(RM4-5) and SA-Qd605 were obtained from Invitrogen. Alexa Fluor 488 anti-LAG3 (C9B7W) was obtained from AbD Serotec. PE anti-Egr-2 (erongr2) was obtained from e-Bioscience. Streptavidin-conjugated microbeads were purchased from Miltenyi Biotec. Recombinant murine IL-2, IL-10, IL-12, IL-21, and IL-27 were obtained from R&D Systems. Recombinant human TGF-β1 was purchased from R&D Systems. Recombinant murine IL-23 was obtained from Biolegend. Zymosan was obtained from Sigma. $E\alpha_{52-68}$ peptide was purchased from Takara (Otsu, Japan). T cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 μg/mL L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μM 2-mercaptoethanol (all purchased from Sigma)..

In vitro T-cell differentiation

Naïve $CD4^+$ T cells ($CD4^+CD45RB^{hi}CD62L^{hi}CD25^-$) from C57BL/6 wild type, Egr-2 CKO or Blimp-1 CKO mice, WSX1 KO mice, and STAT1 KO or STAT3 CKO mice were isolated from their splenocytes. Briefly, single cell suspensions were first purified by negative selection with magnetic-activated cell sorting (MACS; Miltenyi Biotec) using anti-CD8α mAb, anti-CD11b mAb, anti-CD11c mAb, anti-CD19 mAb, anti-CD25mAb, and anti-Ter119 mAb, and were then purified by positive selection with anti-CD62L microbeads. The purity of MACS sorted cells was >90%. Purified cells were cultured in flat-bottomed 24-well plates coated with anti-CD3ε (2 μg/mL) and anti-CD28 (2 μg/mL). Mouse IL-27 (25 ng/mL) was added at the start of culturing. To assess T-cell proliferation, purified naïve $CD4^+$ T cells were labeled with 1 μM carboxyfluorescein diacetate succinimidyl diester (CFSE) (Invitrogen) by incubation for 5 min at 37°C in the dark at a density of $2x10^6$ cells/ml in RPMI medium. Other cytokines used were as follows: IL-2; 20 ng/ml, IL-6; 10 ng/ml, IL-12; 20 ng/ml, IL-23; 20 ng/ml and IFN-γ; 10 ng/ml.

In vitro response of TCR transgenic CD4$^+$ T cells to peptide

A total of 1 x 10^6 cells of CD4$^+$ T cells from Eα_{52-68}/I-A^b-specific transgenic mice were purified by positive selection with anti-CD4 microbeads and cultured with 5 x 10^5 cells of B cells from C57BL/6 wild type mice in the presence of Eα_{52-68} peptide (3 μM) in flat-bottomed 24-well plates. IL-27 (20 ng/ml), TGF-β1 (20 ng/ml), IL-21 (50 ng/ml), IL-10 (50 ng/ml), and zymosan (25 μg/ml) were added respectively.

RNA Isolation, cDNA Synthesis, and Quantitative Real-Time PCR

CD4$^+$ T-cell RNA was prepared using an RNeasy Micro Kit (Qiagen). RNA was reverse-transcribed to cDNA with random primers (Invitrogen) and Superscript III (Invitrogen) in accordance with the manufacturer's protocol (Invitrogen). The cellular expression level of each gene was determined by quantitative real-time PCR analysis using an iCycler (Bio-Rad). The PCR mixture consisted of 25 μL of SYBR Green Master Mix (Qiagen), 15 pmol of forward and reverse primers, and cDNA samples in a total volume of 50 μL. Expression was normalized to the expression of β-actin. Specific primers for each indicated promoter were listed in Supporting Information Table 1.

Flow Cytometry and cell sorting

Cultured T cells were harvested and stained using predetermined optimal concentrations of the respective antibodies. After Fc blocking (anti-mouse CD16/CD32 mAb), prepared cells were stained with the indicated mAbs: Qdot605 anti-CD4, allophycocyanin anti-LAG3, and SA-allophycocyanin Cy7. For intracellular anti-Egr-2 staining, cells were stained using the Foxp3 staining buffer set (e-Bioscience). For co-staining of Egr-2 and IL-10, cells were re-stimulated for 4 h at 37°C with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml; SIGMA), ionomycin (500 ng/ml; SIGMA), and for

final 2 h with GolgiStop (1μl/ml; BD Biosciences), followed by surface staining. Cells were then fixed with 2% paraformaldehyde for 10 min at room temperature and permeabilized with 0.5% saponin (SIGMA) containing anti-Egr-2 and anti-IL-10 antibodies for 30 min at room temperature in the dark. Analysis and cell sorting of $CD4^+$ T cells were performed using FACSVantage with CellQuest (Becton Dickinson). Data were processed with FlowJo software. A full gating strategy was shown in Supporting Information Fig. 1.

