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Stringent control of NFATc1 nuclear occupancy is critical for maintaining balanced immune response

Research Article

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Summary

Many immune and inflammatory diseases still lack a clear mechanistic explanation. NFATc transcription factors are involved in immune homeostasis and response. NFATc1 is rapidly imported into the nucleus upon activation of lymphocytes that leads to its stimulation of a battery of cytokines responsible for immune response and is rapidly removed from the nucleus upon termination of the signaling. We have previously established a tetracycline-regulated transgenic mouse model with a subtle increased nuclear NFATc1 expression. The level of nuclear NFATc1 expression was only 1/7th of the total NFATc1 molecules of a wild type T cell. Here we show that this subtle increase of NFATc1 nuclear occupancy caused a severe disease with multi-organ failure characterized with infiltration of immune cells, elevated auto antibodies, leading to early animal death. Suppression of the transgene expression by doxycycline suppressed and reversed the disease. These results indicate that stringent control of NFATc1 nuclear occupancy is critical for maintaining balanced immune response and may have important clinical implications.

I. Introduction

The mechanism of many immune and inflammatory diseases such as lupus, rheumatoid arthritis, polymyalgia rheumatica, multiple sclerosis, fibromyalgia, inflammatory bowel disease and others is still not completely understood (Janeway et al, 2000; Davidson et al, 2001). Steroid traditionally constitutes the mainstay of therapy of these diseases. More recently strategies such as depletion of TNFα and depleting B cells with anti-CD20 antibody have improved the treatment outcome for many diseases (Pisetsky et al, 2000; Kneitz et al, 2002). However, a portion of patients would only respond minimally or would not respond at all (Kneitz et al, 2002; Pisetsky et al, 2000). In addition, durable response often requires continuous therapy (Pisetsky et al, 2000; Kneitz et al, 2002).

NFATc proteins are involved in the functional regulation of T and B cells as well as cytokine production (Rao et al, 1997; Ranger et al, 1998; Peng et al, 2001; de Gorter et al, 2007). NFATc1 gene is a widely expressed transcription factor and plays critical roles in many biological systems such as cardiac valve morphogenesis, axonal outgrowth in nervous system, in addition to its role in immune system (Crabtree et al, 2002; Graef et al, 2003; Chang et al, 2004). NFATc1 protein contains a number of phosphorylation sites that are normally phosphorylated when it is inactivated and compartmentalized in the cytoplasm (Flanagan et al, 1991; Timmerman et al, 1996; Beals et al, 1997a,b). Activation of T cells leads to its dephosphorylation and rapid shuffling into the nucleus (Flanagan et al, 1991; Timmerman et al, 1996; Beals et al, 1997a,b). NFATc1 has been shown to mediate immune response both in B and T cells (Flanagan et al, 1991; Timmerman et al, 1996; Beals et al, 1997a,b; de Gorter et al, 2007). Activation of lymphocytes causes calcium influx leading to activation of calcineurin, a serine/threonine phosphotase that subsequently dephosphorylates NFATc proteins and leads to their nuclear import (Flanagan et al, 1991; Timmerman et al, 1996; Beals et al, 1997a,b). Activated nuclear NFATc1 protein stimulates production of a battery of cytokines including interleukin-2, interferon, TNFα and many others (Rao et al, 1997). Introduction of calcineurin inhibitors FK506 and

cyclosporine A have revolutionized the treatment for patients with organ transplant (Kunz et al, 1993). Blockade of calcineurin activation by cyclosporine A and FK506 inhibits calcineurin's ability to dephosphorylate and activate NFATc proteins, hence causes inhibition of production of many important cytokines (Clipstone et al, 1994; Kiani et al, 2000).

We have previously established a tetracycline-regulated transgenic mouse model with expression at sub-physiologic level of a nuclear NFATc1 variant (NFATc1^{nuc}) that lacks ability to exit the nucleus (Pan et al, 2007). The level of expression of this nuclear NFATc1 variant was only 1/7th of the total NFATc1 molecules of a wild type T cell yet caused a destabilized positive feedback loop in its own transcription leading to T cell activation independent of CD28 costimuation, partial resistance to cyclosporine A inhibition of T cell proliferation as well as markedly enhanced production of Th1/Th2 cytokines and activation antigens both spontaneously and when activated (Pan et al, 2007). T cell activation of the NFATc1^{nuc} mice were several magnitudes higher than normal T cells in resting state and in activated state and produced increased IgG2α. We have also previously shown using this transgenic mouse model that NFATc1 regulates bone homeostasis (Winslow et al, 2006).

