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Title

Strong TCR-mediated signals suppress integrated stress responses induced by KDEL1 deficiency
in naïve T cells

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Running title: A link between TCR-mediated signals and stress responses

Keywords: TCR signal, T-cell homeostasis, KDEL receptors, Stress responses

Abbreviations used in this study:

APL, altered peptide ligand; eIF2 α , α subunit of eukaryotic initiation factor 2; ER, endoplasmic reticulum; ISR, integrated stress responses; MFI, mean fluorescent intensity; OVA, ovalbumin; PP1, protein phosphatase 1; R, receptor; GT, gene-trap

Abstract

KDEL receptor 1 (KDELRL1) regulates integrated stress responses (ISR) to promote naïve T cell survival in vivo. In a mouse line having nonfunctional KDELRL1, T-Red (naïve T cell reduced) mice, polyclonal naïve T cells show excessive ISR and eventually undergo apoptosis. However, breeding T-Red mice with TCR-transgenic mice bearing relatively high TCR affinity rescued the T-Red phenotype, implying a link between ISR-induced apoptosis and TCR-mediated signaling. Here, we showed that strong TCR stimulation reduces ISR in naïve T cells. In mice lacking functional KDELRL1, surviving naïve T cells expressed significantly higher levels of CD5, a surrogate marker of TCR self-reactivity. In addition, higher TCR affinity/avidity was confirmed using a tetramer dissociation assay on the surviving naïve T cells, suggesting that among the naïve T-cell repertoire, those that receive relatively stronger TCR-mediated signals via self-antigens survive enhanced ISR. Consistent with this observation, weak TCR stimulation with altered peptide ligands decreased the survival and proliferation of naïve T cells, whereas stimulation with ligands having higher affinity had no such effect. These results suggest a novel role of TCR-mediated signals in the attenuation of ISR in vivo.

Introduction

The homeostasis of naïve T cells is mediated by MHC-TCR interactions and cytokines, such as IL-7, whereas that of memory T cells is largely dependent on IL-7 and IL-15 (1). When peripheral T cells are decreased due to involution of the thymus by aging, infections or irradiation, the remaining T cells proliferate, a phenomenon called homeostatic proliferation, which is induced by TCR signaling and/or cytokines (2). Homeostatic proliferation also plays a role in the activation status of peripheral T cells, causing several autoimmune diseases (3-6). The apoptosis of peripheral T cells is regulated by a balance between proapoptotic Bim and antiapoptotic Bcl-2 family member proteins (1,7). Under stress conditions such as endoplasmic reticulum (ER) stress, Bim transcription is controlled by the transcription factor CHOP rather than the forkhead box O family (8). Although it is well known that survival signals from MHC-TCR interactions and cytokines are important for T-cell homeostasis, the role of TCR-mediated signals in stress responses during T-cell homeostasis is not well understood.

We previously performed mouse mutagenesis screening using a DNA alkylating agent, N-ethyl-N-nitrosourea, and identified a mutant strain having very low numbers of naïve T cells, T-Red mice (naïve T-cell reduced) (9). Several T-cell-mediated immune responses, including collagen-induced arthritis, are significantly attenuated in T-Red mice (9). Positional cloning and sequence analysis revealed that T-Red mice have a point mutation in the KDEL receptor 1 (Kdelr1)

gene, which causes an amino acid substitution at serine-123, the residue suggested to be important for KDELR conformation (9,10). KDELR was originally found to act as a chaperone retrieval receptor that recovers soluble endoplasmic reticulum (ER)-resident proteins from the cis-Golgi. This retrograde transport requires the binding of KDELR with a KDEL motif localized at the C-terminal of ER proteins (11-13). In mammals, there are three KDEL receptors, KDELR1, KDELR2 and KDELR3, all of which are localized around ER and Golgi (14). Consistent with their localization, it has been reported that KDELR is involved in ER-stress responses (15). Their involvement in autophagy is also reported (16). Our mechanistic studies using T-Red mice and Kdelr1-deficient mice revealed a novel function of KDELR1 in naïve T cells. KDELR1 was necessary for the termination of integrated stress responses (ISR) in naïve T cells through the control of protein phosphatase 1 (PP1) activity, a key phosphatase for the negative regulation of ISR. In T-Red and T-cell-specific Kdelr1-deficient mice, the homeostasis of naïve T cells is impaired by exaggerated ISR due to reduced PP1 activity resulting from KDELR1 dysfunction, which leads to elevated Bim levels in and apoptosis of naïve T cells (9). However, the relationship between TCR signaling and ISR in naïve T cell homeostasis has not been reported.

ISR are general stress-response programs known to cope with various types of stress signals, including ER stress, amino acid deprivation, infection with double-stranded RNA viruses, heme

deficiency, and oxidative stress, and phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2 α) at serine 51 is induced (17-20). These diverse stress signals activate four kinases: double-stranded RNA-dependent protein kinase R (PKR), RNA-dependent protein kinase-like ER kinase (PERK), general control nonrepressed 2 (GCN2) and heme-regulated eIF2 α kinase (HRI) (21). The activation of these kinases phosphorylates eIF2 α serine 51, which attenuates global translation, while activation of ISR via eIF2 α alteration induces the expression of stress-related genes and those involved in apoptosis induction, including Bim, CHOP and Trib3 (22). While ISR plays a role in reducing cellular stresses to recover homeostasis, prolonged phosphorylation of eIF2 α is known to induce apoptosis (23,24). Dephosphorylation of eIF2 α is mediated by PP1 via interaction with various regulatory proteins such as Bip, GADD34 and CreP (25). Additionally, our recent study revealed that KDELRL1 is associated with PP1 and is required for optimal PP1 activity in naïve T cells (9). Although it has not been shown if TCR signal triggers ISR in naïve T cells, particularly during steady state in vivo, defects in ISR are associated with the development of several important pathologies, including diabetes, Alzheimer's disease and viral infection (26-28).

We recently found that the impaired homeostasis of naïve T cells in T-Red mice, was restored in the offspring from crossing T-Red mice with OT-I TCR transgenic (Tg) mice, which have transgenic expression of ovalbumin (OVA)-specific, MHC class I-restricted TCR, or P14 TCR Tg mice, which

express lymphocytic choriomeningitis virus-specific, MHC class I-restricted TCR. These TCRs have relatively high self-affinity TCRs against endogenous selecting antigens (29,30). These phenomena led us to hypothesize that TCR signals, particularly high avidity ones, suppress ISR. Here we report that surviving naïve T cells in mice show evidence of higher affinity/avidity to self-antigens, suggesting that naïve T cells that receive less TCR-mediated signaling are more susceptible to ISR. Furthermore, using OVA altered peptide ligands (APLs) with various affinities to OT-I TCR (31,32), we revealed that a TCR stimulation below a certain threshold reduces the survival and proliferation of naïve T cells derived from T-Red/OT-I cells in vitro and in vivo, which is consistent with the idea that strong signals from high-affinity TCR overcome ISR triggered by KDELR1 dysfunction. These results uncover a novel role for TCR-mediated signals in the reduction of ISR in naïve T cells at steady state in vivo.