ELISA

Cytokines in culture supernatants of $CD4^+$ T cells were analyzed using ELISA kits according to the manufacturer's instructions (Thermo Scientific and Biolegend).

Luciferase Reporter Assay

The Dual-Luciferase Reporter Assay System was used (Promega). 293T cells were cultured in 96-well plates and transfected with pGL-3-(-1500 Blimp-1) luciferase reporter plasmids and phRL-(thymidine kinase) luciferase control plasmids with either a pMIG vector or pMIG vector containing Egr-2 using Fugene6 (Roche). Cells were harvested 48 hr later and luciferase activity was assessed using MicroLumat Plus LB96V Luminometer (Berthold).

ChIP Assay

Splenocytes from C57BL/6 mice were cultured for 24 h with anti-CD3 antibody (10 μg/ml) and $CD4^+$ T cells were then purified using the MACS system. The ChIP assay was carried out using a Simple ChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology). Briefly, $CD4^+$ T cells were fixed with formaldehyde and quenched with glycine. Crude nuclei were isolated and digested enzymatically using Micrococcal Nuclease and then sonicated to reduce chromatin DNA length to

approximately 500 base pairs (bp). Chromatin solutions was diluted in immunoprecipitation dilution buffer containing protease inhibitor and incubated with anti-Egr-2 antibody (Covance) or normal rabbit IgG. Crosslinks were reversed by incubation overnight at 65°C, and immunoprecipitated chromatin (DNA) was purified by phenol-chloroform extraction and ethanol precipitation. Quantitative PCR analysis was performed using primers; corresponding sequences -3000 bp (Assay position; -2399bp), -2000 bp (Assay position; -1294bp), -1000 bp (Assay position; -388bp) and +1000 bp (Assay position; +601bp) from the transcription start site of Blimp-1, -3000 bp (Assay position; -2386bp), -2000 bp (Assay position; -1388bp), -1000 bp (Assay position; -398bp), and +1000 bp (Assay position; +602bp) from the transcription start site of LAG3, and -1000 bp (Assay position; -399bp) and +1000 bp (Assay position; +605bp) from the transcription start site of IL10. Each primer was obtained from SA Bioscience. The promoter sequence of guanosine monophosphate reductase (Gmpr) was used as a control. PCR products were subjected to gel electrophoresis to check the amplicon size (Supporting Information Fig. 2B).

Statistical analysis

Statistical analysis was performed using the Student's *t*-test. A p-value of <0.05 was considered to indicate a significant difference.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figure 1. IL-27 induces the simultaneous expression of Egr-2 and LAG-3 in $CD4^+$ T cells. (A) Naïve $CD4^+$ T cells from C57BL/6 wild type (WT) mice were cultured with anti-CD3/CD28 mAb. On day5, Egr-2 and IL-10 expression were analyzed by intracellular staining. Data shown are representative of three experiments performed. (B) Naïve $CD4^+$ T cells from TEα TCR transgenic mice were cultured with B cells from WT mice with $E\alpha_{52-68}$ peptide in the presence of indicated factors. Expression of Egr-2, LAG-3, and Foxp3 were measured by quantitative RT-PCR. Data are presented as mean ± SD ($n = 3$; replicate wells). Experiments were performed three times. (C) The induction of Egr-2 in $CD4^+$ T cells stimulated with IL-27. Naïve $CD4^+$ T cells from C57BL/6 WT, Egr-2 CKO, Blimp-1 CKO, and WSX1 KO mice were cultured with anti-CD3/CD28 mAb in the presence or absence of IL-27. Egr-2 expression was detected by intracellular staining on day 5. Data shown are representative of five experiments performed. (D) $CD4^+Egr\text{-}2^+LAG3^+$ T-cell induction was analyzed. Naïve $CD4^+$ T cells from C57BL/6 WT mice were cultured with or without anti-CD3/CD28 mAb under the indicated conditions. Data shown are representative of two or three experiments performed. (E) CFSE-labelled naïve $CD4^+$ T cells from C57BL/6 WT mice were stimulated as in Figure 1C. On day 5, T-cell proliferation and Egr-2 expression was determined by flow cytometry. Data shown are representative of five experiments performed. $*p < 0.05$; Student's *t*-test.