In this manuscript, we show that the subtle increase of nuclear NFATc1 occupancy caused a severe mouse phenotype with multi-organ failure involving lungs, liver, kidneys, muscle, joints characterized with dense infiltration of immune cells including lymphocytes, macrophages and granulocytes leading to early animal death. Serum auto antibodies including anti-ANA, anti-dsDNA, circulating immune complex (CIC) and anti-RNP were elevated and immune complex deposits were detected in the kidneys of the mutant mouse. Treatment of the mutant mouse with doxycycline to suppress the expression of NFATc1^{nuc} prevents the death and reverses the disease (Pan et al, 2007). These results indicate that stringent control of NFATc1 nuclear occupancy is critical for maintaining balanced immune response and may have important clinical implications.

II. Methods

A. Generation of tetracycline-regulated transgemic mouse that contains a constitutively nuclear NFATc1 (NFATc1^{nuc}) and doxycycline treatment of the transgenic mouse

This was described previously (Felsher and Bishop 1999; Winslow et al, 2006; Pan et al, 2007). Briefly, NFATc1^{nuc} was made by site-directed mutagenesis and the DNA was inserted in-frame into pS vector N-terminally to a HA tag. The construct was digested with Bam HI and Acc 65I and inserted into pUD10-3 downstream of Tet-O promoter. The transgene was digested with Spe1, purified and pro-nucleus injection was performed using B6CBAF1/J (Jackson) mice with standard protocol. Tet-O-C1^{wt} mice were generated in similar method. 83 tTA mice (FVB/N) were previously described (Felsher and Bishop 1999). The treatment of NFATc1^{nuc} mouse with doxycycline was performed by feeding the mouse with drinking water containing doxycycline 200 ug/ml that's changed once a week.

B. Western Blot

Cell lysates of lymph nodes, spleen and thymus were prepared, separated on a SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and blotted with anti-HA (16B12, Berkeley antibody company) or anti-actin antibodies (Sigma).

B. Histologic analysis

Tissues and organs were fixed for 24 hours with 10% formalin, dehydrated, embedded in wax, sectioned and processed for H + E staining according to standard protocols.

C. Immunofluorescent staining

Frozen sections of wild type or mutant kidneys were prepared, blocked with buffer containing BSA, NaCl (200mM) and 0.1%triton, stained with FITC-conjugated goat anti-mouse IgG antibody (PharMingen).

D. Assay of auto antibodies

50ul of serum from wild type or mutant mice was used to assay for auto antibodies using ELISA method according to instructions provided by the manufacturer (Alpha Diagnostic International).

III. Results

A. Early death of the NFATc1^{nuc} mice were preventable with suppression of the transgene

We have previously established a tetracycline-regulated transgenic mice model NFATc1^{nuc} that expressed a very low sub-physiologic level of NFATc1^{nuc} (1/7 of the total NFATc1 level of wild type T cells) detectable in T cells and was associated with much increased cytokine production and T cell activation that was independent of CD28 costimulation and partially resistant to cyclosporine inhibition (Pan et al, 2007). No abnormality in T cell development was identified in the NFATc1^{nuc} mutant animals (Pan et al, 2007). We have also shown that NFATc1^{nuc} could be detected in osteoblasts and regulates homeostasis of osteoblasts and bone mass formation (Winslow et al, 2006). NFATc1^{nuc} mice were born at normal Mendelian ratios and were normal at birth but later began showing signs of illness that could be recognized as rough fur, retarded weight gain (cachexia), decreased mobility, joint swelling and joint deformation (**Figure 1A, right**). This gross phenotype appeared coincidence with the expression of NFATc1^{nuc} in the spleen and lymph nodes. As shown in **Figure 1B**, the expression of NFATc1^{nuc} in the peripheral lymph nodes and spleen was only detected when the mutant mice became apparently ill, while it was detectable in the thymus in latent as well as in late stage (**Figure 1A, right**) NFATc1^{nuc} mice. Latent stage was defined as the mutant mice appearing normal or mildly ill by gross observation, while late stage was defined as the mutant mice appearing apparently sick by gross observation. The time to onset of the disease varied from a few days after birth to as long as 6 months with male and female mice being equally affected. The early onset (within 6 weeks of age) mutants normally progressed to death within 2 to 3 weeks while late onset mutants progressed more slowly (after 6 weeks). In contrast, no wild type, Tet-O-NFATc1^{nuc} or mice expressing wild type NFATc1 (NFATc1^{wt}/tTA) developed