Materials and Methods

Mouse strains

C57BL/6 mice were purchased from SLC Japan (Shizuoka, Japan). B6.PL (Thy1.1) and B6.SJL (CD45.1) congenic mice were obtained from the Jackson Laboratory (Bar Harbor, ME). OT-I TCR-Tg mice were kindly provided by Dr. W. R. Heath (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). T-Red mice and Kdelr1^{flox/flox};CD4cre mice were generated as reported previously (9). Kdelr2 gene-trap mice were purchased from the Mutant Mouse Regional Resource Center (Davis, California), and backcrossed to C57BL/6 background more than five generations. All mice were maintained under specific pathogen-free conditions according to the protocols of Osaka University Medical School and Institute for Genetic Medicine, Hokkaido University. All animal experiments were performed following the guidelines of the Institutional Animal Care and Use Committees of the Graduate School of Frontier Bioscience and Graduate School of Medicine, Osaka University, and Institute for Genetic Medicine, Hokkaido University.

Flow cytometry and cell sorting

Single cell suspensions were prepared from the spleen, lymph nodes and peripheral blood, and red blood cells were lysed in 0.165 M NH₄Cl solution. For cell surface labeling, approximately 10⁶ cells were incubated with fluorescence-conjugated antibodies for 30 minutes on ice. After washing,

cells were analyzed with the CyAn flow cytometer (Beckman Coulter, Tokyo). Intracellular staining of phosphorylated ZAP70/Syk was performed using the Cytofix/Cytoperm kit (BD Biosciences, Tokyo) after staining of cell surface molecules. Foxp3 intracellular staining was carried out using the Foxp3 Fixation/Permeabilization kit (eBioscience, Tokyo). Collected data were analyzed using FlowJo software (Tree Star, Ashland, Oregon). To purify naïve CD44^{Low} T cells, splenocytes and lymph node cells were sorted based on their CD44 expression levels using the Moflo cell sorter (Beckman Coulter). Cell purity was routinely > 98%. The following antibodies were used: FITC-conjugated anti-Foxp3, anti-CD5, anti-CD44 and anti-CD45.2; PE-conjugated anti-IL-2R β , anti-CD5, anti-CD62L, anti-CD90.1 and anti-TCR V α 2; PerCP-conjugated anti-CD90.2 and anti-Ly6C; PE-Cy7-conjugated anti-CD8; APC-conjugated anti-CD25, anti-CD45.1, anti-CD90.1 and anti-TCR β ; Alexa Fluor 647-conjugated anti-ZAP70 (pY319)/Syk (pY352); and eFluor450-conjugated anti-CD4 and anti-CD8 antibodies (eBioscience, Tokyo, BD Biosciences, Tokyo and Biolegend, Tokyo).

Retroviral transduction of Bcl-2 in hematopoietic stem cells

pMSCV-IRES-GFP Bcl-2 or mock vectors and T-Red bone marrows were used to generate bone-marrow chimeras, as described previously (9).

In vitro experiments with OVA APLs

For the in vitro survival assays of OT-I cells that used APLs, OT-I (CD45.1⁺ or CD90.1⁺) or T-Red OT-I (CD45.1⁻CD90.1⁻) splenocytes were mixed at 1:1 and cultured with the original ligand, SIINFPEKL (N4), or weaker ligands, SIITFEKL (T4) or SIIVFEKL (V4) (31-33), for 6 hours at 37°C. These peptides were synthesized by Sigma (Tokyo, Japan). The live OT-I cell population (TCR V α 2⁺CD8⁺ and CD90.1⁺ or ⁻) was determined as 7AAD (Biolegend) negative. The Annexin V Apoptosis Detection Kit (Biolegend) was used for Annexin V staining.

In vivo experiments with OVA APLs

Sorted CD44^{Low} OT-I (CD45.1⁺ or CD90.1⁺) and CD44^{Low} T-Red/OT-I (CD45.1⁻CD90.1⁻) cells were mixed at a 1:1 ratio and labeled with 2.5 μ M CFSE (Life Technologies, Tokyo) for 10 min at 37°C. These CFSE-labeled OT-I T cells were transferred i.v. into congenic hosts (CD45.1⁺ or CD90.1⁺). OVA APLs (N4, SIINFPEKL; Y3, SIYNFEKL; T4, SIITFEKL; Q4H7, SIIQFEHL; V4, SIIVFEKL; E1, SIINFPEKL) were synthesized by Sigma (31-33). These peptides were injected i.v. at 100 nmol per mouse, and cell divisions were examined 2 or 4 days post peptide injection at steady state (lympho-replete) or 5 Gy-irradiated lymphopenic conditions, respectively. Intracellular staining of phosphorylated eIF2 α was performed using anti-phospho eIF2 α (S51) (Abcam, Tokyo), Alexa Fluor 647-conjugated goat anti-rabbit IgG (Thermo Fisher, Yokohama), and the Transcription

factor buffer set (BD Biosciences, Tokyo).

Western blotting of eIF2 α

Sorted CD44^{Low} naïve CD4 T cells were stimulated with anti-CD3/anti-CD28-coated beads (Life Technologies) or 20 ng/ml IL-7 (Peprotech, Tokyo) at 37°C. Total cell lysate was prepared in RIPA buffer, and western blotting was performed by a standard method. Anti-phospho eIF2 α (S51) and anti-eIF2 α (Abcam, Tokyo) were used for blotting.

Tetramer dissociation assay

The tetramer dissociation assay was performed as described previously (34,35). Splenic T cells were purified using anti-Thy1.2 magnetic beads (Miltenyi Biotec, Tokyo). Then, T cells were stained with FITC-labeled CD44, PE-labeled H-2Kb/OVA SIINFEKL tetramer (MBL, Nagoya, Japan), Alexa Fluor 647-labeled anti-CD8 (KT15, MBL), Pacific blue-labeled anti-CD4 and anti-MHC class II (for dump, Biolegend) in the presence of anti-CD16/32 antibody (2.4G2) on ice for 60 min. After washing, cells were incubated for 15 min at 37°C in the presence of 50 μ g/ml anti-MHC class I (28-8-6, Biolegend). Cells were fixed and analyzed by the flow cytometer CyAn.

Statistical analysis

Student's t-test (two-tailed) was used for the statistical analysis of differences between two groups.

One-way ANOVA with post-hoc Turkey's test was used for multiple comparisons. P values less than 0.05 were considered statistically significant.