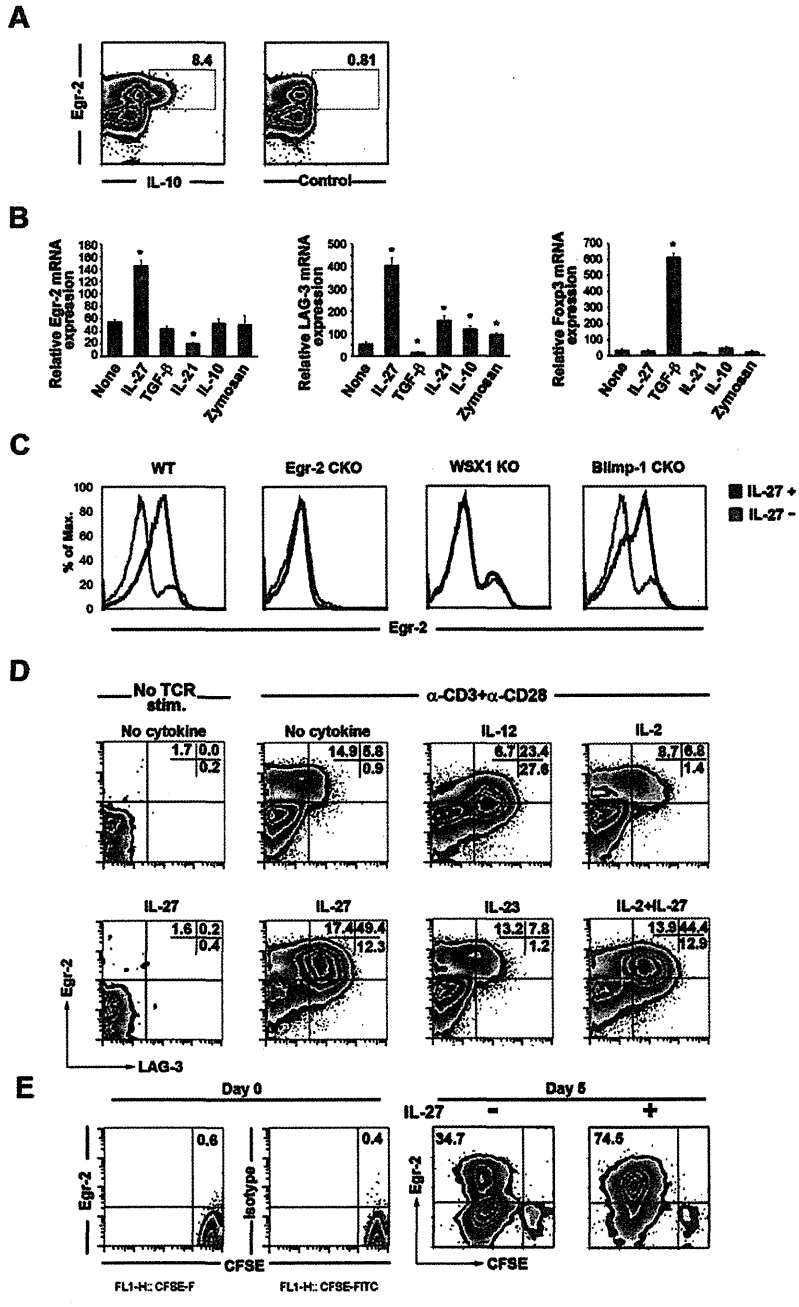


Figure 2. IL-27-mediated induction of IL-10 and Blimp-1. (A) Expression of IL-10 (left) and Blimp-1 (right) mRNA were measured by quantitative RT-PCR. Naïve $CD4^+$ T cells were isolated from C57BL/6 WT, Egr-2 CKO, Blimp-1 CKO, or WSX1 KO mice and cultured with anti-CD3/CD28 mAb in the presence or absence of IL-27. Data are shown as mean ± SD (n = 3; replicate wells). Experiments were performed four times. (B) IL-10 concentrations in culture supernatants of stimulated $CD4^+$ T cells from C57BL/6 WT, Egr-2 CKO, Blimp-1 CKO, or WSX1 KO mice were measured by ELISA. Data are shown as mean ± SD (n = 3; replicate wells). Experiments were performed three times. (C) Expression of IL-10 and Blimp-1 mRNA were measured by quantitative RT-PCR. Naïve $CD4^+$ T cells were isolated from C57BL/6 WT and cultured with anti-CD3/CD28 mAb under the indicated condition. Data are shown as mean ± SD (n = 3; replicate wells). Experiments were performed two times. *$p < 0.05$; Student's t-test.

