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Altered Thymic Selection by Over-Expressing Cellular FLICE Inhibitory Protein in T Cells Causes Lupus-like Syndrome in BALB/c but not C57BL/6 Strain

Guilin Qiao^{1,2}, Zhenping Li¹, Andrew W. Minto¹, Jinru Shia⁴, Lifen Yang^{1,2}, Lihua Bao¹, Jurg Tschopp⁵, Jian-Xin Gao⁶, Jimin Wang⁷, Richard J. Quigg¹, and Jian Zhang^{1,2,3}

¹ Section of Nephrology, Department of Medicine, The University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637, USA ² Committee on Immunology, The University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637, USA ³ Committee on Molecular Medicine, The University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637, USA ⁴ Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA ⁵ Department of Biochemistry, University of Lausanne, Chemin des Boveresses 155, Epalinges CH-1066, Switzerland ⁶ Department of Pathology, The Ohio State University, 1645 Neil Ave., Columbus, OH 43210, USA ⁷ BioSource Cytokines & Signaling, Invitrogen Corporation, 542 Flynn Road, Camarillo, CA, USA

Abstract

Cellular FLICE inhibitory protein (c-FLIP) is an endogenous inhibitor of the caspase-8 pro-apoptotic signaling pathway downstream of death receptors. Recent evidence indicates that the long form of c-FLIP (c-FLIP_L) is required for proliferation and effector T cell development. However, the role of c-FLIP_L in triggering autoimmunity has not been carefully investigated. We now report that c-FLIP_L transgenic (Tg) mice develop splenomegaly, lymphadenopathy, multi-organ infiltration, high titers of autoantibodies, and proliferative glomerulonephritis with immune complex deposition in a strain-dependent fashion. The development of autoimmunity requires CD4⁺ T cells and may result from impaired thymic selection. At the molecular level, c-FLIP_L over-expression inhibits the ZAP-70 activation, thus impairing the signaling pathway derived from ZAP-70 required for thymic selection. Therefore, we have identified c-FLIP_L as a susceptibility factor under the influence of epistatic modifiers for the development of autoimmunity.

Introduction

One of the fundamental properties of the immune system is to distinguish self from non-self which is central to its ability to protect against pathogens and, at the same time, maintain nonresponsiveness to self. This property is established during lymphocyte development

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Correspondence should be addressed to: Jian Zhang, M.D., Section of Nephrology, Department of Medicine, The University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637, USA, jzhang@medicine.bsd.uchicago.edu.

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within the generative lymphoid organs (central tolerance) or in the secondary lymphoid organs (peripheral tolerance) 1,2. Much attention has been devoted to studies of understanding the cellular and molecular basis of tolerance mechanisms, as well as the situations under which these mechanisms break down leading to autoimmune disease. The understanding of these complex biological processes at the molecular levels has been facilitated by various transgenic and knockout mouse strains as well as strains with spontaneous mutations that develop autoimmune phenotype.

Signaling derived from the TCR is required for thymocyte development and thymic selection. The selection of the T-cell repertoire which is essential for establishing self/non-self discrimination of immune recognition occurs in the thymus, and is based on the binding strength (affinity) of the TCR for peptide–MHC complexes that are presented by epithelial and bone-marrow-derived antigen-presenting cells. Thymocytes that bind self peptide–MHC complexes too strongly undergo apoptosis and fail to enter the mature T-cell pool (a process known as negative selection). The thymocytes that express low-affinity TCRs which are specific for self peptide–MHC complexes are the only ones to survive (in a process known as positive selection) and migrate out of the thymus 1,2. Recent studies indicated that a number of TCR signaling molecules are involved in both positive and negative selection in the thymus 3. A defect in thymic selection results in autoimmunity 2,3.

Cellular FLICE inhibitory protein (c-FLIP) is an endogenous inhibitor of the Fas-associated death domain protein (FADD)-caspase-8 pro-apoptotic signaling pathway downstream of death receptors 4. The 55-kDa-long-form of c-FLIP (c-FLIP_L). is structurally similar to caspase-8 and caspase-10, since it contains two death effector domains (DEDs) and a caspase-like domain. However, this caspase-like domain lacks residues which are important for the catalytic activity of caspase-8 5. Thus, c-FLIP can inhibit death receptor-mediated caspase-8 activation 4. Intriguingly, when expressed at a low concentration, c-FLIP_L can act as an activator of pro-caspase-8 possibly by heterodimerizing with caspase-8 6. It appears that the expression levels of c-FLIP_L may be crucial for its biological functions. Although mice deficient for both c-FLIP_L and c-FLIP_S in T cells exhibit severe defects in thymocyte development 7,8, c-FLIP_L deficiency in T cells does not disturb thymocyte development. Rather, c-FLIP_L may mediate T cell activation and proliferation via an unidentified mechanism 7. c-FLIP_L may therefore serve as a modulator between apoptotic and proliferative responses after T cell receptor (TCR) stimulation 9. However, it has not been shown that over-expression of c-FLIP_L in T cell lineage triggers autoimmunity.

In this report, we describe that mice over-expressing c-FLIP_L in T cells in the BALB/c background spontaneously develop an autoimmune lymphoproliferative syndrome exhibiting features of systemic lupus erythematosus (SLE) in humans. In contrast, c-FLIP_L transgenic (Tg) mice in the C57BL/6 (B6) background maintain tolerance to nuclear antigens, and are resistant to the development of lupus-like disease. The development of lupus-like disease in c-FLIP_L Tg mice is due to altered thymic selection in the BALB/c background by interfering with the signaling pathway derived from the ζ chain-associated protein tyrosine kinase of 70 kDa (ZAP-70).

Results

c-FLIP_L transgene product is specifically expressed in T cells but not B cells and dendritic cells

c-FLIP_L was expressed transgenically in the T cell compartment as previously reported 6. Briefly, FLAG-tagged mouse FLIP_L cDNA was inserted into a target vector containing the β -globin promoter and a downstream human CD2 locus enhancer element. To confirm that c-FLIP_L transgene product is not expressed in other cell types such as B cells and dendritic cells (DCs), c-FLIP_L transgene expression in thymocytes, splenic T cells, B cells, and DCs of BALB/c and B6 backgrounds was detected using anti-FLAG Ab. As shown in Supplementary Figure 1, c-FLIP_L transgene product was detected only in thymocytes and T cells but not in B cells and DCs from BALB/c and B6 c-FLIP_L Tg mice but not their non-Tg littermate controls (NLC), and was comparable between BALB/c and B6 strains. Consistent with the previous report 6, in addition to the p55 kDa form of c-FLIP_L, an additional 43 kDa cleavage product of transgenic c-FLIP_L was found in both thymocytes and splenic T cells from BALB/c and B6 c-FLIP_L Tg mice (Supplementary Figure S1).

BALB/c but not B6 c-FLIP_L Tg mice spontaneously develop lupus-like disease

c-FLIP_L Tg mice in the B6 background (B6 c-FLIP_L Tg mice) do not develop any autoimmune disease 6. In keeping with this data, we did not observe any sign of autoimmunity in B6 c-FLIP_L Tg mice until 10 months of age (data not shown). The absence of auto-antibodies (auto-Abs) or spontaneous autoimmune disease suggested that additional genetic or environmental factors might be necessary to trigger autoimmunity in these susceptible mice. To investigate the contribution of genetic background and modifier loci to the development of autoimmunity, we have backcrossed these mice to BALB/c background for fourteen generations. While new-born BALB/c c-FLIP_L Tg mice appeared normal, these mice were smaller in size than NLC when they grew up to 3–5 wk of age (Figure 1a). Almost all BALB/c c-FLIP_L Tg mice developed a generalized lymphadenopathy and splenomegaly starting from 3 wks of age and being evident by 6–10 wks of age, of which 20–30% BALB/c c-FLIP_L Tg mice exhibited diarrhea (Figure 1a). In contrast, there was an age-dependent severe thymic atrophy in these mice (Figure 1b). The spleens from 7-wk-old BALB/c c-FLIP_L Tg mice showed that there was a significant increase in lymphoid cells, which formed aggregates in the white pulp and spread into the red pulp, filling in the cords and sinuses. PNA staining revealed spontaneous germinal center (GC) formation in BALB/c c-FLIP_L Tg mice but not in NLC (Figure 1b).