Results

T-Red mice have excessive T cells with the memory/activated phenotype (9). We found a relative increase in the CD44^{High} memory-phenotype T-cell population and a reduction in the CD44^{Low} naïve T-cell population (Fig. 1A and data not shown). Other markers for memory-phenotype T cells, such as IL-2R β and Ly6C, were also upregulated, whereas CD62L levels were unchanged in mutant T cells (Fig. 1A). The upregulation of these memory markers was a relative phenomenon due to the significant reduction of naïve T cell numbers in T-Red mice and T-cell-specific Kdelr1-deficient mice (Figs. 1B and 1D). The loss of naïve T cells in T-Red and Kdelr1-deficient mice is progressive; the phenotype becomes more prominent > 7-9 weeks old (9), suggesting cellular stresses in the periphery may accumulate over time to cause naïve T cell apoptosis in mice that lack functional KDELR1. We found that T cells expressed KDELR1 and KDELR2, but not KDELR3 (Supplementary Fig. 1). The KDELR family members are highly homologous in terms of amino acid sequences (14). We showed that compared to KDELR1 deficiency, the number of naïve T cell was further decreased in mice with homozygous deficiency of KDELR1 plus heterozygous of KDELR2 in T cells (Figs. 1C and 1D). Thus, we consider that not only KDELR1, but also KDELR2 is involved in the regulation of ISR in naïve T cells in vivo. We crossed T-Red mice with TCR Tg mice for immunological studies and surprisingly found that CD44 levels in and the

absolute numbers of T cells in T-Red/TCR Tg mice were not significantly reduced compared with those in control/TCR Tg mice (Figs. 1E-1G, and (9)).

We next investigated how naïve T cells from T-Red mice having TCR Tg are rescued from the ISR-mediated reduction *in vivo*. Because the V(D)J recombination events of TCR α and β chains were not fundamentally impaired in T-Red mice (9), we hypothesized that TCR affinity/avidity to self antigens might be involved. Consistent with this notion, it is known that T cells receive a low but certain level of constant TCR-mediated signaling from endogenous antigens even at steady state (30,36,37). CD5 is an activation marker and up-regulated by TCR stimulation, and thus is used as a reliable surrogate marker for the self-reactivity of polyclonal T cells, particularly those with the naïve phenotype (30,38-40). For example, the transgenic expression of GFP under Nur77 promoter showed that the Nur77-GFP signal is higher in the CD5^{High} population than CD5^{Low} population in both CD4 and CD8 polyclonal naïve T cells (30). In addition, CD5 levels on naïve CD8⁺ T cells are decreased after adoptive transfer to MHC class I deficient mice (41). It is also reported that compared to the CD5^{Low} population, the CD5^{High} population shows higher levels of CD3 ζ phosphorylation in polyclonal naïve T cells (39). We confirmed that the CD5^{High} population has higher ZAP70/Syk phosphorylations than the CD5^{Low} population in polyclonal naïve CD8⁺ T cells (Supplementary Fig. 2). These lines of evidence support a correlation between CD5 expression

levels and self-reactivity in naïve T cells in vivo. It is known that OT-I and P14 T cells express higher levels of CD5 than WT polyclonal CD8⁺ T cells (29,30,39). Therefore, we hypothesized that relatively stronger TCR-mediated signals from the high affinity OT-I and P14 TCRs might attenuate ISR and protect T-Red naïve CD8⁺ T cells from apoptosis. If this theory is true, then the surviving naïve T cells in T-Red mice would bear higher self-affinity/avidity TCRs and therefore express more CD5 than WT naïve CD8⁺ T cells. Indeed, CD5 levels in CD44^{Low} naïve CD4⁺ and CD8⁺ T cells from T-Red mice were significantly higher than those from WT mice (Figs. 2A-2C). We found that OT-I T cells expressed higher CD5 levels than did WT or T-Red CD8⁺ T cells, while CD5 expressions were similar between control OT-I and T-Red/OT-I cells (Figs. 2B-2C). Moreover, it is known that the dissociation rate of peptide-MHC tetramer from T cells positively correlates with affinity/avidity between the TCR and peptide-MHC (34,35). We used a foreign peptide-loaded tetramer (H-2Kb/SIINFEKL) to estimate self-reactivity by tetramer dissociation assay, because it is reported that the strength of self-reactivity is directly correlated to the strength of TCR binding to foreign antigens including SIINKFEL in naïve CD8⁺ T cells (39,42,43). Consistent with the higher CD5 levels (Fig. 2A-2C), the dissociation of H2-Kb/SIINFEKL tetramer in the polyclonal T-Red CD8⁺ T cell population was reduced as compared with that in the WT CD8⁺ T cell population (Figs. 2D-2E). These results indicated that the surviving naïve T cells in T-Red mice have higher self-affinity/avidity.

The results above led us to examine the relationship between TCR signal strength and ISR-mediated apoptosis. Therefore, we employed ovalbumin (OVA) altered peptide ligands (APL) as a model antigen (31-33) and used them to stimulate control OT-I and T-Red/OT-I T cells to assess survival in vitro. We found that the original OVA peptide, SIINFEKL (N4), which triggers a high avidity, strong TCR signal, increased the survival of both control and T-Red/OT-I T cells similarly (Fig. 3A), while stimulation with the much weaker OVA APL, SIIVFEKL (V4), had no effect on either (Fig. 3B). Importantly, the OVA APL, SIITFEKL (T4), which triggers an intermediate avidity, middle level TCR signal (approximately 8 times lower K_d value than N4 and >2.3 times higher K_d value than V4 (33)), did not promote the survival of T-Red/OT-I T cells as efficiently as it did that of control OT-I T cells (Fig. 3C). Similar results were obtained with 7AAD/Annexin V double staining for apoptosis (Fig. 3D). Furthermore, in this culture condition, no cell divisions were observed (Fig. 3E). Thus, these results suggested that a high TCR-mediated signal promotes the survival of T-Red T cells with enhanced ISR in vitro.

We next performed similar experiments in vivo by injecting various OVA APLs, including N4, T4, V4, SIYNFEKL (Y3), SIIQFEHL (Q4H7) and EIINFEKL (E1) plus CFSE-labeled CD44^{Low} control OT-I and T-Red/OT-I T cells that had been co-transferred in congenic WT hosts (Fig. 4A). We found

that the TCR signal strength induced robust cell divisions in control OT-I and T-Red/OT-I T cells in vivo and that this induction was greater than that from T4 stimulation (i.e. N4, Y3 and T4) (Fig. 4B and C). On the other hand, the weaker ligand, Q4H7, induced significantly different cell divisions, as control OT-I T cells divided more than did T-Red/OT-I T cells (Fig. 4B and D). OVA APLs with affinities weaker than Q4H7 (i.e. V4 and E1) or vehicle treatment did not induce cell divisions under this experimental condition in vivo (Fig. 4B).