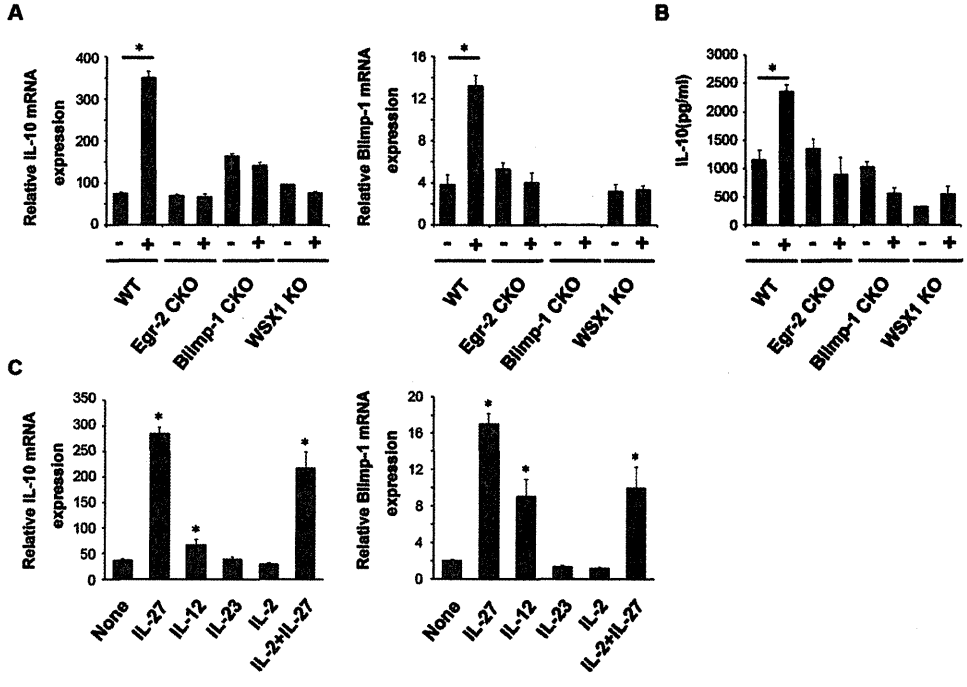


Figure 3. Blimp-1 promoter activity is directly regulated by Egr-2. (A) Egr-2 enhanced luciferase activity regulated by the Blimp-1 promoter. 293T cells were co-transfected with pGL-3-(-1500 Blimp-1)-Luc vector or pGL-3-Basic-Luc control vector and a pMIG-Egr-2 vector or a pMIG mock vector. Luciferase activity was measured 24 h after transfection. Data are shown as mean ± SD of duplicates, from one experiment representative of at least three performed. (B) ChIP-coupled quantitative PCR analysis of Egr-2 binding to promoter regions in mouse $CD4^+$ T cells. Normal IgG and anti-Egr-2 antibodies were used for immunoprecipitation assays. The Gmpr locus was used as a negative control. The enrichment of Egr-2 binding to each promoter was determined. Mean ± SD of triplicates done in one experiment representative of at least three performed are shown.