Histological analysis showed evident lymphocytic infiltration in portal tracts of the liver, heart valves, peri-bronchiolar spaces of the lung, and the mucosa and submucosa of the stomach and the large and small intestines. Salivary glands from BALB/c c-FLIP_L Tg mice also displayed dense lymphocyte infiltration (Figure 1c). Kidney sections from BALB/c c-FLIP_L Tg mice exhibited a diffuse proliferative glomerulonephritis in BALB/c c-FLIP_L Tg mice characterized by enlarged glomeruli, loss of Bowman's capsule space, thickening of the mesangium, and hypercellularity. Immunofluorescence staining displayed IgG and C3 deposition in glomeruli (Figure 1d–e). Serological analysis showed that BALB/c c-FLIP_L Tg mice had markedly elevated levels of anti-nuclear, anti-ssDNA and anti-dsDNA Abs,

whereas B6 c-FLIP_L Tg mice were negative for these auto-Abs (Figure 1f). Taken together, our results suggest that over-expressing c-FLIP_L in T cells leads to the development of systemic lupus-like disease, which was contingent upon the BALB/c background. The susceptible c-FLIP_L Tg background requires the host background to trigger the autoimmunity.

c-FLIP_L over-expression leads to impaired thymic selection in BALB/c background

The difference observed in susceptibility to disease development in BALB/c c-FLIP_L Tg mice as compared to B6 c-FLIP_L Tg mice may result from the differences in T cell development in these two strains. Development of autoimmunity could be due to breakdown of self-tolerance in the thymus and/or changes in the activation thresholds of peripheral lymphocytes. It has been documented that thymocyte development is not altered in B6 c-FLIP_L Tg mice ⁶. To investigate whether a defect in T cell development might be responsible for the disease development in BALB/c mice over-expressing c-FLIP_L in T cells, we stained thymocytes from BALB/c c-FLIP_L Tg and NLC (1 wk of age) with fluorescence-conjugated Abs against CD4 and CD8 in combination with CD25 and CD44 to define thymocyte development. BALB/c c-FLIP_L Tg thymocytes developed successfully from the double negative (DN) to the double positive (DP) stage (data not shown). Although total thymocyte numbers in BALB/c c-FLIP_L Tg mice were normal at 1 wk of age, there were significant reductions in the percentages and absolute numbers of CD4 single positive (SP) thymocyte populations (Figure 2a; table-1), suggesting that their differentiation from DP to CD4 SP thymocytes was compromised. At 7 wks of age the total thymocyte numbers of BALB/c c-FLIP_L Tg mice were reduced to about 20 % that of the NLC. The reduced thymocyte numbers were the result of smaller DP (31% of control) and CD4⁺ SP (65% of control) populations (Figure 2a; Table 1). The involuted thymus in older BALB/c c-FLIP_L Tg mice may be secondary to stress. In sharp contrast, B6 c-FLIP_L Tg mice showed normal distributions of DP and SP thymocytes (Supplementary Figure S2a) and no thymic atrophy (data not shown). These data suggest that over-expression of c-FLIP_L impairs thymocyte development in a strain-dependent manner.

To obtain further insight into the mechanism underlying the altered thymic development, we investigated whether the reduction in the numbers of CD4 SP thymocytes was caused by a defect in positive selection. During positive selection, CD69 expression is induced, and CD5 is up-regulated ¹⁰. As shown in Figure 2b, BALB/c c-FLIP_L Tg thymocytes failed to up-regulate the expression of CD69 and CD5, suggesting that thymic positive selection is impaired in the presence of c-FLIP_L over-expression. To further facilitate the examination of positive selection, we established a TCR Tg system to specifically study the effect of the over-expression of c-FLIP_L on the development of these cells. This was accomplished by breeding the I-A^d-restricted DO11.10 TCR transgene which is specific for a peptide from chicken ovalbumin (OVA) (amino acids 323-339; OVA_{p323-339}) ^{11,12} onto BALB/c c-FLIP_L Tg mice. Positive selection of CD4 lineage was investigated with the use of KJ1-26 anti-idiotypic mAb which recognizes DO11.10 TCR. The analysis indicated that there was a significant decrease in the percentage of CD4 SP thymocytes in DO11.10.c-FLIP_L Tg mice (Figure 2c, upper panel), further demonstrating that positive selection of CD4 lineage was impaired.

Negative selection of thymocytes is a major mechanism of central tolerance. It is possible that the autoimmune lupus-like disorder observed in c-FLIP_L Tg mice is due to the breakdown of central tolerance. To substantiate the role of c-FLIP_L in thymic negative selection, we systemically administrated OVA_{p323-339} peptide into OVA-specific TCR transgenic DO11.10 and DO11.10.c-FLIP_L Tg mice. Injection of OVA_{p323-339} peptide into DO11.10 mice reduces both the percentage and the absolute number of immature transgenic thymocytes 13. We found that administration of OVA_{p323-339} peptide did induce deletion of DP thymocytes in NLC in a dose-dependent manner, but this deletion was significantly impaired in BALB/c c-FLIP_L Tg mice (Figure 2c, middle and lower panels), suggesting that thymic negative selection is also impaired in BALB/c c-FLIP_L Tg mice.

To confirm the effect of c-FLIP_L over-expression in thymic selection in a strain-dependent manner, we first measured the expression of CD5 and CD69 in DP thymocytes from B6 c-FLIP_L Tg mice and their NLC. There was no difference in CD5 and CD69 expression between B6 c-FLIP_L Tg and their NLC DP thymocytes (Supplementary Figure S2b). To further verify c-FLIP_L over-expression in thymic selection in B6 strain, we bred B6 c-FLIP_L Tg mice onto OT-II mice in which T cells express a TCR specific for peptide 323–339 of OVA in the context of I-A^b12. We then treated OT-II and OT-II.c-FLIP_L Tg mice with OVA_{p323-339} peptide or with PBS, and monitored positive and negative selection of thymocytes in these mice. As shown in Supplementary Figure 2c, over-expressing c-FLIP_L in OT-II T cells did not result in any significant impairment in both positive and negative selection. Our data indicate that c-FLIP_L over-expression may affect the threshold of TCR avidity for self-peptide/MHC ligands required for each selection event under BALB/c but not B6 background, leading to aberrant thymic selection in BALB/c strain.

The model of negative selection described above depends upon the activation of exogenous TCR ligand in the system. To determine whether negative selection induced by endogenous antigens is also affected by c-FLIP_L transgene, we analyzed the repertoire of T cells reactive to endogenous mouse mammary tumor virus (MMTV) products presented on I-E molecule. BALB/c mice have the integration of MMTVs (Mtv types 6, 8 & 9) in the genome and normally delete the viral super-antigen (VSAg)-reactive TCR Vβ3-, 5-, 11-, and 12-positive T cells, whereas B6 mice fail to delete them due to the lack of MHC class II I-E molecules 14,15. As shown in Figure 2d, TCR Vβ3-, 5-, 11-, and 12-positive cells were significantly deleted in the thymi from NLC but this deletion was inhibited in BALB/c but not B6 c-FLIP_L Tg mice. Taken together, our data suggest that thymic negative selection is impaired in BALB/c mice over-expressing c-FLIP_L. Note that DO11.10.c-FLIP_L Tg mice developed lupus-like syndrome at high incidence (Supplementary Figure S3), suggesting that the disease development in these mice is possibly due to the expression of endogenous TCRs.

BALB/c c-FLIP_L Tg T and B cells display activated phenotypes

It has been shown that the c-FLIP_L Tg mice in the B6 background do not display activated phenotype in their T and B cells 6. Having examined thymocyte development/selection in BALB/c c-FLIP_L Tg mice, we then wanted to determine the phenotype of peripheral T cells in BALB/c c-FLIP_L Tg mice. Although the spleens and lymph nodes were enlarged in BALB/c c-FLIP_L Tg mice, T cells were significantly reduced in the spleen and lymph nodes

from BALB/c c-FLIP_L Tg mice compared to NLC at 7 wks of age (Figure 3a and b; Table-1). The ratio of T to B cells was abnormally increased in the spleens and lymph nodes, suggesting that enlarged secondary lymphoid tissues are not caused by expanded T cell compartments of BALB/c c-FLIP_L Tg mice. A significant fraction of CD4⁺ and CD8⁺ T cells from spleens and lymph nodes of BALB/c c-FLIP_L Tg mice at 7 wks of age expressed high levels of CD25, CD69, and CD44, but low levels of CD62L (Figure 3a and b), suggesting that they had recently been stimulated by antigens, and were activated effector T cells. Therefore, splenomegaly and lymphadenopathy observed in BALB/c c-FLIP_L Tg mice are likely secondary to aberrant effector T cell activation. In support of this notion, the percentages and numbers of B cells, granulocytes, macrophages, and DCs were significantly increased in BALB/c c-FLIP_L Tg mice compared to their NLC (Table-1). We did not detect any B220⁺CD3⁺CD4⁻CD8⁻ DN T cells which are typically present in *lpr* mice 16 (data not shown). In addition, the expression of B7-1, B7-2, CD69, I-A^d, and Fas was increased in BALB/c c-FLIP_L Tg B cells, suggesting that BALB/c c-FLIP_L Tg B cells are also activated in vivo (Figure 3c). In contrast, B6 c-FLIP_L Tg mice showed reduced CD8⁺ T cells in their spleens and lymph nodes (Supplementary Figure S4a), consistent with the previous report 6. In addition, we also found that expression of activation markers such as CD25, CD62L, CD69, and Fas was comparable between B6 c-FLIP_L Tg T cells and their NLC T cells (Supplementary Figure S4b). Therefore, development of autoimmunity requires susceptible genetic background.