We also examined the effect of APLs under lymphopenic condition in which endogenous antigens stimulated more TCR-mediated signals due to the increase of relative MHC numbers caused by the reduction of total T cells and subsequent homeostatic proliferation ((2) and Fig. 4E). In this condition, we found no difference in the degree of cell divisions between control OT-I and T-Red/OT-I T cells with T4, Q4H7 or vehicle alone (Fig. 4F and data not shown), suggesting that the signal threshold that differentiates cell divisions under the lymphopenic condition may be below the average TCR signal strength from endogenous self-antigens. These results suggested that high affinity TCR-mediated signaling transduced a survival signal that makes enhanced ISR in T-Red T cells ineffective, but that low affinity TCR signaling leaves T-Red T cells sensitive to enhanced ISR in vivo.

Discussion

It has been suggested that the homeostasis of naïve T cells is maintained by TCR signals from endogenous self-peptides/MHC complexes and cytokines such as IL-7 in steady state in vivo. In addition to these survival signals, our previous study suggested that cellular stress responses, collectively termed ISR, adversely contribute to the survival of naïve T cells in vivo through the association of KDELR1 with PP1 (9). In mice with KDELR1 dysfunction, such as T-Red mice and Kdelr1-deficient mice, naïve T cell survival is impaired (9). A key event that triggers ISR is the phosphorylation of eIF2 α doctrinally induced by one of four kinases, PERK, GCN2, PKR and HRI, depending on the stress type (21). Freshly isolated naïve T cells and thymus contain a certain level of eIF2 α phosphorylation (9,44), suggesting that T cells sense cellular stresses even at steady state in vivo. However, the signals that counteract ISR to promote naïve T cell survival have not been identified.

In this study, we revealed that the TCR signal strength determines the survival of naïve T cells in the presence of ISR enhanced by the dysfunction of KDELR1. The results obtained from OT-I TCR Tg system suggested that enhanced ISR were suppressed in naïve T cells that received TCR-mediated signals above certain thresholds (i.e. > T4 in vitro and > Q4H7 under steady state in vivo). Consistent with these results, CD5, which is highly expressed on naïve T cells that have high affinity/avidity

TCRs against endogenous antigens (30,38-40), was elevated in CD44^{Low} naïve T cells from T-Red mice compared with the same cells from WT mice, while CD5 expression on CD44^{High} memory-phenotype T cells was comparable between the two (Fig. 2A-2C). Moreover, a pMHC tetramer dissociation assay showed enrichment of CD44^{Low} naïve T cells with higher affinity/avidity TCRs in T-Red mice (Fig. 2D and 2E). Although we performed detailed assays using OT-I cells and CD8⁺ T cells in this study, T-Red naïve CD4⁺ T cells also show enhanced ISR (9) and higher CD5 levels than WT naïve CD4⁺ T cells (Fig. 2A), suggesting that the same phenomenon would occur in both CD8⁺ and CD4⁺ T cells. It is known that Foxp3⁺ regulatory CD4⁺ T cells receive stronger TCR signals and express higher CD5 levels than naïve CD4⁺ T cells (45,46). As expected, these Treg cells were relatively resistant to KDELR1 mutation and not significantly reduced in T-Red mice. (Supplementary Fig. 3). All these data support the idea that enhanced ISR induced by the T-Red mutation selectively depletes low self-affinity naïve T cells in vivo.

We found that APC plus APLs and/or IL-7 treatment, which transduce signaling pathways in naïve T cells at steady state in vivo, did not induce eIF2 α phosphorylation in naïve T cells in vitro, although anti-CD3 and anti-CD28 antibody stimulation, which induces an artificially high TCR signal, did (Supplementary Fig. 4 and data not shown). Therefore, the physiological stressors that trigger ISR in naïve T cells in vivo remain unclear. We hypothesize that cell divisions

(Supplementary Fig. 6) and/or the in vivo presence of T cells itself increase ISR in naïve T cells, because short term in vitro culture alone was found to significantly decrease the phosphorylation of eIF2 α (9). On the other hand, TCR-mediated signals, particularly strong ones, act as a negative regulator of ISR, as revealed by the present study.

How does TCR-induced signaling interfere ISR-mediated apoptosis in naïve T cells? One possible explanation is the upregulation of anti-apoptotic Bcl-2 family members by TCR signaling (48-50), since Bim is increased in naïve T cells in T-Red mice (9) and Bim-mediated T cell death is suppressed by Bcl-2 (51). Consistently, retroviral overexpression of Bcl-2 in bone-marrow cells from T-Red mice partially rescued the T-Red phenotype in naïve T cells, as judged by the relative reduction of CD44^{High} memory-phenotype T cell populations in the bone-marrow chimera (Supplementary Fig. 5). However, other explanations, such as direct crosstalk between TCR signaling and ISR, are also possible. Future study is needed to identify the precise mechanism responsible.

Because we have not successfully identified stressors that cause ISR in naïve T cells in vivo, we are currently unable to perform detailed biochemical studies using appropriate stimulations that replicate the in vivo setting. We here examined whether stronger TCR signals inhibit ISR, in vivo by using phosphorylation of eIF2 α as a marker. CFSE labeled OT-I cells were transferred to wild

type mice, and then a stronger T4 OVA altered peptide ligand or a weaker Q4H7 peptide was injected. The phosphorylation of eIF2 α was induced during cell divisions. The stronger T4 peptide significantly suppressed the phosphorylation of eIF2 α compared to the weaker stimulation with Q4H7, suggesting strong TCR signals can inhibit ISR in vivo (Supplementary Fig. 6). These results also suggest that the inhibition of ISR by strong TCR signals may be mediated by an upstream event of eIF2 α phosphorylation, an ISR regulator, in vivo.

In summary, using OT-I T cells and OVA APLs, we showed evidence that TCR-mediated signals, particularly strong ones, can reduce ISR. The results of this study indicate a possible link between TCR-mediated signaling and ISR for the maintenance of naïve T cell homeostasis in vivo.

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The authors declare they have no conflicting financial interests.

Figure legends

Figure 1. Reduction of naïve CD8⁺ T cells in T-Red mice is reversed by crossing with OT-I TCR Tg mice.