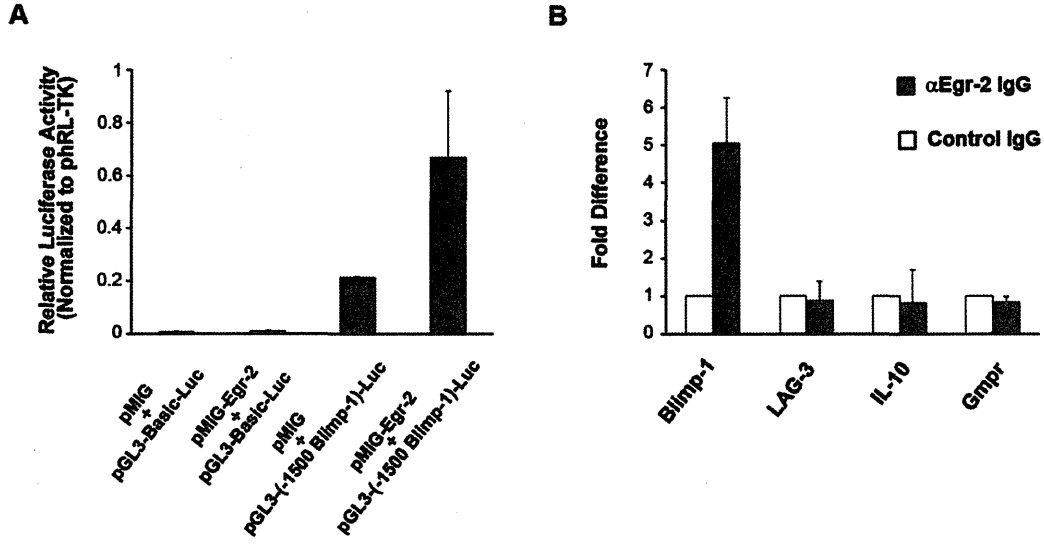


Figure 4. IL-27-induced Egr-2 expression in STAT1- and STAT3- deficiencies. (A) Naïve $CD4^+$ T cells from STAT1 KO, STAT3 CKO, and control mice were cultured with or without IL-27 in the presence of anti-CD3/CD28 mAb. Egr-2 expression was analysed by flow cytometry on day 5. Plots are gated-on $CD4^+$ cells. Data shown are representative of three experiments performed. (B) Naïve $CD4^+$ T cells were cultured as shown in (A). The percentage of Egr-2-expressing cells in $CD4^+$ T cells was analysed by flow cytometry on day 5. Data are shown as mean ± SD (n = 3; replicate experiments). (C) The expression of IL-10 mRNA was measured by quantitative RT-PCR. Naïve $CD4^+$ T cells were isolated from STAT1 KO, STAT3 CKO, or control mice and cultured with anti-CD3/CD28 mAb in the presence or absence of IL-27. Data are shown as mean ± SD (n = 3; replicate wells). Experiments were performed two times. (D) IL-10 concentrations in culture supernatants of stimulated naïve $CD4^+$ T cells as in (C) from STAT1 KO, STAT3 CKO, or each control mice were measured by ELISA. Data are shown as mean ± SD (n = 3; replicate wells). Experiments were performed three times. (E) Naïve $CD4^+$ T cells were cultured as shown in (A) under the indicated condition. Egr-2 expression was analyzed by intracellular staining. Data shown are representative of three experiments performed. (F) Expression of IL-10 and Blimp-1 mRNA were measured by quantitative RT-PCR. Naïve $CD4^+$ T cells were cultured as shown in (A) under the indicated condition. Data are shown as mean ± SD (n = 3; replicate wells). Experiments were performed two times. $^*p < 0.05$; Student's t-test.

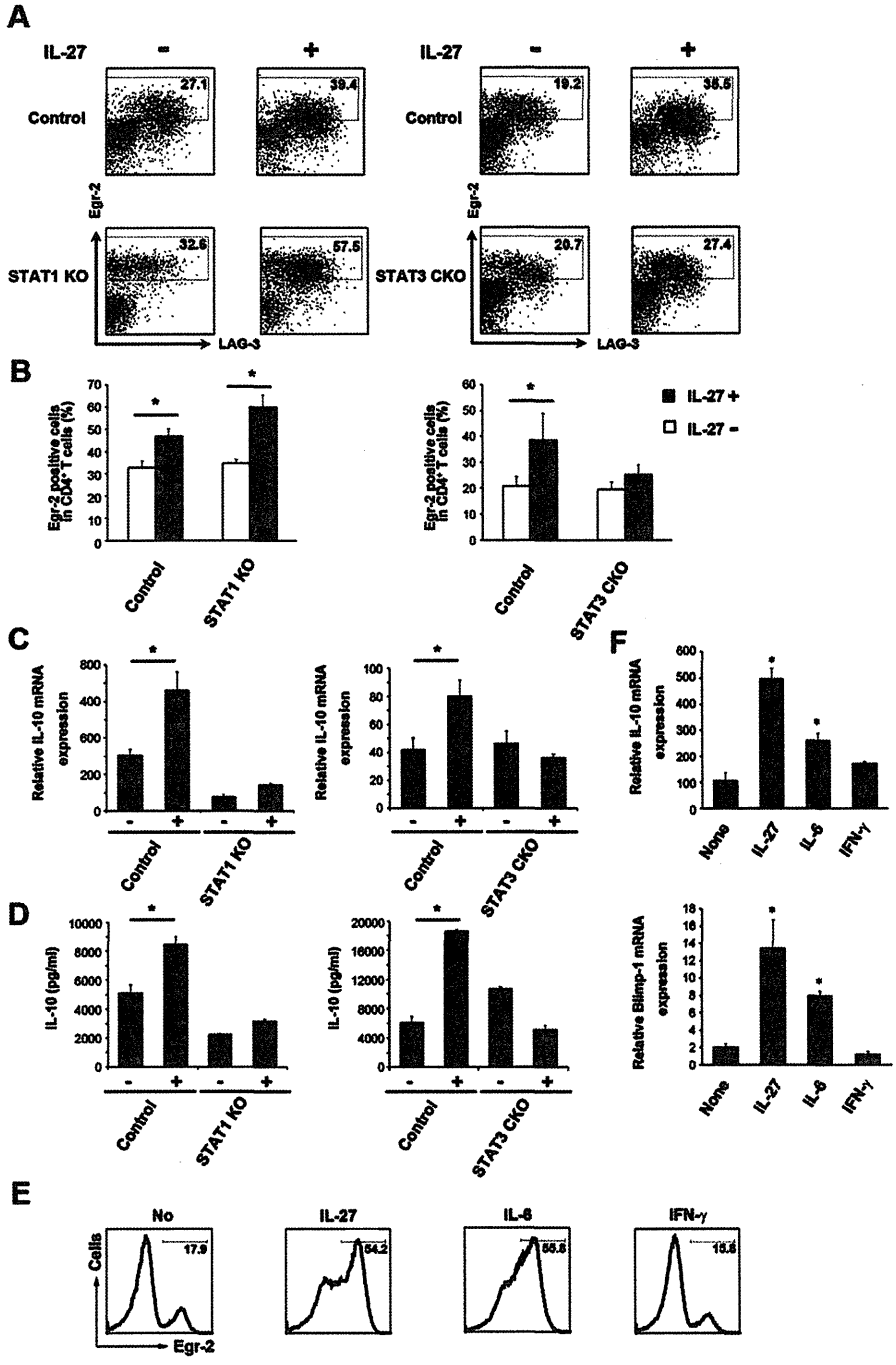
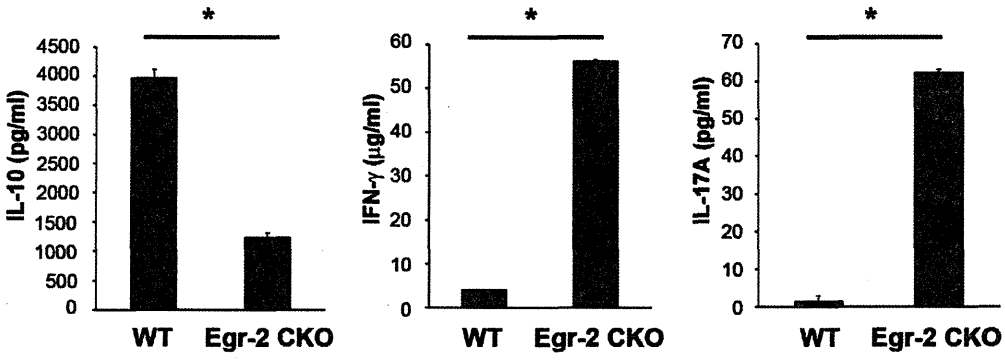