To test whether the CD4⁺ T cells are autoreactive in BALB/c c-FLIP_L Tg mice, CD4⁺ T cells isolated from either NLC or c-FLIP_L Tg mice at 2 wk of age (before disease onset) were adoptively transferred in conjunction with either c-FLIP_L Tg or NLC B cells into Rag-1^{-/-} hosts (BALB/c background). Seven weeks later Rag-1^{-/-} mice were monitored for anti-dsDNA Ab production and lupus-like syndrome. Rag-1^{-/-} mice reconstituted with BALB/c c-FLIP_L Tg CD4⁺ T cells and BALB/c c-FLIP_L Tg or NLC B cells displayed an increase in anti-dsDNA Ab production and lymphocyte infiltration in multi-organs (Figure 4a and b). However, transfer of NLC CD4⁺ T cells in conjunction with either c-FLIP_L Tg or NLC B cells failed to result in high anti-dsDNA Ab titers and remained disease-free. FACS analysis clearly identified B cell populations in Rag-1^{-/-} hosts, suggesting that transfer of B cells together with CD4⁺ T cells could survive long enough in Rag-1^{-/-} mice 17,18. Note that B cell populations were increased in Rag-1^{-/-} recipients receiving c-FLIP_L Tg CD4⁺ T cells compared to those receiving CD4⁺ T cells from NLC (Figure 4c).

Over-expression of c-FLIP_L inhibits T cell proliferation by promoting apoptosis, and leads to heightened Th2 cytokine production

The development of lupus-like syndrome in BALB/c c-FLIP_L Tg mice could be due to aberrant autoreactive T cell proliferation in the periphery. It has previously been shown that over-expression of c-FLIP_L in T cells in the B6 background may result in an increase in T cell proliferation 6,19 (Supplementary Figure S5a and b). To verify the roles of c-FLIP_L in T cell proliferation, splenic T cells from BALB/c c-FLIP_L Tg mice and NLC at 2 wk of age before disease onset were stimulated for 72 h with different concentrations of plate-bound anti-CD3 or anti-CD3 plus anti-CD28. Interestingly, the proliferation rate of BALB/c c-FLIP_L Tg splenic T cells in response to TCR or TCR/CD28 stimulation was significantly

lower than that in NLC splenic T cells (Figure 5a). Regardless of hypo-responsiveness to TCR stimulation because of the over-expression of c-FLIP_L, c-FLIP_L Tg CD4⁺ T cells were twice as proliferative as NLC CD4⁺ T cells in the physiological state, as shown with in vivo BrdU incorporation (Figure 5b). Over-expressing c-FLIP_L in T cell lineage did not affect CD4 SP thymocyte proliferation, indicating that the proliferating T cells may be due to homeostasis of peripheral T cells.

We found that BALB/c c-FLIP_L Tg CD4⁺ T cells are hypo-proliferative in vitro but not in vivo, and CD4⁺ T cells are dramatically reduced in BALB/c c-FLIP_L Tg mice. These observations raised a possibility that BALB/c c-FLIP_L Tg CD4⁺ T cells are highly susceptible to apoptosis. To assess proliferation and apoptosis simultaneously, we labeled CD4⁺ T cells of BALB/c c-FLIP_L Tg and NLC mice with carboxyfluorescein succinimidyl ester (CFSE), and stimulated these T cells with anti-CD3. The apoptosis in proliferating or non-proliferating T cells was determined by staining them with PE-conjugated Annexin V. As shown in Figure 5c, a significant portion of BALB/c c-FLIP_L Tg CD4⁺ T cells that did not enter cell cycle underwent apoptosis. Consistent with [³H] thymidine incorporation results, BALB/c c-FLIP_L Tg CD4⁺ T cells proliferated poorly in response to CD3 stimulation (Figure 5c). Therefore, the reduced peripheral T cells may be at least in part due to cell cycle arrest and increased sensitivity to apoptosis in BALB/c c-FLIP_L Tg mice. In sharp contrast, B6 c-FLIP_L Tg T cells highly proliferated in response to lower doses of anti-CD3 stimulation, although they displayed a similar sensitivity to apoptosis (Supplementary Figure S5b), in agreement with the previous reports 6,19.

One of the principal responses of activated T cells is the production of cytokines. To test whether activated T cells from c-FLIP_L Tg mice were able to secrete effector cytokines, we stimulated CD4⁺ T cells from c-FLIP_L Tg mice and NLC with anti-CD3, or anti-CD3 plus anti-CD28 for 72 h as described before, and the production of Th1, Th2, and Th17, as well as proinflammatory cytokines and chemokines was measured by Cytokine 20-Plex in the supernatants collected. As shown in Figure 5d and Supplementary Figure S5c, over-expression of c-FLIP_L in T cells selectively led to a significant increase in the production of Th2 cytokines, especially IL-4, detected upon TCR or TCR/CD28 stimulation, consistent with the previous reports 20,21. In support of this observation, serum IgG1 and IgE production was significantly elevated in BALB/c c-FLIP_L Tg mice (data not shown).

Over-expressing c-FLIP_L in T cells in BALB/c background impairs TCR-induced ZAP-70 activation and downstream signaling pathways in thymocytes

Several studies reveal that a point mutation at the ZAP-70 (ZAP-70^{W163C}) or LAT (LAT^{Y136F}) gene leads to development of autoimmune arthritis or systemic autoimmunity as a result of altered thymic selection 22,23. To test whether over-expression of c-FLIP_L interferes with proximal TCR signaling, we measured activation of Lck, ZAP-70, TCR- ζ , and LAT in thymocytes from BALB/c and B6 c-FLIP_L Tg and NLC in response to TCR stimulation. Intriguingly, we found that although Lck Y394 phosphorylation was comparable between BALB/c c-FLIP_L Tg and NLC thymocytes, phosphorylation of ZAP-70, TCR- ζ , and LAT was significantly reduced (Figure 6a). However, we did not observe a similar reduction in phosphorylation of ZAP-70, TCR- ζ , and LAT in B6 c-FLIP_L

Tg thymocytes following TCR stimulation (Supplementary Figure 6a). These observations suggest that over-expression of c-FLIP_L may selectively impair the formation of TCR signalosome in BALB/c background which connects events on the plasma membrane to distal signaling cascades 24. To test this idea, we assessed the assembly of TCR signalosome in thymocytes from BALB/c c-FLIP_L Tg mice and their NLC. As expected, TCR-induced signaling complex formation consisting of ZAP-70, TCR- ζ , LAT, and SLP-76 was defective in thymocytes over-expressing c-FLIP_L in BALB/c mice (Figure 6b). These data suggest that c-FLIP_L over-expression may constrain ZAP-70 in the cytosol, thus inhibiting the translocation of ZAP-70 from cytosol to plasma membrane. To verify this possibility, we isolated cytosol and membrane fractions from BALB/c c-FLIP_L Tg mice and NLC before and after TCR stimulation by subcellular fractionation 25. The amounts of ZAP-70, LAT, SLP-76, and PLC- γ 1, were significantly reduced in the membrane fraction from BALB/c c-FLIP_L Tg thymocytes compared with that from NLC thymocytes after TCR stimulation (Figure 6c). Therefore, our data support the notion that over-expressing c-FLIP_L in T cells may impair ZAP-70-mediated signaling pathway, thus attenuating TCR signaling responsible for thymic selection.

The signaling events downstream of the TCR play a critical role in positive and negative selection. To further determine the cause of the defect in positive and negative selection of BALB/c c-FLIP_L Tg thymocytes, we assessed their responses to TCR stimulation. Particularly, we examined MAPK- and NF- κ B-pathways, as these TCR responses play an essential role in thymic selection 26–29. We found a significant reduction in phosphorylation of ERK and I κ B α , upon TCR stimulation, whereas phosphorylation of JNK and p38 MAPK was comparable between BALB/c c-FLIP_L Tg and NLC thymocytes in response to TCR stimulation (Figure 7). These data suggest that c-FLIP_L over-expression impairs TCR-induced activation of ZAP-70-mediated signaling pathway which subsequently results in altered activation of ERK and NF- κ B responsible for thymic selection, thus eliciting lupus-like disease in BALB/c mice. In contrast, activation of MAPKs including ERK, JNK, and p38 as well as NF- κ B was comparable in the presence or absence of c-FLIP_L transgene in B6 background (Supplementary Figure S6b).

Discussion

In this study, we first report that BALB/c c-FLIP_L Tg mice spontaneously develop lupus-like disease starting from 3–4 wks of age, characterized by splenomegaly, lymphadenopathy, multi-organ infiltration including liver, lung, gastrointestinal, heart, and salivary gland, proliferative glomerulonephritis with immune complex deposition, and high titers of auto-Abs (Figure 1). These features are clinically similar to human SLE patients. BALB/c c-FLIP_L Tg T and B cells display activated phenotypes (Figure 3), suggesting they are hyper-activated in vivo. The development of the disease requires CD4⁺ T cells, and may be due to impaired thymic selection. Thus, the BALB/c c-FLIP_L Tg strain provides evidence of a key role for c-FLIP_L signals in the maintenance of immunological self-tolerance. Our data are consistent with the reports that the elevated c-FLIP expression was observed in human T cells from patients with SLE 30,31 although the exact role of c-FLIP in SLE development in human remains to be elucidated.