A) Phenotype of CD8⁺ T cells in the peripheral blood from wild-type (WT) or T-Red mice. Histograms are gated on the CD8⁺TCRβ⁺ population. B) Absolute numbers of CD44^{Low} naïve (N) and CD44^{High} memory-phenotype (M) CD4⁺ and CD8⁺ T cells in the spleen. C) CD44 levels in CD4⁺ and CD8⁺ T cells in peripheral blood. Percentages shown in the histograms are CD44^{High} populations. Histograms are gated on the CD3ε⁺CD19^{Neg}CD4⁺ (top) or CD8⁺ (bottom) population. GT, gene-trapped mice. D) Absolute numbers of naïve (N), memory-phenotype (M) CD4⁺ and CD8⁺ T cells in the spleen. E) CD44 levels of OT-I or T-Red/OT-I mice in the spleen. Histograms are gated on the CD8⁺TCRVα2⁺ population. F) Absolute number of total splenocytes in OT-I and T-Red/OT-I mice. G) Absolute number of CD44^{Low}CD8⁺TCRVα2⁺ OT-I and T-Red/OT-I T cells in the spleen. The data in the bar graphs represent the mean + SEM. ** P < 0.01 and *** P < 0.001.

Figure 2. Remaining naïve T cells in T-Red mice show evidence of higher self-affinity/avidity.

A) CD5 levels of naïve (N) or memory-phenotype (M) CD4⁺ or CD8⁺ T cells in wild-type (WT) or T-Red mice. Mean fluorescence intensity (MFI) is shown. B) CD5 levels of wild-type B6 (B6), T-Red, OT-I or T-Red/OT-I T cells. Histograms are gated on the CD44^{Low}CD8⁺ population. C) MFI

of CD5 levels in (B). D) Tetramer dissociation assay using splenic T cells in wild-type (WT) or T-Red mice. The contour plots are gated on CD4-negative, MHC class II-negative, CD8+ population. E) Tetramer positive populations at 0 min incubation are indicated as 100%. The data in the bar graphs indicate the mean + SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. NS, not significant.

Figure 3. Survival responses to OVA APLs in vitro.

Splenocytes from OT-I and T-Red/OT-I mice were mixed and stimulated with OVA APL N4 (A), V4 (B), or T4 (C) for 6 hours at 37°C. Percentages of 7AAD-negative live OT-I or T-Red/OT-I T cell populations are shown. Affinities of the APLs to OT-I TCR are known to be N4 (original) > T4 > V4 (31-33). D) Co-staining of Annexin V with 7AAD. E) CFSE-labeled splenocytes from OT-I and T-Red/OT-I mice were co-cultured with OVA APLs for 6 hours. CFSE levels in OT-I and T-Red/OT-I T cells are shown. There was no cell division during the culture period. The data in the bar graphs represent the mean + SD. ** $P < 0.01$, and *** $P < 0.001$.

Figure 4. Proliferation responses to OVA APLs in vivo.

A) Schematic diagram of the in vivo experiment under the lymphoreplete condition. B) CFSE dilutions in OT-I or T-Red/OT-I T cells 2 days after peptide injection. The percentage of the cell

population that underwent cell divisions is shown. The order of affinity of APLs is N4 (original) > Y3 > T4 > Q4H7 > V4 > E1 (31-33). C) Relative frequencies of cell divisions in response to T4 treatment. Control OT-I T cell populations that underwent cell divisions are indicated as 100%. D) Relative frequencies of cell divisions in response to Q4H7 treatment. E) Schematic diagram of the in vivo experiment under the lymphopenic condition. F) CFSE dilutions in OT-I or T-Red/OT-I T cells 4 days after peptide injection. The data in the bar graphs represent the mean + SEM. *** P < 0.001.

Supplementary Figure 1. Naïve T cells express KDELR1 and KDELR2.

RT-PCR of KDELR family members using cDNA from naïve T cells.

Supplementary Figure 2. CD5^{High} naïve CD8⁺ T cells contain higher levels of ZAP70/Syk phosphorylation.

A) ZAP70/Syk phosphorylation levels in CD5^{Low} and CD5^{High} population (25% from each side) of CD44^{Low} naïve CD8⁺ T cells are shown. B) The mean fluorescent intensity of ZAP70/Syk phosphorylation levels is shown. Each column represents the mean + SEM. *P < 0.05.

Supplementary Figure 3. Foxp3⁺CD25⁺CD4⁺ regulatory T cells are not significantly reduced

in T-Red mice.

A) Foxp3+CD25+CD4+ regulatory T cell population in the spleen is shown. Pseudo-color plots are gated on CD8-negative, CD4+ population. B) Absolute numbers of CD44^{Low} naïve CD4+, CD8+ T cells, and Foxp3+CD25+CD4+ regulatory T cells in the spleen. *P < 0.05, **P < 0.01 and NS, not significant.

Supplementary Figure 4. TCR stimulation by anti-CD3/CD28 induces eIF2 α phosphorylation.

Sorted naïve CD4+ T cells from WT mice were stimulated with anti-CD3/CD28 or IL-7 for the indicated hours (hrs). Phospho-eIF2 α S51 and total eIF2 α were detected by Western blotting.

Supplementary Figure 5. Forced expression of Bcl-2 rescues the T-Red phenotype.

Retroviral transduction of Bcl-2 or mock transduction was performed in bone-marrow cells from T-Red mice. CD44^{High} population (%) within CD4+ and CD8+ T cells in the bone-marrow chimera are shown. The data represent the mean + SEM. * P < 0.05, and ** P < 0.01.

Supplementary Figure 6. Stronger TCR stimulation inhibits eIF2 α phosphorylation in vivo.

CFSE labeled OT-I cells were transferred to naïve congenic mice on day 0, and then a stronger T4 OVA altered peptide ligand or a weaker Q4H7 peptide was injected on day 1. Intracellular staining

of phosphor-eIF2 α in donor OT-I cells in the spleen was performed on day 3. A) The CFSE profiles of donor OT-I cells after T4 or Q4H7 peptide injection. B) The mean fluorescent intensity (MFI) of phosphor-eIF2 α staining in donor OT-I cells at each cell division is shown. Data represent the mean + SEM. *P < 0.05, and **P < 0.01 vs. Q4H7; #P < 0.05 and ##P < 0.01 vs. 0 division.

