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## Interleukin-6 expands homeostatic space for peripheral T cells

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### Abstract

T cell homeostasis and survival is dependent on interleukin-7 (IL-7). Immune activation, however, downregulates IL-7 receptor expression on T cells so that T cell survival during activation must be maintained independently of IL-7. The pro-inflammatory cytokine IL-6 shares common signaling pathways with IL-7 and can promote T cell survival *in vitro*. But whether IL-6 promotes T cell survival and homeostasis *in vivo* is not clear. Notably, IL-6 overexpression results in massive plasmacytosis and autoimmunity so that an IL-6 effect on *in vivo* T cell survival has remained untested. To overcome this limitation, here we generated IL-6 transgenic mice on an immunoglobulin heavy chain (IgH) deficient background which rendered them B cell deficient. Notably, such IgH<sup>KO</sup>IL6<sup>Tg</sup> mice were free of any signs of inflammation or autoimmunity and remained healthy throughout the course of analysis. In these mice, we found that IL-6 overexpression significantly increased peripheral T cell numbers, but importantly without increasing thymopoiesis. Moreover, IL-6 signaled T cells maintained their naïve phenotype and did not express activation/memory markers, suggesting that increased T cell numbers were due to increased T cell survival and not because of expansion of activated T cells. Mechanistically, we found that IL-6 signaling induced expression of pro-survival factors Mcl-1 and Pim-1/-2 but not Bcl-2. Thus, IL-6 is a T cell homeostatic cytokine that expands T cell space and can maintain the naïve T cell pool.

### Keywords

Apoptosis; Cytokine; Survival; Proliferation; Thymus

## 1. Introduction

T cells are generated in the thymus and then migrate into peripheral tissue for immune surveillance and protection. Maintaining T cells in the periphery is referred to as T cell homeostasis, and cytokines play essential roles in this process [1]. Specifically, interleukin-7 (IL-7) is a non-redundant cytokine in T cell homeostasis, and *in vivo* availability of IL-7 sets the size of the peripheral T cell pool [2–4]. IL-7 sustains T cell survival by providing anti-apoptotic signals, inhibiting pro-apoptotic activities, and promoting cell metabolism. To do

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so, IL-7 signaling upregulates Bcl-2, inhibits Bax and Bad, and induces expression of glucose transporter-1 [5–8]. Collectively, IL-7 is an essential pro-survival signal that maintains the size and composition of the T cell pool under steady state conditions.

IL-7 is a member of the common  $\gamma$ -chain ( $\gamma$ ) cytokine family that also includes IL-2, IL-4, IL-9, IL-15 and IL-21 [9].  $\gamma$  cytokines share the  $\gamma$  receptor for ligand binding and signaling, and have common characteristics in their signaling pathways. All  $\gamma$  cytokines, including IL-7, induce activation of receptor bound Janus kinases (JAK) which leads to phosphorylation and nuclear translocation of STAT molecules. PI3-kinase/Akt activation is another major pathway induced by all  $\gamma$  cytokines [10–12]. Because of such similarities in their downstream signaling effects, it has been a longstanding question what makes IL-7 unique in its ability to drive T cell homeostasis. Also, it has remained unclear if cytokines other than IL-7 can act redundantly to IL-7 in T cell homeostasis. Interestingly, overexpression of most  $\gamma$  cytokines failed to maintain naïve T cell homeostasis *in vivo* [13–16]. Transgenic expression of IL-2 or IL-4 resulted in severe inflammation and loss of naïve T cells due to aberrant T cell activation [15, 16]. IL-15 transgenic mice showed dramatic expansion and accumulation of memory phenotype CD8 T cells with minimal contribution to naïve CD8 T cell survival [14]. IL-21 overexpression increased the CD8 memory T cell pool concomitant to significantly reduced naïve T cell numbers [13]. Thus so far, no  $\gamma$  cytokine other than IL-7 has been found to promote naïve T cell homeostasis.