The development of autoimmunity may result from a breakdown of central or peripheral tolerance. Autoreactive T cells are deleted in thymus by negative selection. Recently, the defect in positive selection was reported to cause autoimmunity 32. Moreover, a point mutation at the ZAP-70 (ZAP-70^{W163C}) or LAT (LAT^{Y136F}) gene alters both positive and negative selection in the thymus, eliciting either autoimmune arthritis or systemic autoimmunity 22,23. In keeping with this, targeted disruption of *Gads* as well as deletion of the *Gads* binding site in SLP-76 impair both positive and negative selection 33. Moreover, in the *Lcp2^{wp}* strain, an ENU-induced mutation in a splice donor site reduces the amount of full-length mRNA encoding SLP-76 promotes autoimmunity 3. The failure of up-regulating CD69 and CD5 in DP thymocytes of BALB/c c-FLIP_L Tg mice suggests a defect in positive selection. Indeed, a similar reduction of CD4 SP thymocytes was also seen in DO11.10.c-FLIP_L Tg mice (Figure 2c, upper panel), supporting a defect in positive selection in these mice. Using two model systems, VSAg-mediated deletion and systemic administration of OVA peptide to DO11.10.c-FLIP_L Tg mice, we demonstrated that thymic negative selection is also impaired in BALB/c mice over-expressing c-FLIP_L (Figure 2c, middle and lower panels). Taken together, our data indicate that c-FLIP_L over-expression in T cells in the BALB/c background leads to altered thymic selection, resulting in positive selection of otherwise negatively selecting self-reactive T cells in the thymus.

Mechanistically, over-expressing c-FLIP_L in T cells may impair ZAP-70-mediated signaling pathway, thus attenuating TCR signaling responsible for thymic selection. Consistent with altered thymic selection in BALB/c c-FLIP_L Tg mice, activation of ERK and NF-κB was impaired in c-FLIP_L Tg thymocytes following TCR stimulation (Figure 7). The attenuated activation of ERK and NF-κB would impair both positive and negative selections of T cells in BALB/c c-FLIP_L Tg mice 33,34. As c-FLIP_L may heterodimerize with caspase-8 upon TCR stimulation, and caspase-8 may mediate NF-κB activation in a CARMA-1-dependent manner 35,36, it is presumed that TCR-induced c-FLIP_L-caspase-8 heterodimer formation may interfere with the role of caspase-8 in NF-κB activation. However, c-FLIP_L-mediated inhibition of NF-κB activation in the thymus remains to be further determined, as deficiency for caspase-8, CARMA-1, Bcl-10, and MALT-1 does not perturb thymic selection 37,38.

It has been documented that c-FLIP_L inhibits FasL-induced cell death 39. However, c-FLIP_L has also been suggested to be crucial for T cell proliferation and differentiation 6–8,21. As c-FLIP_L is an endogenous inhibitor of caspase-8, it was expected that mice over-expressing c-FLIP_L in T cells would lead to development of lupus-like disease as seen in *lpr* mice. Unexpectedly, mice over-expressing c-FLIP_L in T cells in B6 background do not succumb to lupus-like syndrome 6,21,39. In contrast to this, retrovirus-mediated over-expression of c-FLIP_L in T and B cells blocks Fas-induced apoptosis of activated T and B cells, which leads to the production of auto-Abs and to the development of autoimmune diseases 40; however, the contribution of c-FLIP_L-over-expressing T cells vs. B cells in disease development is not defined. We found that c-FLIP_L Tg mice in the BALB/c background spontaneously develop lupus-like disease (Figure 1). The development of the disease is mediated by CD4⁺ T cells from BALB/c c-FLIP_L Tg mice because adoptive transfer of CD4⁺ T cells from BALB/c c-FLIP_L Tg mice but not NLC together with B cells from either BALB/c c-FLIP_L Tg or NLC mice into Rag-1^{-/-} recipients causes the disease (Figure 4a and b). Our data also suggest that

c-FLIP_L over-expression does not inhibit T cell apoptosis in vitro, but rather results in cell cycle arrest and aberrant apoptosis, thus suppressing CD3-triggered T cell proliferation (Figure 5a and c). The reduced export of CD4⁺ SP thymocytes from BALB/c c-FLIP_L Tg mice to the periphery may result in heightened homeostatic proliferation in the periphery as shown by increased BrdU incorporation in splenic CD4⁺ T cells in these mice (Figure 5b).

The restrictive function of c-FLIP_L further explains the requirement for genetic modifiers for the emergence of autoimmunity in mice over-expressing this molecule in T cells. Autoimmunity appears when multiple immunological pathways are perturbed. In general, single gene knockouts or transgenes that result in autoimmunity have pleiotropic effects on T and B cell function as well as antigen presentation. Specific genetic backgrounds may provide the additional coactivating components necessary to the development of autoimmune disease. This notion is supported by the fact that FcγRIIB^{-/-} mice develop lupus-like autoimmune diseases in B6 but not BALB/c background 41, suggesting that spontaneous autoimmune disease in FcγRIIB^{-/-} mice results from strain-specific epistasis. Our data indeed support the notion that over-expression of c-FLIP_L in T cells is not sufficient to trigger autoimmunity, and the development of lupus-like autoimmune phenotype requires the trigger from host genetic background. We believe that these two factors together would affect the threshold for thymic selection which results in aberrant positive and negative selection in the thymus in BALB/c but not B6 background. Indeed, thymic selection is intact in OT-II.c-FLIP_L Tg mice (Supplementary Figure S2). Moreover, c-FLIP_L transgene does not perturb proximal TCR signaling in B6 background as seen in BALB/c c-FLIP_L Tg mice (Supplementary Figure S6a). Taken together, these data suggest that c-FLIP_L over-expression in T cells may alter the threshold for T cell activation in BALB/c but not B6 strain required for thymic selection. The nature of these genetic factors that influence c-FLIP_L function is currently under investigation.

In conclusion, BALB/c c-FLIP_L Tg mice spontaneously develop an autoimmune disorder exhibiting many similarities to human SLE, providing a novel model of autoimmune disease in a strain-specific epistasis. The development of the disease is due to altered thymic selection. c-FLIP_L is thus a susceptibility factor in the development of systemic autoimmune disease, contributing in a combinatorial fashion through the presence of interacting genetic factors to the emergence of disease. Further study of BALB/c c-FLIP_L Tg mice will improve our understanding of the complex network of signals that serve to maintain self-tolerance and will improve our insights into the genetic basis of autoimmune disease.

Materials and Methods

Mice

c-FLIP_L was expressed transgenically in the T cell compartment as previously reported 6,39. Briefly, FLAG-tagged mouse c-FLIP_L cDNA was inserted into a target vector containing the β-globin promoter and a downstream human CD2 locus enhancer element. B6 c-FLIP_L Tg mice were have been backcrossed onto BALB/c background for 14 generations. DO11.10 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c c-FLIP_L Tg mice were crossed onto DO11.10 mice to generate DO11.10.c-FLIP_L Tg mice. B6 c-FLIP_L Tg mice were crossed onto OT-II mice which were purchased from the Jackson Laboratory

to generate OT-II.c-FLIP_L Tg mice. All the animals were used in accordance with the National Institutes of Health guidelines. The experiments described in this study were reviewed and approved by the University of Chicago Institutional Animal Care and Use Committee. Mice were housed at the University of Chicago Animal Care facility. The expression of the c-FLIP_L transgene in thymocytes, splenic T cells, B cells, and DCs was detected by Western blotting with anti-FLAG and anti-c-FLIP_L (Dave-2).

Histopathology

Tissue specimens for histopathology were fixed in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) or snap frozen. Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) and evaluated by light microscopy. Immunohistochemical analysis on frozen kidney sections was performed on acetone-fixed sections. In brief, sections were blocked with 10% goat serum and, in some cases, with 0.1% Triton X-100 followed by fluorescein-conjugated goat anti-mouse IgG (Jackson Immunosciences, West Grove, PA) for 1 hr at room temperature. After washing, immunofluorescence analysis was performed with a Nikon Eclipse E600 microscope.

Purification of T cells, B cells, and DCs

CD4⁺ T cells were purified from the spleen by negative selection (>90% CD4⁺) (Miltenyi Biotec, Auburn, CA). B cells were isolated (purity > 95% as determined by FACS analysis of B220 cell surface expression) using a B cell isolation kit using negative selection (Miltenyi Biotec.). Contaminating T cells were less than 1% as determined by CD3 staining. DCs were isolated from spleen by positive selection (>90% DCs as determined by FACS analysis of CD11c) (Miltenyi Biotec.).