disease during this time (**Figure 1C**). By week 5 to 6, approximately half of all the mutant mice have died (**Figure 1C**). By age six months, ninety five percent of all mutant mice have died of the disease (data not shown).

To examine if the expression of NFATc1^{nuc} was responsible for the disease, we treated the mutant mice with docycyline which suppresses the expression of the transgene (Pan et al, 2007). As shown in **Figure 1D**, when treated with doxycycline during the perinatal or disease latent period, no NFATc1^{nuc} mice developed the illness. However, only 70% of the adult mutant mice that had developed a full-blown onset of the disease during p19-35 had the disease reversed with docycycline suppression of the NFATc1^{nuc} expression, while 30% of these mutant mice still progressed to death. This suggests that 30% of the adult mutant mice had developed a disease that's no longer dependent on the expression of NFATc1^{nuc} protein, or the disease had caused multi-organ failure that's no longer reversible. Treatment of the mutant mice with doxycycline promoted weight gain consistent with their recovery from the disease, indicating that cachexia was associated with the cytokine production (Pan et al, 2007) and the disease (**Figure 1E** green). The mutant mice not treated with doxycycline continued to show retarded weight gain until death (**Figure 1E** orange). Histological examination of the doxycycline-treated mutant mice showed normal organs and tissues (**Figure 2C and 2F**) comparable to the wild type (**Figure 2A and 2D**). However, three to six months after NFATc1^{nuc} expression is reactivated (by discontinuing doxycycline treatment), these once cured NFATc1^{nuc} mice developed the disease again and died (data not shown).

