN, proteinuria or hematuria. In this study's treatment group, mice were in the early stage of lupus development and there was no detectable renal dysfuntion. The costimulatory blockades established by

rAAV8-mediated gene delivery decreased serum autoantibody titer, indicating suppression of the activation and expansion of autoreactive T and B cells. Thereafter, the autoantibody titers were stable at low levels, which suggests that the development of autoimmunity was kept in check. Accordingly, the onset of proteinuria was delayed by 2 months, and mice in the treatment group experienced prolonged survival. These data clearly indicate that early intervention can slow down the development of lupus. I also evaluated the therapeutic efficiency of rAAV8-mCTLA4Ig on three mice with advanced lupus. One mouse died 6 days after gene delivery, another died at the age of 12 month, and the third one had to be sacrificed at the age of 13 month because of the animal facility shut down. The two long-surviving mice did not show significant decreases in autoantibody titers. Moreover, severe proteinuria persisted until death (data not shown). These results suggest that rAAV8-mediated costimulatory blockades are able to slow down the disease progress and improve survival even after mice have developed advanced lupus and have severe target organ damage. The explanation for the persistent high titers of autoantibody could be the already-established autoreactive T cell and B cell clones, as well as the self-renewal of memory T cells, whose activation is independent of the costimulatioin pathways (291). Once severe proteinuria is established, the damage of kidney tissue is irreversible. Even though costimulatory blockades were able to affect the upstream of the autoimmune process, there were other mechanisms involved in the downstream pathophysiological process, such as inflammation and complements, which were not affected by costimulation. In an effort to protect target organs, studies using anti-inflammation drugs have demonstrated retardation of GN development in lupus mice (292). Clinically, it is rational to treat disease flares with multiple drugs targeted at different molecules, or with different mechanisms of action.

4.5.2. The dose-effect relationship in lupus treatment

In lupus prevention, the two vector combination achieved maximum preventive effects, which was partly due to the synergistic effect between the two costimulatory blockades. In this chapter, two groups were set up with 10 fold differences in vector particle number in order to study the dose-effect relationship. The results showed that a high dose significantly decreased serum anti-dsDNA IgG titers 2 months after gene delivery and kept them at low levels for at least 6 months. While in the low dose treatment group, serum autoantibody titers decreased for only 6 weeks, then slowly rose up to a high level. Therefore, low dose treatment was less efficient than high dose in suppressing autoantibody production. Consistent with the change in autoantibody titers, the onset of proteinuria occurred 2 months earlier in the low dose group. When 80% of the mice in the low dose group suffered from proteinuria at the age of 12 months, only 33.33% of the mice in the high dose group were tested positive. However, when considering the survival rate at the age of 12 months, there was not much difference between these two groups. This result could be because of the smaller sample size of the low dose group, which had only 4 mice alive by that age. Another evidence of a dose-effect relationship came from the rAAV8-mCTLA4Ig single vector prevention group. One mouse had high levels of mCTLA4Ig (188μg/ml) in the circulation by 5 months of age, and by 6 months of age, levels had dramatically dropped to 27.1μg/ml. This mouse developed proteinuria by the age of 7 months and died at the age of 8 months. The other three long-term surviving mice had high levels of serum mCTLA4Ig for at least 9 months. Another study of Ad-mCTLA4Ig on lupus prevention in B/W mice also indicates a dose-effect relationship, where the low dose fails to prevent autantibody production and to inhibit proteinuria development (293). For clinical applications, the optimal dose is the one with sufficient efficacy, but minimal side effects.

5. FUTURE STUDIES

5.1. Regulation of gene therapy

For a chronic disease like lupus, long-term intervention is necessary and gene therapy offers an advantageous solution by achieving long-lasting and stable transgene expression and convenient delivery. However, on the other hand, the disease status is not constant and it goes through flare ups and remissions, which can last for years. Even though gene therapy has been shown here to be an efficient disease treatment for lupus, continuous transgene expression will inevitably bring side effects during the remission periods. Therefore, these transgenes need to be expressed at the right site, at the right time and at the appropriate level, which makes regulation of transgene expression a practical consideration for the clinical application of gene therapy. So far, several regulatory strategies have been adopted for control over the delivery and expression of transgenes, including targeting delivery and transcriptional regulation.