Cell activation and lysis

Thymocytes were maintained on ice in RPMI-1640 supplemented with 20 mM Hepes until stimulation. Quiescent thymocytes (4×10^7 /ml) were stimulated with hamster anti-mouse CD3 mAb (1 μg/ml) on ice for 30 min, and washed in pre-warmed medium, and crosslinked with anti-hamster IgG (10 μg/ml) for various times. The cells were lysed in 1% Triton X-100 lysis or 0.5% NP-40 lysis buffer as indicated.

Adoptive transfer

CD4⁺ T cells (5×10^6) and B cells (10^7) from BALB/c c-FLIP_L Tg mice and NLC at 2 wks of age were i.v. injected into Rag-1^{-/-} recipients (n=5) with different combinations as indicated. The mice were sacrificed seven weeks later, and serum anti-dsDNA Abs were determined by ELISA. The different organs were collected for histopathological analysis.

Detection of auto-Abs by ELISA

Anti-nuclear Abs (ANA) were detected by ELISA. Auto-Abs to double-stranded DNA (dsDNA), ssDNA, and nuclei were determined by ELISA as described previously 42.

Flow cytometry

The following Abs (Pharmingen, San Diego, CA) were used: anti-CD4 (clone GK1.5), anti-CD8 (53-6.7), KJ1-26, anti-V β 3, anti-V β 5, anti-V β 11, anti-V β 12, anti-B7-1 (16-10A1), anti-B7-2(GL1) and anti-B-220 (RA3-6B2) for lineage markers; anti-CD69 (H1.2F3), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-Fas (Jo2), anti-CD5 (53-7.3), and anti-CD25 (PC81) for activation markers. Acquisition of samples was performed on a FACScan flow cytometer, and the data was analyzed using FlowJo software V.4.4.3 (Tree Star Inc.).

In vivo administration of OVA_{p323-339}

DO11.10 and DO11.10.c-FLIP_L Tg or OT-II and OT-II.c-FLIP_L Tg mice (2 wk of age) were injected intraperitoneally with PBS or OVA_{p323-339} once a day at doses of 30 μ g or 300 μ g/mouse. Mice were sacrificed after 72 h, and their thymi were removed for analysis.

Assays for cell proliferation and apoptosis

T cells were stimulated with anti-CD3 or anti-CD3 and anti-CD28 for the indicated times. Proliferation was assayed by measuring [³H]thymidine incorporation. Alternatively, purified CD4⁺ T cells were labeled with CFSE (Molecular Probes; Eugene, OR) at 5 μ M in PBS for 10 min at 37°C, washed with RPMI containing 10% FCS, and incubated with plate-bound anti-CD3 for 72 h. The cells were then washed, stained with PE-labeled Annexin V, and analyzed by flow cytometry. For in vivo BrdU labeling, mice were i.p. injected with 1.0 mg BrdU (Sigma-Aldrich) every 12 h twice and given 0.8 mg/ml BrdU in drinking water for three days until flow cytometric analysis.

Cytokine assay by luminex

The mouse cytokine 20-plex assay kits (Invitrogen) were used to measure the concentrations of the cytokines in the supernatants collected from T cell cultures according to the manufacturer's instruction. Concentrations of analyte in samples were determined by interpolation from 20 individual standard curves, using the 5 parameter curve fitting provided in Bio-Plex ManagerTM software version 3.0.

Subcellular fractionation

Thymocytes (10⁸) were resuspended and lysed by brief sonication in ice-cold 10 mM Tris, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 2 mM EGTA hypotonic buffer containing the above-described mixture of protease and phosphatase inhibitors (buffer A). Lysates were adjusted to 150 mM NaCl, centrifuged to remove nuclei and debris, and particulate membrane-containing (P100) and soluble cytoplasm-containing (S100) fractions were separated by differential centrifugation for 30 min at 100,000 g. Membrane fractions were washed with ice-cold buffer A and solubilized by sonication in buffer A supplemented with 150 mM NaCl and 1% Triton X-100.

Immunoprecipitations of cellular proteins

Precleared postnuclear fractions obtained from 4 \times 10⁷ cells were normalized for protein concentration levels and immunoprecipitated (3 h at 4°C) with the specific polyclonal Abs or control isotype-matched preimmune Ig precoupled to 30 μ l of protein A-Sepharose

CL-4B (GE Healthcare Systems). This was followed by four washes of the precipitates with ice-cold lysisbuffer.

Western blot analysis

The thymocyte lysates from NLC and BALB/c c-FLIP_L Tg mice were blotted with phospho-Abs against Lck (Y394), ZAP-70 (Y493), LAT (Y191), PLC- γ 1 (Y783), ERK (T202/Y204), JNK (T183/Y185), p38 MAPK (T180/Y182), or I κ B (S32) Cell Signaling Technology, Inc., Beverly, MA), and anti-actin (Sigma-Aldrich, St. Louis, MO). Precipitated proteins or the membrane and cytosol fractions were solubilized in 2 \times Laemmli sample buffer containing 2-ME, 20 mM EDTA, and 2 mM Na₃VO₄, resolved by SDS-PAGE under reducing conditions, transferred to Immobilon (Millipore, Bedford, MA) or nitrocellulose (Schleicher & Schuell, Keene, NH) membranes, and immunoblotted with the indicated Abs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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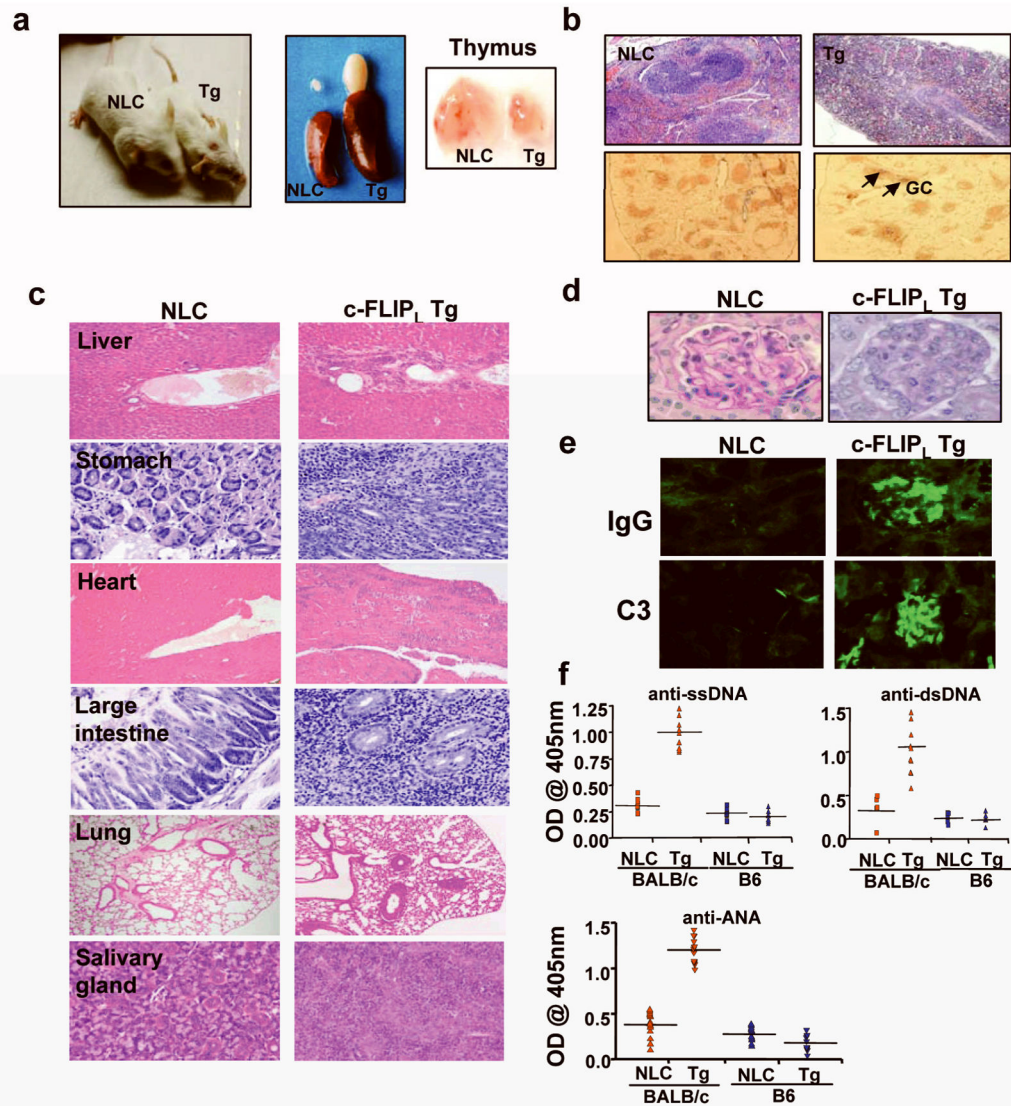


Figure 1.