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Figure 1

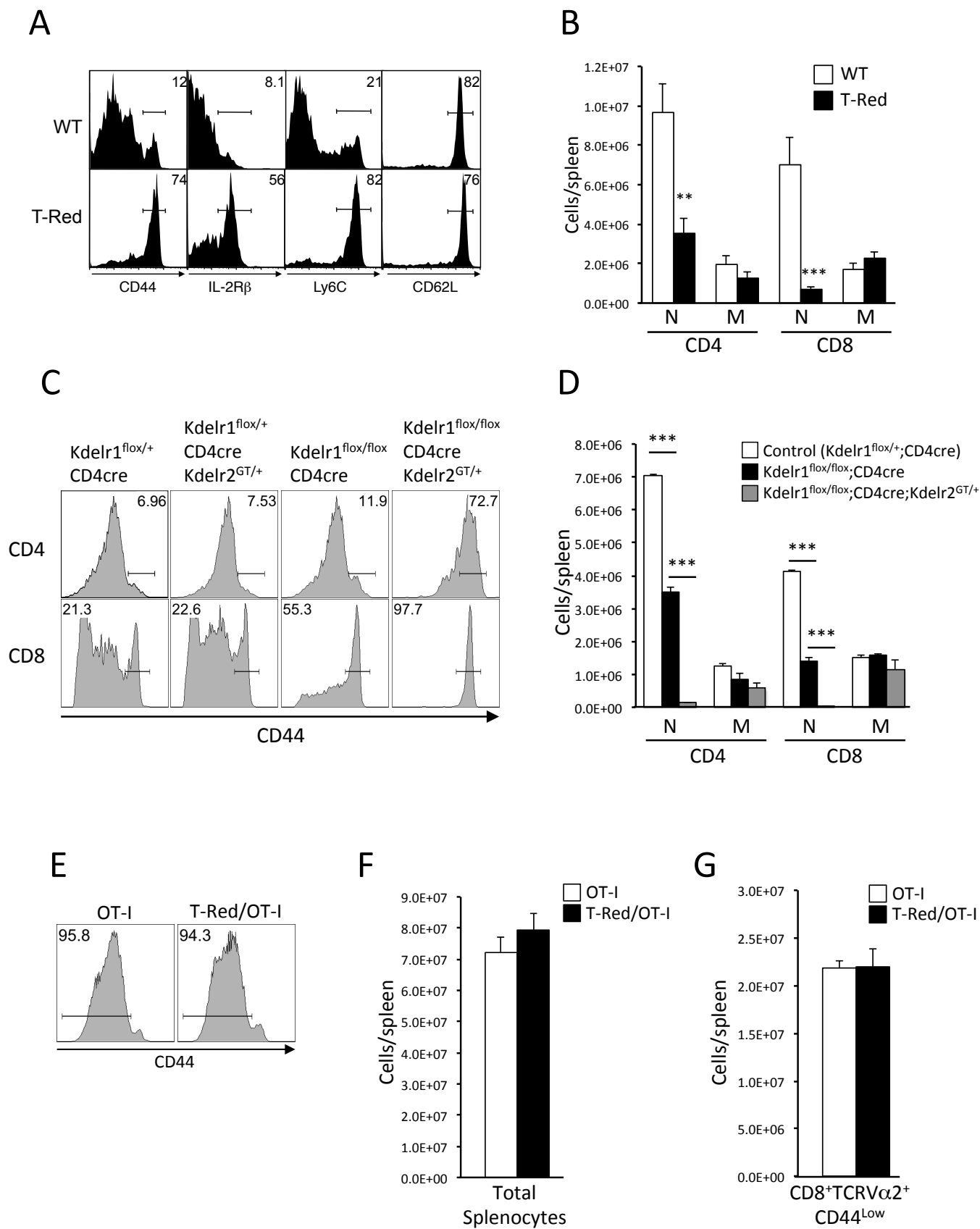


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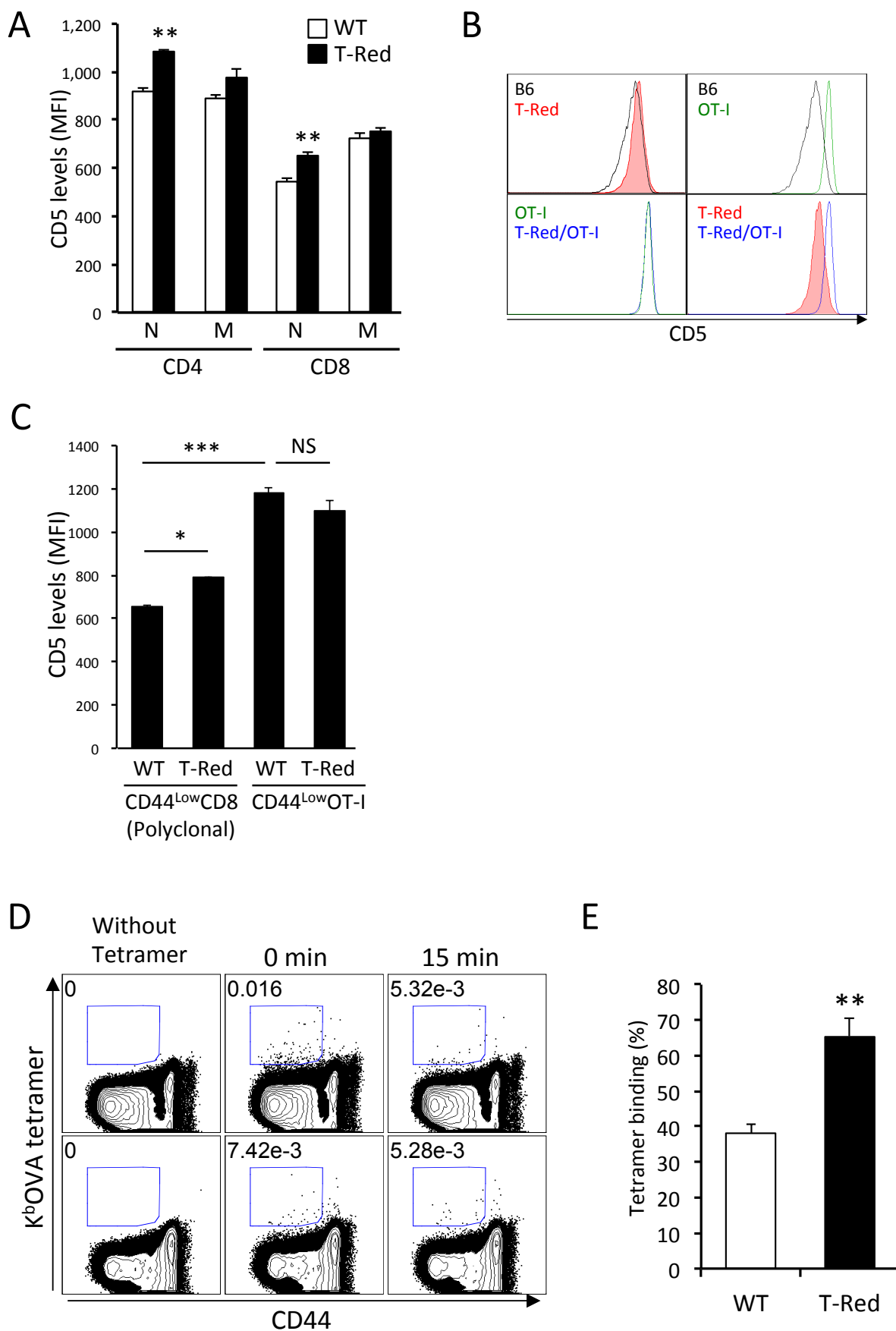