A unique feature of IL-7 signaling is downregulating expression of its own receptor [17, 18]. We have previously shown that this mechanism maximizes the availability of limited *in vivo* IL-7 and that it increases the size of the naïve T cell pool [18]. On the other hand, signaling of other  $\gamma$  cytokines upregulates expression of their own receptors, resulting in further reinforcement of  $\gamma$  cytokine signaling and expansion of memory/activated phenotype cells, presumably at the expense of naïve T cells [19, 20]. As such, downregulating expression of its own receptor contributes to the molecular basis of a homeostatic cytokine.

In the current study, we made the serendipitous finding that the non- $\gamma$  cytokine IL-6 also downregulates expression of its own receptor. IL-6 is a pro-inflammatory cytokine that is produced by many cell types, including stromal cells, endothelial cells, and lymphocytes [21]. IL-6 is largely known for its inflammatory effects and its involvement in cancer and autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, and Crohn's disease [22, 23]. Consequently, IL-6 deficiency ameliorates a series of experimental autoimmune diseases, including induction of Experimental Autoimmune Encephalomyelitis (EAE) [24, 25], collagen-induced arthritis [26], and colitis [27]. Along this line, recent studies revealed a role for IL-6 on the generation of the pro-inflammatory T cell subset, Th17 cells [28–30], and its suppressive effect on FoxP3<sup>+</sup> regulatory CD4 T cells [28, 31]. Thus, IL-6 signaling is a central component of a pro-inflammatory response. IL-6 signals presumably through a hexameric complex composed of two heterotrimers of IL-6, IL-6R $\alpha$ , and gp130 [32]. Previous studies have shown that IL-6 can promote T cell survival *in vitro* utilizing the same pathways as IL-7 by activating JAK/STAT and PI3-K/Akt, but whether IL-6 can act as a homeostatic cytokine *in vivo* has remained unresolved [33–35]. Addressing this question *in vivo* is further complicated because IL-6 overexpression induced severe plasmacytosis so that IL-6 transgenic mice (IL6<sup>Tg</sup>) developed massive lymphoid organ infiltrates of plasma cells, resulting in premature death due to glomerulonephritis-induced renal failure [36, 37].

To circumvent this problem, here we generated B cell-deficient IL6<sup>Tg</sup> mice by introducing the IL6<sup>Tg</sup> onto an immunoglobulin heavy chain (IgH) deficient (IgH<sup>KO</sup>) background. Such B cell-deficient IgH<sup>KO</sup>IL6<sup>Tg</sup> mice survived more than one year without developing any immunopathology and autoimmune diseases. Notably, IL-6 overexpression did not affect T cell development in the thymus and did not induce activation of mature T cells in the

periphery. In fact, composition of the peripheral T cell compartment of IgH<sup>KO</sup>IL6<sup>Tg</sup> mice was indistinguishable to that of control IgH<sup>KO</sup> mice. However, naïve T cell numbers in IgH<sup>KO</sup>IL6<sup>Tg</sup> mice were significantly increased, and T cells expressed increased levels of survival factors such as Pim-1/2 and Mcl-1. Collectively, these results identify IL-6 as a novel homeostatic cytokine for T cells that can expand the peripheral T cell space and potentially contributes to maintaining the naïve T cell pool under inflammatory conditions.

## 2. Materials and methods

### 2.1 Mice

C57BL/6 (B6) mice were obtained from the Frederick Cancer Research and Development Center, Frederick, MD. IgH-deficient (IgH<sup>KO</sup>) mice and IL-6 transgenic (IL6<sup>Tg</sup>) mice, expressing human IL-6 under the control of the MHC-I promoter, have been previously described [36, 37] and purchased from the Jackson Laboratory (Bar Harbor, ME). Animal experiments were approved by the NCI Animal Care and Use Committee, and all mice were cared for in accordance with NIH guidelines.