BALB/c c-FLIP_L Tg mice develop lupus-like disease. **(a)** Splenomegaly and lymphadenopathy in BALB/c c-FLIP_L Tg mice. BALB/c c-FLIP_L Tg mice were smaller than NLC in size and displayed splenomegaly and lymphadenopathy at 6–10 wks of age. Pictures are representative of five mice analyzed for each group. **(b)** Disorganized structure of spleen in BALB/c c-FLIP_L Tg mice. Expansions of the white pulp were observed in the spleen of a BALB/c c-FLIP_L Tg mouse with spontaneous GC formation. Sections of spleens from 6-wk-old BALB/c c-FLIP_L Tg mice and NLC were stained with HE, or immunostained with biotinylated-PNA followed by ABC-AP for GC (blue) and FITC-labeled anti-B220 followed by HRP-conjugated anti-FITC for B cells (brown). **(c)** Multi-organ infiltration. Lymphocyte infiltrations were observed in liver, heart, stomach, large intestine, lung, and salivary gland of BALB/c c-FLIP_L Tg mice at 7 wks of age. Original magnification, $\times 100$ **(d)** Histopathological analysis of kidneys. Sections of kidneys from 6-wk-old BALB/c c-FLIP_L Tg mice and NLC were stained with PAS and observed under

microscopy. Representative histopathology of c-FLIP_L-transgenic glomeruli exhibiting proliferative glomerulonephritis. Original magnification, × 400. **(e)** Immunohistochemical analysis of kidneys. Frozen sections of kidneys from the same animals as in D were stained with FITC-conjugated anti-mouse IgG Ab or FITC-conjugated anti-mouse CD3 Ab. Sections were observed under fluorescence microscopy. Original magnification, × 400. **(f)** Spontaneous autoAb production in BALB/c but not B6 c-FLIP_L Tg mice. Sera were obtained from 6–10-wk-old BALB/c or B6 c-FLIP_L Tg mice and NLC. Anti-ssDNA, anti-dsDNA, and anti-ANA Abs were measured by ELISA.

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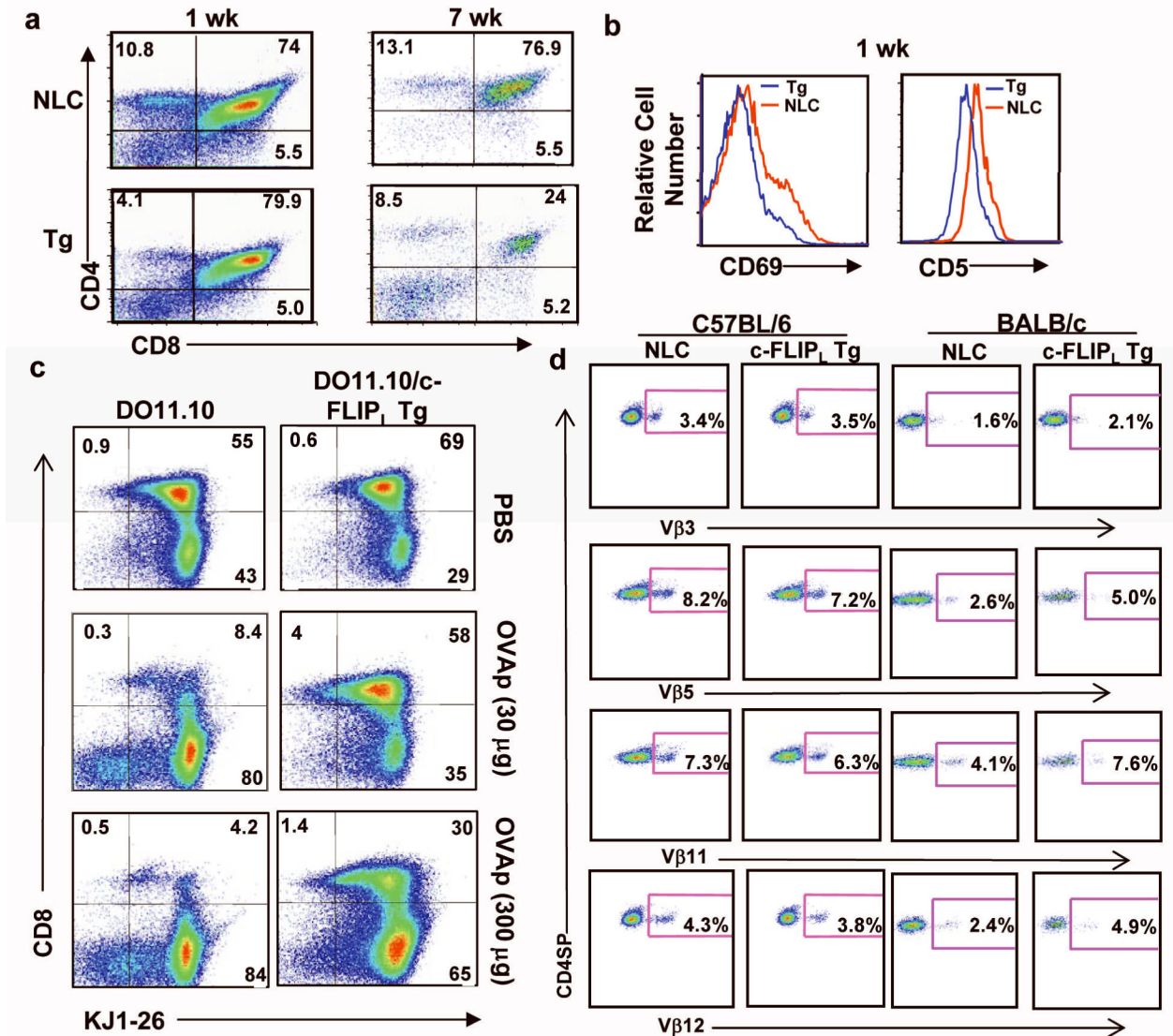


Figure 2.

Thymic selection is impaired in BALB/c c-FLIP_L Tg mice. **(a)** CD4⁺ SP thymocytes are selectively reduced in BALB/c c-FLIP_L Tg mice. The freshly isolated thymocytes from c-FLIP_L Tg mice and their NLC at 1 and 7 wk of age were stained with Abs against CD4 and CD8. **(b)** BALB/c c-FLIP_L Tg thymocytes failed to up-regulate CD69 and CD5. Thymocytes from BALB/c c-FLIP_L Tg mice and NLC at 1 wk of age were stained with Abs against CD4, CD8, CD69, and CD5. The expression of CD5 and CD69 in DP thymocytes from BALB/c c-FLIP_L Tg mice and NLC was determined. **(c)** Over-expression of c-FLIP_L impairs both positive and negative selection in the thymus. DO11.10.c-FLIP_L Tg mice and DO11.10 mice (at 2 wk of age) were injected i.p. with 30 or 300 μg OVAp₃₂₃₋₃₃₉ peptide every day for three days. Thymocytes from these mice were stained with KJ1-26 and anti-CD8, and analyzed by flow cytometry. **(d)** VSAg-mediated deletion is impaired in BALB/c but not B6 c-FLIP_L Tg mice. Thymocytes from B6 and BALB/c c-FLIP_L Tg and their NLC

at 1 wk of age were stained with Abs against CD4, CD8, V β 3, V β 5, V β 11, and V β 12, and analyzed by flow cytometry.

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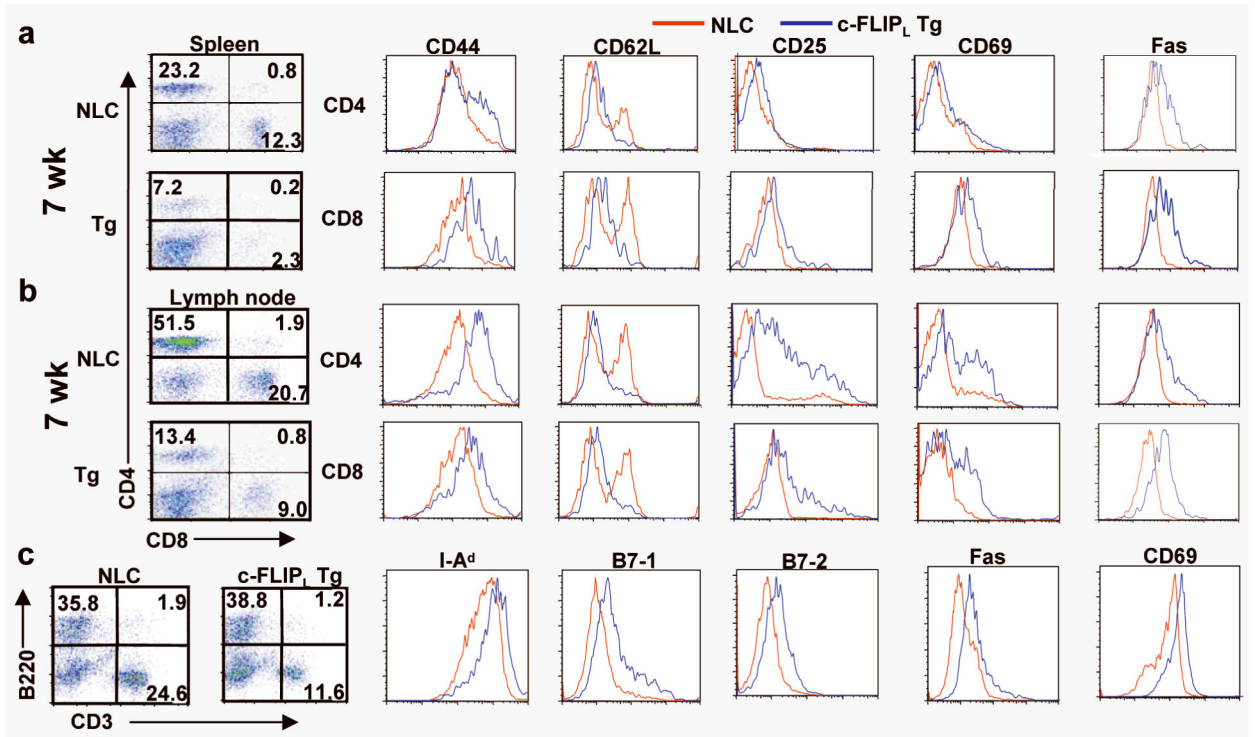