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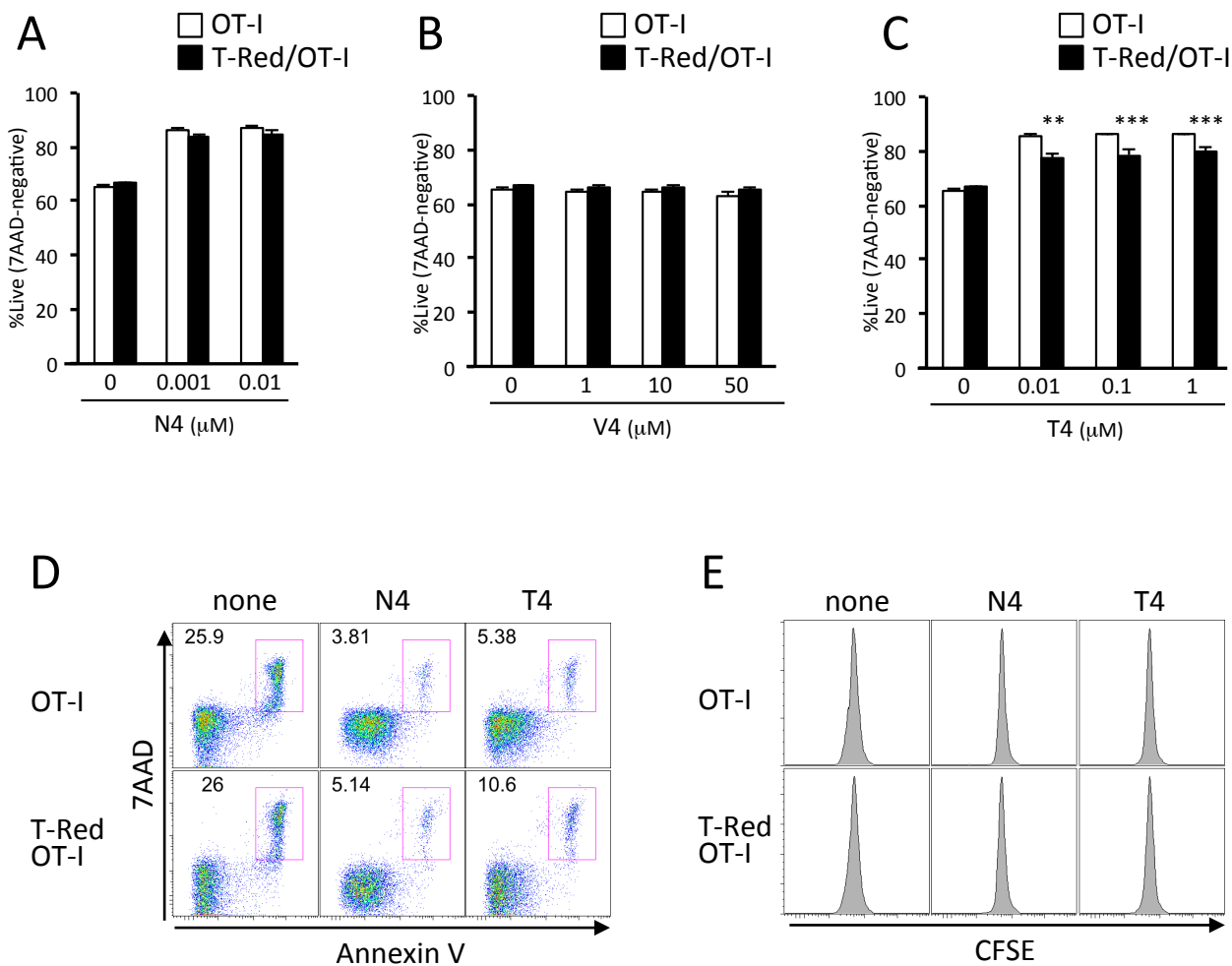
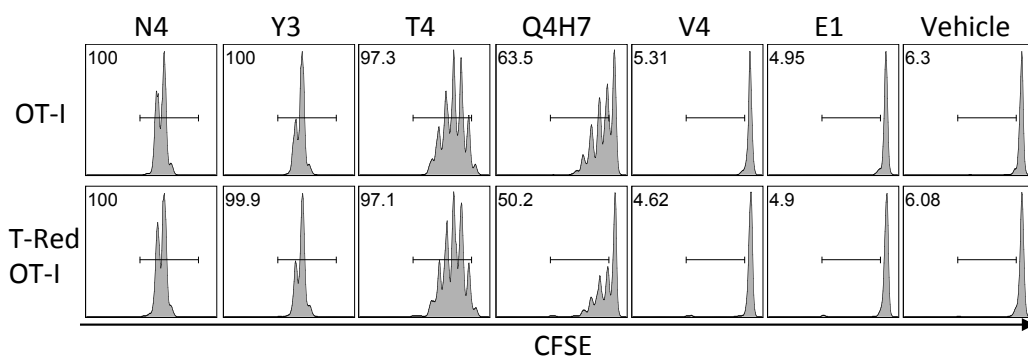


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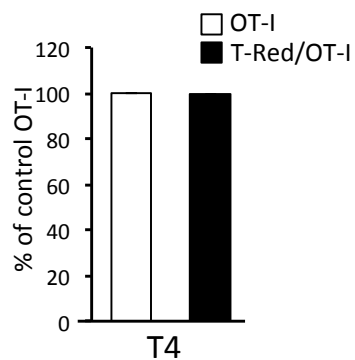
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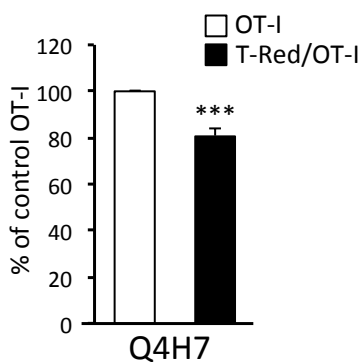
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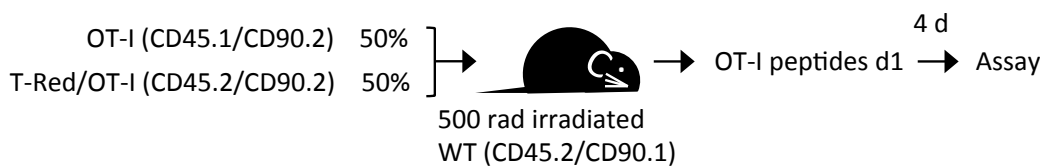
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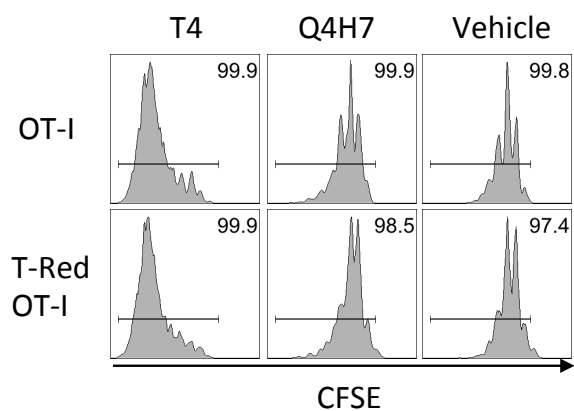
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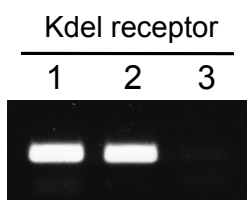
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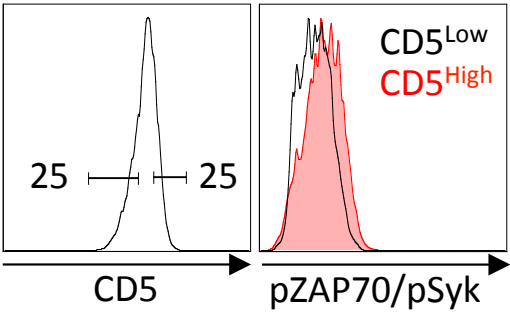