### 2.2 Cell isolation and cell culture

Lymph node (LN) T cells from B6 mice were isolated by depleting B cells with anti-mouse IgG beads (Qiagen). LN T cells were cultured in media or recombinant murine IL-6 (10 ng/mL; PeproTech) and IL-7 (10 ng/mL; PeproTech) for RNA isolation or viability assay. CD4<sup>+</sup> LN T cells from IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> mice were isolated by depleting CD8<sup>+</sup> cells with anti-CD8 antibody (2.43) and anti-rat IgG BioMag beads (Qiagen).

### 2.3 Quantitative reverse transcription PCR

Total RNA was isolated with the RNeasy kit (Qiagen). RNA was reverse transcribed into cDNA by oligo(dT) priming with the QuantiTect Reverse transcription kit (Qiagen). Quantitative reverse transcription PCR (qRT-PCR) was performed with an ABI PRISM 7900HT Sequence Detection System and the QuantiTect SYBR Green detection system (Qiagen) with the primers for the following molecules: IL-6R (F: 5 -GCAGGAATCCTCTGGAACCC-3 , R: 5 -CAGAAGGAAGGTCGGCTTCA-3 ), IL-7R (F: 5 -CACACAAGAACAACAATCCCACA-3 , R: 5 -GATCCCATCCTCCTTGATTCTTG -3 ), Bcl-2 (F: 5 -TGTAATTGCCGAGAAGAAGGG-3 , R: 5 -TCCCCGTTGGCATGAGAT-3 ). Bcl-xL (F: 5 -GCGGCTGGGACACTTTTG-3 , R: 5 -ACTTCCGACTGAAGAGTGAGCC-3 ), Mcl-1 (F: 5 -AGACGGCCTTCCAGGGC-3 , R: 5 -CCAGTCCCCTTTCGTCTT-3 ) Pim-1 (F: 5 -ACCTGAGCCGCGGCGAAATC-3 , R: 5 -GCCGTGGTAGCGATGGTAGCG-3 ), Pim-2 (F: 5 -CACCGTCTTCGCGGGACACC-3 , R: 5 -CCACCTCCACAGCAGCGCA-3 ). Gene expression values were normalized to those of HPRT (F: 5 -GCGATGATGAACCAGGTTATGA-3 , R: 5 -ACAATGTGATGGCCTCCCAT -3 ) in the same sample.

### 2.4 Flow cytometry

Single cell suspensions were prepared from thymus or LN, and then stained and analyzed on an LSRII, FACSria or FACSCalibur (BD Biosciences). Dead cells were excluded by forward light scatter gating and propidium iodide (PI) staining for viability assays and for phenotyping. Annexin V/PI staining was performed according to the manufacturer's instructions (BD Biosciences). Total caspase activity was assessed using a CaspGLOW fluorescein active caspase staining kit (eBioscience). Antibodies with the following specificities were used for staining: CD4 (GK1.5 and RM4.5), CD25 (PC61.5), CD8 (53-6-7), TCR (H57-597), IL-7R (A7R34), Ki-67, and Foxp3 (FJK-16s; all from eBiosciences), CD69 (H1.2F3), IL-6R (M5), CD44 (IM7), CD62L (MEL-14) and pSTAT3

(pY705; all from BD Biosciences), Helios (22F6) and CD122 (5H4; all from BioLegend). STAT3 phosphorylation was determined by methanol/acetone fixation for 30 min after IL-6 stimulation [38]. For intra-nuclear Ki-67, Foxp3 and Helios staining, cells were fixed and permeabilized with Foxp3 fixation/permeabilization buffer (eBiosciences). Data were analyzed using software designed by the Division of Computer Research and Technology at the NIH.