Figure 5. IL-10, IFN-γ, and IL-17 production in response to IL-27 stimulation. Naïve $CD4^+$ T cells from C57BL/6 WT and Egr-2 CKO mice were cultured with anti-CD3/CD28 mAb in the presence of IL-27. On day 5, IL-10, IFN-γ, and IL-17 concentrations in culture supernatants were measured by ELISA. Data are shown mean ± SD ($n = 3$; replicate wells). Experiments were performed two times. *$p < 0.05$; Student's t-test.



CD3 ζ defects in systemic lupus erythematosus

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ABSTRACT

The prototype autoimmune disease, systemic lupus erythematosus (SLE), has been known to be associated with deficiency of ζ chain, a component of the T-cell receptor–CD3 complex. Comprehensive analysis has shown that expression of the CD3 ζ chain is attenuated or absent in over half of SLE patients. Furthermore, aberrant transcripts of the CD3 ζ chain, including spliced variants lacking exon 7 or having a short 3′-untranslated region, have been detected in SLE T cells. Although attenuated expression of the CD3 ζ chain is also observed in cancer patients, infections and other autoimmune diseases, sustained attenuation of the CD3 ζ expression accompanied with aberrant transcripts are only observed in SLE. In this study, the authors review the unique features of CD3 ζ defects observed in SLE and discuss the molecular basis of the defects by recent findings in animal models, single-nucleotide polymorphisms and genome-wide association studies.

Systemic lupus erythematosus (SLE) is a prototype autoimmune disease with a wide spectrum of clinical manifestations.[1] While abundant production of autoantibodies and subsequent formation of immune complexes lead to tissue damage such as glomerulonephritis,[2 3] a particularly crucial role in SLE pathogenesis is played by SLE T cells.[2 4] In addition, comprehensive messenger RNA expression analysis has revealed that type I interferon (IFN)-related genes are upregulated in peripheral blood cells, partly through the increased production of IFN.[5–7] Although all of these events may interact with each other, the detailed molecular characterisation of SLE T cells is required. Here, we focus on the abnormal expression of the ζ chain of T-cell receptor (TCR)–CD3 complexes (CD3 ζ chain, TCR ζ chain, CD247) in patients with SLE, and discuss how the molecular basis of the defects affect developing autoimmune diseases.