Supplementary Figure 1

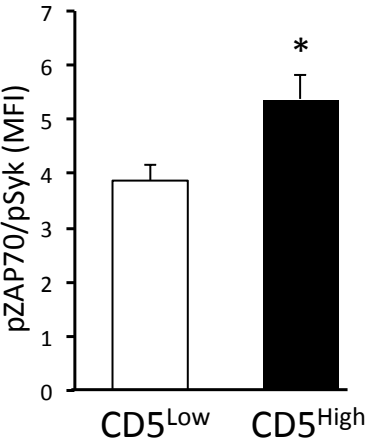


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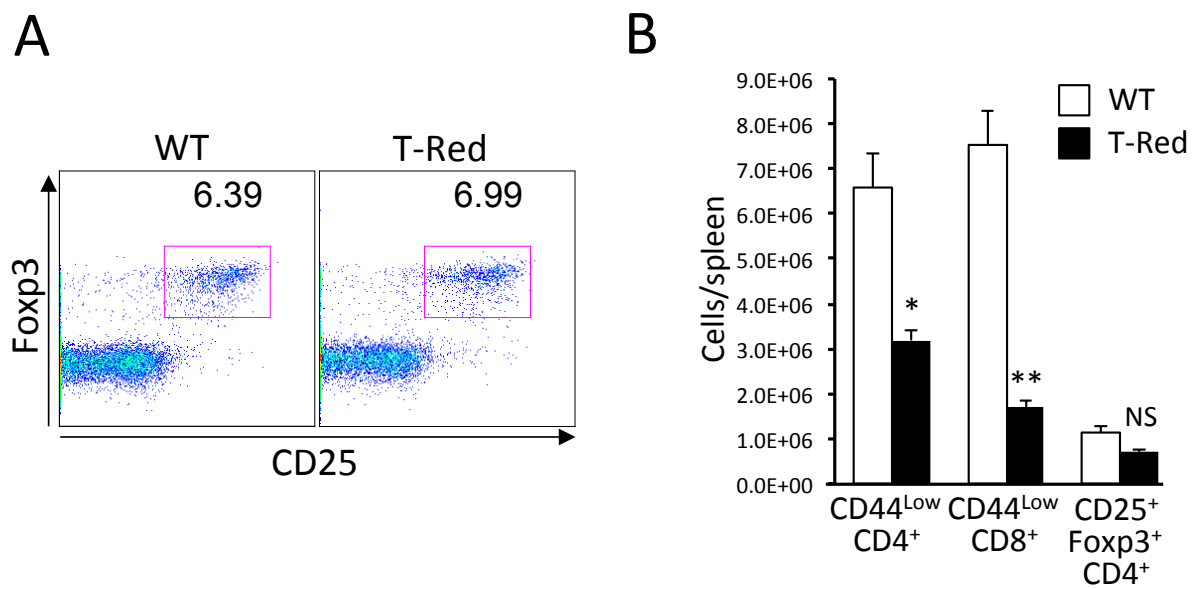
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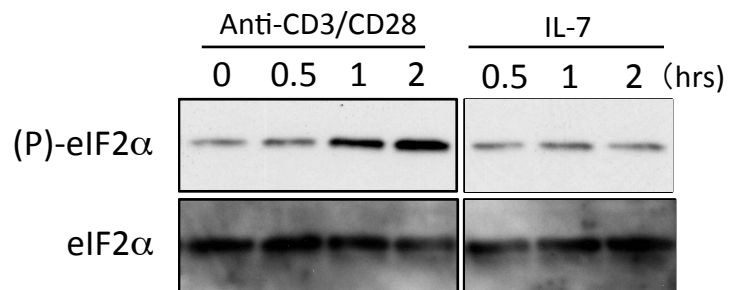
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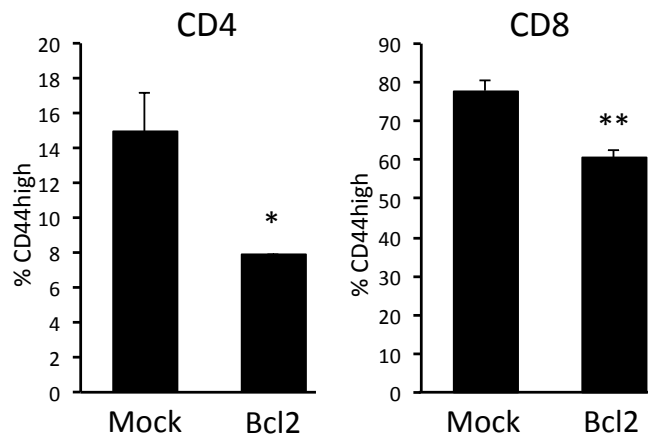
Supplementary Figure 3



Supplementary Figure 4

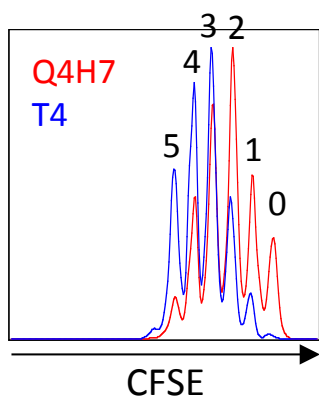


Supplementary Figure 5



Supplementary Figure 6

A



B