5.1.1. Targeting delivery and adoptive cellular gene therapy

There are many hurdles that gene therapy must surmount before it becomes an effective and safe tool for the disease treatment. One major hurdle is improving the specificity and efficiency of gene delivery, which is determined by the interaction between the vector and the cell surface. The wide host range of AAV appears to be disadvantageous for systemic gene therapy. Many methods have been invented to achieve selective transduction by using genetic modification of viral capsids, pseudotyped viruses, and antibodies as mediators for restrict viral infection to specific cell types. Bispecific antibodies recognize both the AAV2 viral capsid and a specific surface receptor of the target cells (294). Another way to change the AAV tropism is by manipulating its capsid. AAV display technology, in which random peptide libraries displayed in the viral capsid, enables the selection of AAV vectors for targeted gene delivery (295, 296). A

most recent attempt is to generate rAAV with a novel phenotype and tropism by transcapsidation of different AAV capsid mixed at different ratios (297). In SLE, rAAV need to be modified to target at autoreactive T and B cells in order to deliver therapeutic transgene to induce tolerance in these cells.

When the above strategies can not guarantee sufficient specificity for transduction, another way to reduce the chance of infecting non-target cells is adoptive cellular gene therapy. Autoimmune disorders represent inappropriate immune responses directed at self-tissue. $CD4^+$ T cells and dendritic cells (DCs) are important mediators in the pathogenesis of autoimmune disease, and thus, are ideal candidates for adoptive cellular gene therapy, through an *ex vivo* approach to therapeutic gene transfer. In animal models of multiple sclerosis, arthritis and diabetes, studies have demonstrated that transduced T cells and DCs rapidly and preferentially home to the sites of inflammation and deliver the immunoregulatory proteins to the inflamed lesions (298, 299). It is also rational to transduce hematopoietic stem cells (HSC) *in vitro* by rAAV and transfuse them back to the host. The downstream B cells and T cells derived from the HSC will carry the transgene and be directly affected by the transgene product. This strategy has been proven effective in the retrovirus-mediated gene therapy of diabetes mice (300).

5.1.2. Transcriptional regulation

Transcriptional regulation can generally restrict the expression of the therapeutic transgenes to the appropriate cells, or in certain conditions, through tissue specific promoters, inducible promoters, or switch-on/off systems. Construction of viral vectors harboring cell-specific promoters has been applied for controlling gene regulation. A liver-specific promoter, such as CB promoter, is widely used for hepatocyte-specific transgene expression. In the case of lupus, B lymphocyte-specific promoter and enhancer of immunoglobulin heavy chain may be more

applicable. It has been found to induce transgene expression only in B cells and not HeLa cells (301). For the inducible promoter, the most commonly used are stress-gene promoters. For example, irradiation-responsive promoter Egr-1 is used for tumor gene therapy following radiation treatment (302). The HSP70 promoter from the heat shock protein family is induced by a variety of environmental stresses, namely heat, irradiation, photobeam irradiation, hypoxia, acidosis, hypoglycemia, and osmotic changes (303). Because environmental factors play an important role in lupus initiation and activation, HSP70 promoter may be a good candidate for transgene regulation in the gene therapy of lupus. Moreover, the Tet-controlled transcription system is comprised of Tet-Off and Tet-On transcriptional regulation, derived from the Escherichia coli Tet-resistance operon. It has been used to suppress and induce transgene expression and has achieved tight positive regulation in Ad-mediated gene therapy (304). The same strategy in a rAAV-mediated gene delivery system could be adopted to control transgene expression at the time of lupus flare ups.

5.2. Comprehensive treatment

Since lupus is a disease with multiple steps of development, it is necessary to design treatments working through different mechanisms. In this study, soluble IL-10R does not show any protective effect on lupus, which is explained by the low secretion level of the transgene product. However, it remains an interest of further study, based on the mounting evidence of successful lupus prevention by anti-IL-10 mAb. Moreover, other cytokine inhibitors, such as anti-IL-6 and anti-TNFα, may also be applied to lupus treatment for their anti-inflammatory effects (305). A comprehensive treatment strategy with multiple targets is more promising than the treatment with single agent. For example, for lupus nephritis, costimulatory blockades fail to reverse the renal diseae and proteinuria persists. However, the combination of CTLA4Ig and

cyclophosphamide (CTX) reverses proteinuria and prolongs survival (165). On the other hand, it decreases the CTX dosage, resulting in less adverse effects. Therefore, in the application of gene therapy in lupus, rAAV delivering anti-inflammatory cytokines and the costimulatory blockades could be expected to achieve maximal therapeutic results.

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