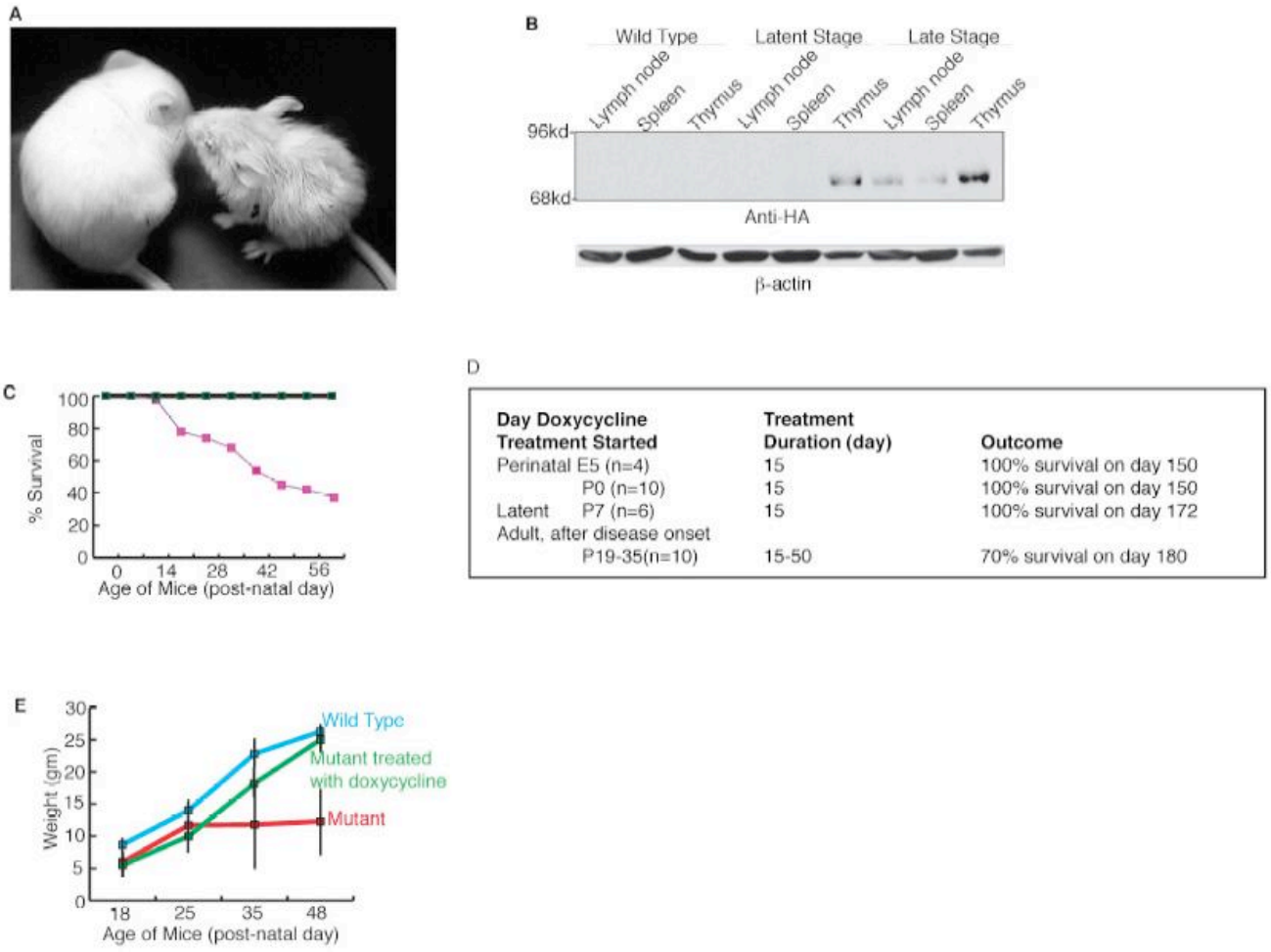

Figure 1. Early death of the NFATc1^{nuc} mutant mice was preventable with doxycycline treatment. **A**. Gross appearance of a day 35 wild (left) and mutant mouse (right). **B**. Expression of NFATc1^{nuc} in periphery of wild type mice, latent, late stage NFATc1^{nuc} mice performed with anti-HA antibody western blot. Tissues, molecular weight and actin control were indicated. The data is representative of three experiments with similar results. **C**. Survival curves of wild type (black, n=35) and NFATc1^{nuc} mice (pink, n=66). **D**. Treatment of mutant mice with doxycycline during perinatal and disease latent period. Time period that doxycycline was treated and survival rate were indicated. Day 0 is designated as the birth date (P0). E5 represents embryonic day 5. n, number of mice treated. Treatment duration and outcome of the treated mice are shown. **E**. Weight measurement of the mutant mice treated or not treated with doxycycline. NFATc1^{nuc} mice untreated (red, n=10); NFATc1^{nuc} treated with doxycycline (green, n=4); wild type mice (blue, n=12); Doxycycline treatment was started on day 19 and continued for 15 days.