Figure 3. T and B cells of BALB/c c-FLIP_L Tg mice display an activated phenotype. **(a and b)** The spleen and lymph node cells from c-FLIP_L Tg mice and their NLC at 7 wk of age were stained with Abs against CD4, CD8, CD25, CD44, CD62L, CD69, and Fas. **(c)** c-FLIP_L Tg and spleen cells of NLC (7-wk-old) were stained with Abs against B220 and CD3, or B220 with I-A^d, B7-1, B7-2, Fas, or CD69. The percentages of CD4 and CD8 in spleen and lymph node cells and B cells in spleen, as well as the expression of activation markers in T cells and B cells were detected by flow cytometry.

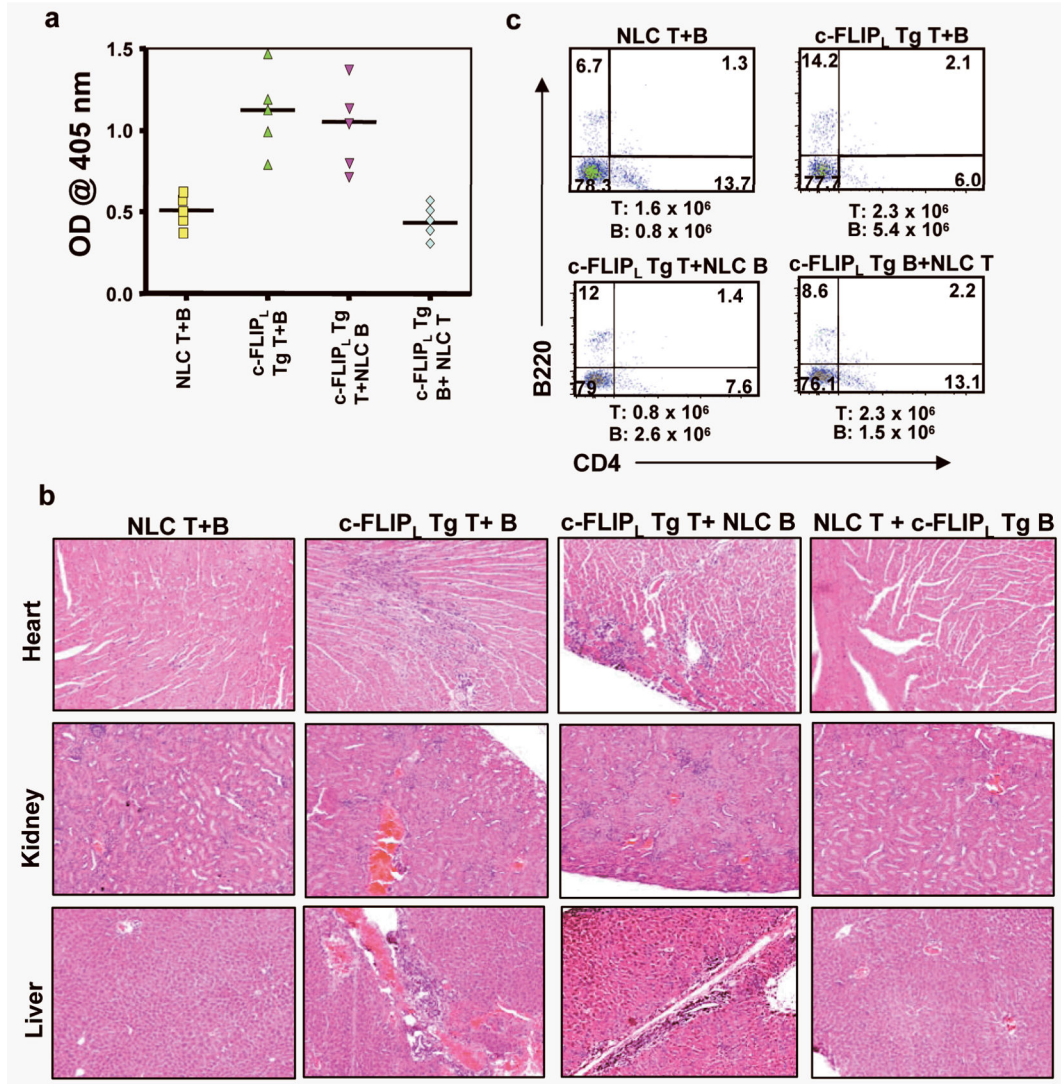


Figure 4.

Production of auto-Abs and autoimmune syndrome in BALB/c c-FLIP_L Tg mice requires CD4⁺ T cells. CD4⁺ T cells and B cells from BALB/c c-FLIP_L Tg mice and NLC at 2 wk of age were injected into Rag-1^{-/-} recipients (n=5) with different combinations as indicated. The mice were sacrificed seven weeks later, and serum anti-dsDNA Abs were determined by ELISA (a). The sections of liver, lung, and kidney were stained with H&E and observed under microscope. Original magnification, × 100. (b). The presence of T and B cells in spleens of Rag-1^{-/-} recipients was determined by flow cytometry (c).

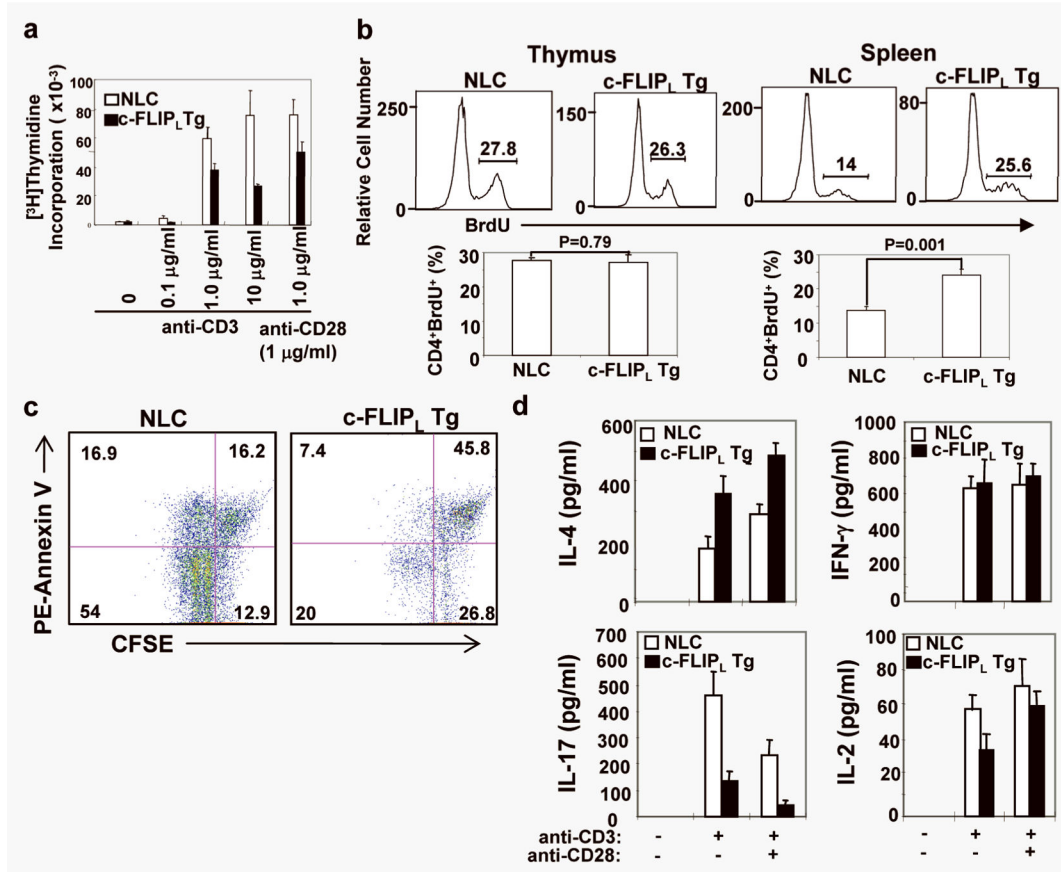


Figure 5. c-FLIP_L over-expression inhibits T cell proliferation and leads to a Th2-biased cytokine production