DEFECTIVE EXPRESSION OF THE CD3 ζ CHAIN IN SLE PATIENTS

When SLE T cells are stimulated in vitro or in vivo through TCR–CD3 complexes, the response is usually attenuated in SLE T cells, compared with normal T cells.[8 9] However, the direct activation of protein kinase C with phorbol ester and ionomycin leads to normal or enhanced response in SLE T cells, raising a hypothesis that defects might reside in the proximal signal transduction molecules between the TCR–CD3 complexes and protein kinase C[9] (figure 1). Indeed, the tyrosine phosphorylation of cellular proteins, particularly the CD3 ζ chain, was diminished in SLE T cells in response to anti-CD3 and anti-CD4 treatment.[10–12] Surprisingly, the protein expression of the CD3 ζ chain was diminished in SLE patients.[10 11] While reduced protein expression of the CD3 ζ chain in cancer patients, infections and other autoimmune diseases such as rheumatoid arthritis has been reported to be transient,[13] the reduction was maintained throughout the course of the disease in more than half of the SLE patients.[12 14 15]

MECHANISM OF REDUCED PROTEIN EXPRESSION OF THE CD3 ζ CHAIN IN SLE PATIENTS

Several mechanisms are responsible for the decreased expression of the CD3 ζ chain, including low transcription activity,[11] generation of spliced variants,[10 16 17] increased ubiquitination,[18] increased caspase-3-dependent proteolysis,[19] redox status,[20] oxidative stress,[21] heat stress,[22] chronic exposure to pro-inflammatory cytokines[23] and direct contact with activated macrophages.[24] No mutations or deletions have been identified in the 5′-flanking region of the CD3 ζ gene in patients with SLE,[25] while other researchers have found those in the 5′-flanking region.[16] On the other hand, we and others have detected abnormal transcripts of the CD3 ζ chain, such as the splice variants including those lacking exon 7 and with the 3′-untranslated region (UTR).[10 16 26 27] The η (exon 1–7 plus exon 9) and the ι (exon 1–7 plus exon 10) variants have been shown to be generated by alternative splicing of the CD3 ζ chain on human and mouse chromosome 1q22–23[28–32] (figure 2); however, the functions of these splice variants are not fully understood. The CD3 ζ chain lacking exon 7 and short 3′-UTR variants, which are exclusively observed in SLE patients, are new class spliced variants[9] (figure 2).

ROLE OF UNIQUE SPLICE VARIANTS IN DEFECTIVE EXPRESSION IN THE CD3 ζ CHAIN

A 562-bp region containing the consensus sequence for mRNA stabilisation and a 31-nucleotide conserved sequence is missing from the short 3′-UTR splice variant.[17] As this conserved region is important for stabilisation, transportation and localisation of the CD3 ζ chain,[33] we speculated that this short 3′-UTR splice variant accounts for the downregulation of protein expression. To test this hypothesis, mRNA from the spliced variants was transfected into mouse T-cell hybridomas lacking CD3 ζ, resulting in instability of CD3 ζ mRNA and thereby leading to reduced protein expression.[27]

The 3′-UTR region of mRNA is known to control the turnover rate of presynthesised mRNA through interactions with trans-acting factors by altering mRNA stability and affecting its transportation and localisation.[34] mRNA 3′-UTR contains cis-acting, adenosine–uridine-rich elements that bind to trans-acting proteins and participate in either the stabilisation or destabilisation of transcripts. Adenosine–uridine-rich elements are located at positions +735, +803 and +1646 of the CD3 ζ mRNA,

and the second element is found within the deleted sequence in the short 3′-UTR variant (figure 3). This notable absence may affect the stability of the short 3′-UTR variant mRNA. Using deletion mutants we found that the regions +871 to +950 and +1070 to +1136, which contain conserved regions one and two, respectively, are necessary to maintain the stability of CD3 ζ mRNA (figure 3). Similar transcript instability has been shown in another variant lacking exon 7,[35] suggesting that exon deletion and exon skipping also lead to the downregulation of protein expression through mRNA instability.

DEFECTS OF PROXIMAL SIGNAL TRANSDUCTION MOLECULES IN T CELLS IN MODEL ANIMALS

The SKG mouse, which models human autoimmune arthritis, exhibits a ζ-associated protein 70-kDa loss-of-function mutation, suggesting that one cause of the disease is defective proximal signalling molecules in T cells.[36] It is speculated that the thymic selection process has been altered by defective signal transduction, resulting in positive, but not negative, selection of autoreactive clones. More comprehensive data have been obtained by a study that tested whether the loss of tyrosine residues in each immunoreceptor tyrosine-based activation motif (ITAM) domain of a TCR–CD3 complex leads to autoimmunity.[37] That study demonstrated that scalable defects in signalling capability of the TCR–CD3 complex lead to multi-organ systemic autoimmune diseases such as interstitial pneumonitis, bowel inflammation and liver inflammation. Interestingly, a defect in two ITAM domains is sufficient to allow the development of autoimmune diseases and produces skewed cytokine production from interleukin (IL) 2 to IFNγ.