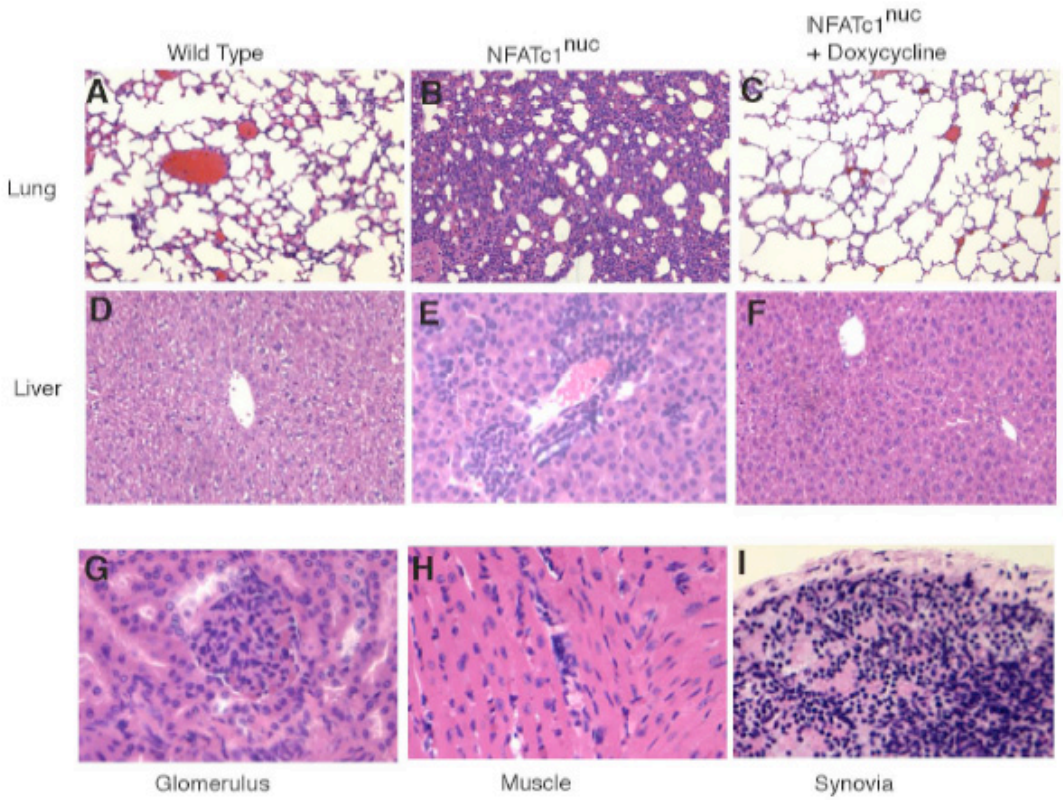

Figure 2. H&E examination of organs and tissues of the NFATc1^{nuc} mice. A. H&E of wt lung. **B**. H&E of the mutant lung. **C**. H&E of the lung from the mutant treated with doxycycline. **D**. H&E of wt liver. E, H&E of the mutant liver. F. H&E of the liver from the mutant treated with doxycycline. G. H&E of the mutant kidney. H. H&E of the mutant muscle. I. H&E of the mutant synovium. Note the dense cellular infiltrates of the mutant organs and tissues. Mice of postnatal age 3-6 weeks were used.

B. Dense immune cell infiltration of multiple organs and tissues in NFATc1^{nuc} mice

We examined all organs of the mutant mice compared to the wild type mice and found abnormalities in several organs including skin, bones, lungs, liver, kidneys, muscle, joints and others (Winslow et al, 2006; Pan et al, 2007). We have reported that NFATc1 regulates bone mass previously (Winslow et al, 2006). Histological examination of the mutant mice skin showed non-specific inflammation in the dermis and in the hair follicles consistent with a response to increased cytokine production (data not shown). However, in contrast to the lungs of the wild type mouse (**Figure 2A**), histological examination of the NFATc1^{nuc} mice with H&E staining revealed dense infiltrates in the lungs with thickening of interstitial spaces and focal destruction of the epithelium (**Figure 2B**). Compared to the wild type mouse liver (**Figure 2D**), diffuse as well as focal cellular infiltration surrounding vessels (focal vasculitis) and billiary ducts were consistently demonstrated in the mutant liver coupled with necrosis of the liver tissue (**Figure 2E**). The liver infiltrate was found by flow cytometry to be a mixture of approximately 50% CD4$^+$ or CD8$^+$ T cells, 30% macrophages (Mac-1$^+$) and 20% granulocytes (Gr-1$^+$) (data not shown). We have also analyzed the synovial fluid of the mutant mice and found similar cellular infiltration (data not shown). In the kidneys there was marked hypercellularity and obliteration of the subcapsular space in the glomeruli (**Figure 2G**). Occasional wire loop formation and hyaline scars or lupus bodies were evident in the mesangium. The mutant mice progressively developed muscle weakness correlated with the presence of severe cellular infiltrates in the muscle indicating presence of myositis (**Figure 2H**). In addition, the mutant mice showed redness, swelling and deformation of multiple joints including knees, hips, as well as digital joints and histological examination of the joints found dense cellular infiltration of immune cells present in the synovial space (**Figure 2I**) and fluid (data not shown) indicating presence of synovitis. This disease of multi-organ damage is clearly responsible for the death of the mutant mice. In addition, expression of NFATc1^{nuc} is responsible for this disease because suppression of its expression prevents and reverses the disease (**Figure 2C and 2F; Figure 1D and 1E**).