(a) Splenic CD4⁺ T cells from BALB/c c-FLIP_L Tg mice and their NLC (2-wk-old) were stimulated with different concentrations of anti-CD3, or anti-CD3 (1 µg/ml) plus anti-CD28 (1 µg/ml) for 72 h and proliferation of T cells was measured by [³H]thymidine incorporation. (b) BALB/c c-FLIP_L Tg mice and NLC at 2 wk of age were administered with BrdU for three days, and percentages of BrdU-stained cells among CD4⁺ SP thymocytes and CD4⁺ T cells are shown. (c) Splenic CD4⁺ T cells from BALB/c c-FLIP_L Tg mice and their NLC (2-wk-old) were labeled with CFSE, and cultured in the plate pre-coated with anti-CD3 for 72 h. The cells were washed, and stained with PE-Annexin V. (d) T cells from BALB/c c-FLIP_L Tg mice and their NLC were stimulated with anti-CD3, anti-CD3 plus anti-CD28 as in A. The supernatants were collected, and the production of IL-2, IL-4, IFN-γ, and IL-17 was detected by mouse cytokine 20-plex assay.

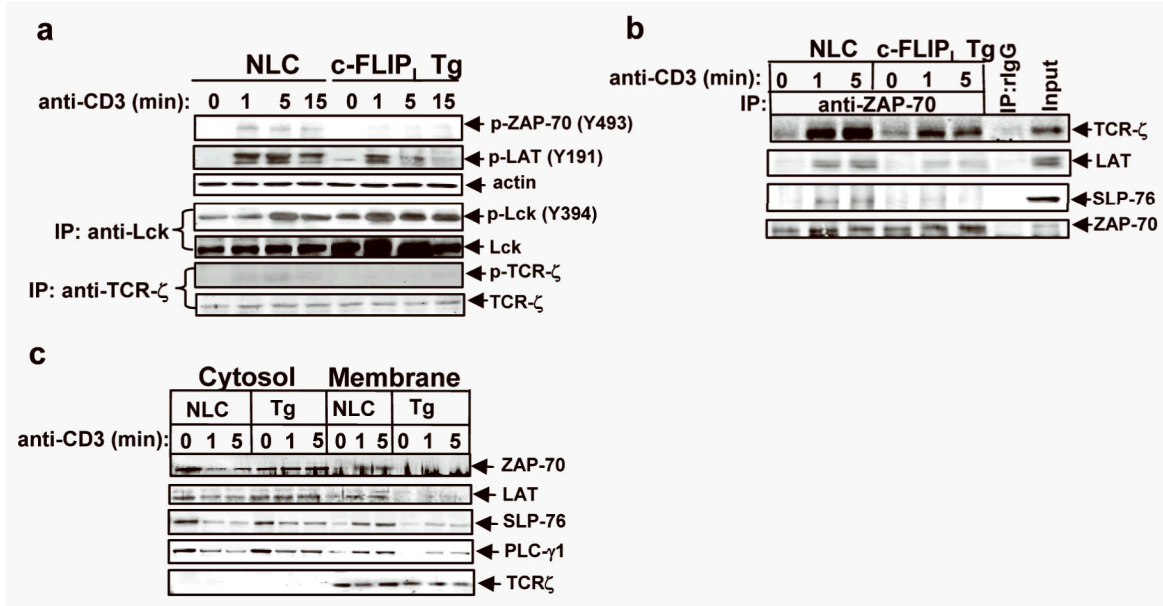


Figure 6. c-FLIP_L over-expression impairs the signaling pathway derived from ZAP-70 BALB/c c-FLIP_L Tg thymocytes. **(a)** c-FLIP_L over-expression results in defective phosphorylation of ZAP-70, TCR-ζ, and LAT in thymocytes of BALB/c c-FLIP_L Tg mice. Thymocytes from BALB/c c-FLIP_L Tg mice and their NLC (2 wk of age) were stimulated with anti-CD3, and lysed. The cell lysates were immunoblotted with phospho-Abs against ZAP-70 (Y493), and LAT (Y191), or immunoprecipitated with anti-Lck or anti-TCR-ζ, and blotted with anti-phospho-src-PTK (Y416) which recognizes Lck Y394 or anti-anti-phospho-tyrosine mAb (4G10). **(b)** c-FLIP_L over-expression impairs the formation of TCR signalingosome. Thymocytes from BALB/c c-FLIP_L Tg mice and their NLC (2 wk of age) were stimulated with anti-CD3, and lysed in 0.5% NP40 lysis buffer. The cell lysates were immunoprecipitated with anti-ZAP-70, and blotted with anti-TCR-ζ, anti-LAT, and anti-SLP-76, respectively. **(c)** Over-expression of c-FLIP_L in T cells inhibits the recruitment of ZAP-70, LAT, SLP-76, and PLC-γ1 to the plasma membrane. Thymocytes from BALB/c c-FLIP_L Tg mice and their NLC (2 wk of age) were stimulated with anti-CD3, and the membrane and cytosol fractions were isolated. The expression of TCR-ζ, ZAP-70, LAT, SLP-76, and PLC-γ1 in the membrane and cytosol fractions was detected by immunoblotting.

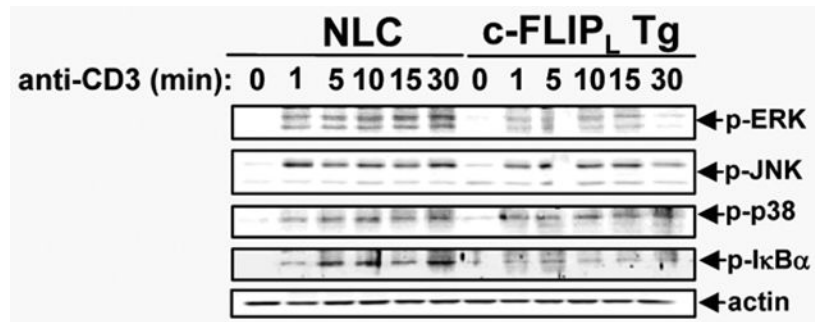


Figure 7.

TCR-induced activation of ERK and NF- κ B is defective in BALB/c c-FLIP_L Tg thymocytes. Thymocytes from BALB/c c-FLIP_L Tg mice and their NLC (2 wk of age) were stimulated with anti-CD3, and lysed. The cell lysates were blotted with phospho-Abs against ERK (Thr282/Tyr284), JNK (T183/Y185), p38 MAPK (T180/Y182), and I κ B α (S32). The membranes were stripped and reprobbed with anti-actin as loading controls.

Table 1

Cell numbers in thymuses and spleens of c-FLIP_L Tg mice and NLC at 1 and 7 wk of age

		Thymus											
Total cell number (×10 ⁶)		CD4 ⁺ CD8 ⁺		CD4 ⁺		CD8 ⁺		CD3 ⁺ B220 ⁺					
		1W	7W	1W	7W	1W	7W	1W	7W	1W	7W	1W	7W
NLC		62.4±12.5	201.5±9	47.5±4.3	155±11	6.4±1.2	26.4±5.4	3.1±0.5	11.1±3.5	0.2±0.1	1.4±0.34		
c-FLIP _L Tg		52.6±9.8	19±9.5	42.4±5.5	7.0±2.4	2.2±0.6	1.3±0.53	2.3±1.1	1.1±0.45	0.4±0.15	8.5±4.5		

		Spleen															
Total cell number (×10 ⁶)		CD3 ⁺		CD4 ⁺		CD8 ⁺		B 220 ⁺		CD11b ⁺		CD11c ⁺		Gr-1 ⁺			
		1W	7W	1W	7W	1W	7W	1W	7W	1W	7W	1W	7W	1W	7W		
NLC		30.6±5.5	111±65	0.9±0.3	40.6±6.4	0.4±0.2	23.5±3.4	0.4±0.1	13.7±2.5	4.4±1.0	55.2±5.5	1.9±0.3	6.2±2.3	0.1±0.04	5.7±1.5	1.02±0.04	3.60±0.64
c-FLIP _L Tg		30.9±4.6	335±125	0.6±0.4	19.9±3.4	0.4±0.12	13.9±2.5	0.3±0.2	5.3±2.3	3.5±1.8	117±35	2.1±0.6	37.2±5.6	0.1±0.03	39.2±3.4	0.85±0.2	89.445±31

mean±SD, n=6