CONSEQUENCES OF EXPRESSING THE UNIQUE CD3 ζ CHAIN SPLICE VARIANTS

The evidence obtained from the model animal introduced an attractive hypothesis that defective signal transduction in T cells can be a cause of autoimmunity, partly through the altered thymic selection. One may raise the question as to whether other mechanisms may be responsible for developing skewed cytokine production and multi-organ disease. In this regard, it is interesting to know the common upregulated and downregulated genes after introducing two unique spliced variants of the CD3 ζ chain. After transfecting the short 3′-UTR and exon 7-lacking spliced variants from SLE patients into mouse T-cell hybridoma defective for CD3 ζ, DNA microarray analysis has been performed in two transfectants.[38] While only 16 common genes were upregulated in both the short 3′-UTR and exon 7-lacking variants, 36 shared genes were downregulated. We further supported that these results using real-time PCR, showing that expression levels of IL-2, IL-13, IL-15, IL-18 and transforming growth factor β-2 were significantly reduced in both spliced variants compared with those found after transfection with wild-type CD3 ζ.[38] In contrast, levels of Gsta4, Gzma, Lcn2, Mad3, Pmm1, Ptp4a3, Pvrl2, Sdc1, Selenbp1, Slc4a8, Tcf7 and Wasl were significantly increased. The possibility of whether these molecules may be involved in tissue inflammation or damage in SLE remains to be elucidated in the future.

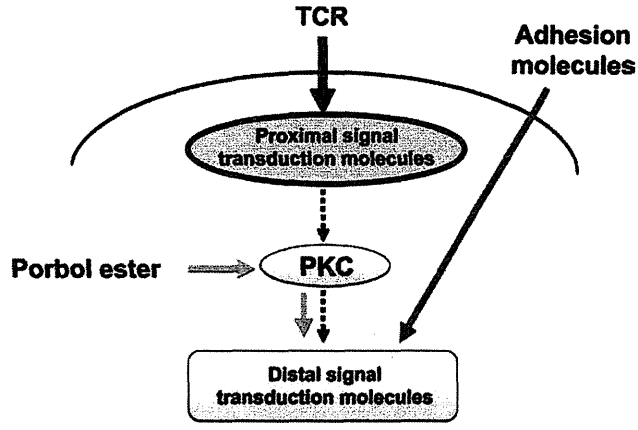

Figure 1 Functional defects of systemic lupus erythematosus (SLE) T cells. Phorbol ester can restore defects associated with SLE, indicating that proximal signal transduction molecules may be responsible for these defects. In contrast, adhesion molecules and their downstream signalling molecules are upregulated, raising the possibility that signals via adhesion molecules can bypass the proximal transduction molecules. PKC, protein kinase C; TCR, T-cell receptor.

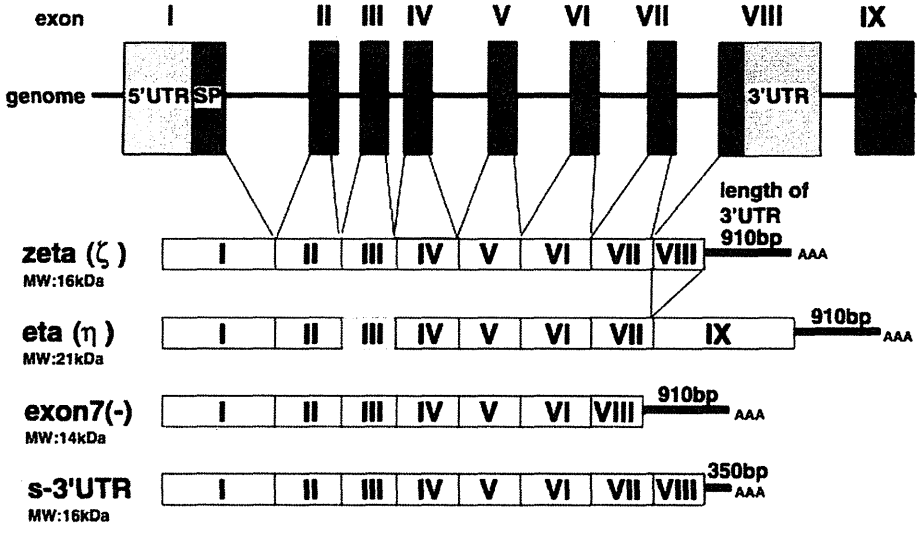

Figure 2 Structure of spliced variants of the human CD3 ζ chain. Exon–intron organisation of CD3 ζ chain genes and their transcripts for wild-type and spliced variants found in systemic lupus erythematosus (lacking exon 7 and short 3′-untranslated region; UTR).