C. Detection of immune deposits in the kidneys and elevated serum auto antibodies in the NFATc1^{nuc} mice

To investigate if autoimmune response might be present in the mutant mice, we studied the immune deposits of the kidneys in wild type and the mutant mice as well as serum level of four commonly elevated auto antibodies seen in autoimmune disease. We found that immune deposits could be detected in the mutant glomeruli (**Figure 3A, B**). This is consistent with the findings with the H&E examination of the kidneys (**Figure 2G**). We also examined the serum autoantibody titers of the wild and mutant mice. As shown in **Figure 3C-F**, the serum titers of anti-nuclear antibody, anti-RNP, anti-dsDNA and circulating immune complexes were elevated several folds in the mutant mice compared to the

wild type mice. The higher titer was found in the mutant animals with more severe illness. These results are consistent with our previous finding that IgG2α level was elevated in the serum of the mutant mice (Pan et al, 2007). While in humans these auto antibodies are characteristic of both lupus erythematous and mixed collagen tissue disorder. These increased autoantibody titers in the mutant animals could reflect an autoimmune-like but non-specific inflammatory response, because the mutant mice still died in the absence of T cells when crossed to the TCRα and Rag1-deficient background. The mechanism of the disease is not clear and requires further investigation.

IV. Discussion

We show here that expression of an NFATc1 variant NFATc1^{nuc} in a very small sub-physiologic level in a transgenic mouse model caused a severe disease characterized with multi-organ failure leading to early death of the animals. The skin, lungs, liver and kidneys, as well as muscle and synovium were all involved with a full-blown immune response characterized with dense cellular infiltrates of T lymphocytes, macrophages and granulocytes (**Figure 2**), coincidence with the expression of the transgene in the peripheral lymphoid organs (**Figure 1B**). Majority of these mutant mice died within days or weeks after birth with cachexia, muscle weakness, arthritis and with elevation of several auto antibodies including anti-ANA, Anti-dsDNA, anti-RNP and circulating immune complex in the serum (**Figure 3C-F**). The elevated autoantibody titers could be a result of a non-specific autoimmune-like inflammatory response of the mutant mice, rather than a classic autoimmune disease, because the mutant mice still died in the absence of T cells when crossed to a TCR- and Rag1- deficient background (Pan et al, 2007). We still do not understand the specific cell type that's responsible for the disease because no expression of NFATc1^{nuc} has been detectable in cells other than T lymphocytes and osteoblasts (Pan et al, 2007; Winslow et al, 2006). We speculate that NFATc1^{nuc} is expressed in a very subtle level undetectable with our quantitative PCR techniques and this subtle expression well below its physiological level could cause a severe disease characterized with full-blown immune response in multiple organs and tissues.

When treated with doxycycline to suppress the expression of NFATc1^{nuc}, the disease could be prevented perinatally or in the latent period. However, only 70% of the adult mutant mice with severe illness were cured with doxycycline (**Figure 1C**), indicating that at the time of the treatment, these mice might have developed to a disease stage that was no longer dependent on NFATc1^{nuc} gene expression, or the mice had developed end-stage multi-organ failure that's not clinically reversible.

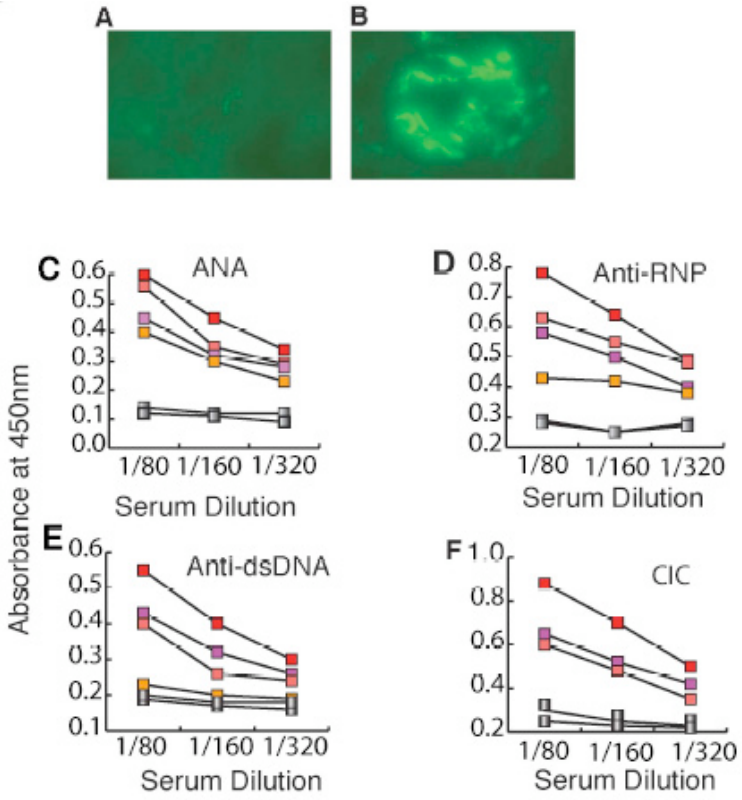

Figure 3. Immune complex deposit of glomerulus and serum auto antibodies in the mutant mice. **A**. Anti-mouse IgG Immunofluorescent staining of wild type kidney. **B**. Anti-mouse IgG Immunofluorescent staining of the mutant kidney. **C**. Serum titer of anti-nuclear antibody (ANA); D. Anti-RNP autoantibody titers. **E**. Anti-dsDNA (anti-double-stranded DNA) titers. **F**, Serum circulating immune complex (CIC) titers. Two wild type controls are shown in gray, NFATc1^{nuc} mice are shown in color. Serum was diluted 1 to 80, 160 and 320. Mice of postnatal age 3-6 weeks were used.

This is reminiscent of many human immune and inflammatory disorders (Janeway et al, 2000; Davidson et al, 2001). This also appears similar to the transgenic mice model expressing a c-Myc gene that caused T cell lymphoma (Felsher and Bishop 1999). The suppression of c-Myc transgene by doxycycline only reversed a portion of the mice with lymphoma while some continued to progress with lymphoma and died (Felsher and Bishop 1999). Reactivation of the NFATc1^{nuc} expression by discontinuing doxycycline caused disease again after the mutant mice is cured of the disease. This is similar to the c-Myc transgenic mice model of cancer that relapses again from dormancy following the reactivation of the transgene (Felsher and Bishop 1999; Shachav et al, 2004).

A subtle increase of nuclear NFATc1 expression caused a full-blown disease with multi-organ failure highlights the critical significance of the stringent control of NFATc1 nuclear occupancy. This is clinically relevant because many autoimmune and inflammatory diseases such as lupus, psoriasis, polymyalgia rheumatica, multiple sclerosis, inflammatory bowel disease as well as fibromyalgia and others still lack a clear mechanistic explanation (Janeway et al, 2000; Davidson et al, 2001). NFATc transcription factors are involved in lymphoid homeostasis and development (Rao et al, 1997; Ranger et al, 1998; Peng et al, 2001; David et al, 2007) and it is possible that a subtle increase of NFATc1 nuclear occupancy might be associated with these diseases. Subtle increase of nuclear NFATc1 destabilizes a positive feedback loop that could easily play a role in human immune and inflammatory diseases. One clinical example is the drug procainamide that induces lupus and disrupts Na^+ channel activity leading to secondary changes in intracellular Ca^{2+}level that activates calcineurin and NFATc proteins (Kretz-Rommel and Rubin 2000).

V. Conclusion

We have shown that a subtle increase of nuclear NFATc1 expression can cause a severe disease of multi-organ failure with immune response characterized by extensive immune cell infiltration. These results indicate that stringent control of NFATc1 nuclear occupancy is critical for maintaining balanced immune response and may have important clinical implications.

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