## 2.5 TREC analysis

Cell lysates were prepared from either  $3 \times 10^6$  LN cells using DNAzol (Molecular Research Center, Inc.), or  $5 \times 10^6$  LN cells using the QIAmp Blood Mini Kit (QIAGEN), as per manufacturer's instructions. Isolated DNA from cell lysates was then amplified using the TaqMan real-time PCR assay (Applied Biosystems) specific for the m Rec primer (5'-GGGCACACAGCAGCTGTG-3'), the J primer (5'-GCAGTTTTTGTAAAGGTGCTCA), and the m Rec-J fluorescent probe (5'-FAM-CACAAGCACCTGCACCCTGTGCA-TAMRA-3'). Primers for the single-copy CD8 gene include the forward primer (5'-CAGGACCCCAAGGACAAGTACT-3'), the reverse primer (5'-CACTTTCACCATACAAAACCTCTTTG) and probe (5'-FAM-TGAGTTCCTGGCCTCCTGGAGTTCTTC-TAMRA-3'). Primers were obtained from Invitrogen, and probes were ordered from Eurofins MWG Operon. Standard curves were generated per experiment by cloning the signal joint m Rec-J TREC or the CD8 gene into a pCR-XL TOPO vector. TREC copy numbers were determined for 50,000 cells, normalized to CD8, and then analyzed relative to littermate control.

## 2.6 IL-6 ELISA

Serum samples were obtained from IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> mice. Serum human IL-6 levels were determined by ELISA Ready-SET-Go kit according to manufacturer's instructions (eBioscience).

## 2.7 Immunoblotting

T cells were lysed in Cell Lytic-M (Sigma) supplemented with protease inhibitors (Roche). Whole-cell lysates were electrophoresed in 10% Tri/glycine gels and transferred to PVDF membranes (Invitrogen). Blots were incubated with anti-Mcl-1 (Rockland Immunochemicals) or anti-actin antibodies (Santa Cruz Biotechnology), and detected using horseradish peroxidase conjugated anti-rabbit IgG antibodies. Reactivity was visualized by enhanced chemiluminescence (Pierce).

## 2.8 BrdU incorporation assay

IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> mice were given an initial intraperitoneal injection of BrdU (1 mg dissolved in PBS) and then kept on BrdU containing drinking water (1mg/ml) for 3 days. Thymocytes and LN T cells were first stained with anti-CD4, anti-CD8, and anti-TCR antibodies, and then fixed and permeabilized with Cytotfix/Cytoperm and Cytotfix/Cytoperm Plus for intranuclear anti-BrdU staining according to the manufacturer's protocol (BD Biosciences).

## 2.9 Statistical analysis

Data are shown as mean  $\pm$  SEM. Two-tailed Student's *t*-test was used to calculate *P*-values for all experiments except in the calculation of MFI in which paired *t*-test was used. A value of *P* < 0.05 was considered statistically significant.

### 3. Results

#### 3.1 IL-6 promotes T cell survival *in vitro*

To assess a role for IL-6 in T cell homeostasis, first we analyzed IL-6R expression on T cells under steady state conditions. All T cells expressed IL-6R, but CD4 T cells expressed significantly higher levels of IL-6R than CD8 T cells (Fig. 1A). Such difference in IL-6R levels, however, did not result in distinct IL-6 signaling as IL-6-induced STAT3 phosphorylation *in vitro* was identical between CD4 and CD8 T cells (Fig. 1B). Whether such is also the case *in vivo* remains untested. Nevertheless, these results suggest that both CD4 and CD8 T cells are IL-6 signaling competent.

Analysis of IL-6 stimulated CD4 and CD8 T cells showed that IL-6 induced downregulation of its own receptor (Fig. 1C). This is in contrast to T cell activating cytokines, such as IL-2 and IL-4, which upregulate expression of their own receptors [19, 20]. Thus, IL-6 signaling is more in line with homeostatic IL-7 signaling, which downregulates expression of its own receptor to maximize *in vivo* IL-7 availability [18]. To further determine the downstream effects of IL-6, we assessed the phenotype of IL-6 treated T cells. We found that IL-6 stimulation significantly suppressed pro-apoptotic caspase activities (Fig. 1D), and that IL-6 promoted viability and increased cell size (Fig. 1E, F, and Suppl. Fig. 1A). These results demonstrate anti-apoptotic and pro-metabolic functions of IL-6 on resting T cells.

To understand the pro-survival effect of IL-6, we analyzed mRNA contents of IL-6 treated LN T cells. We found that IL-6 upregulated expression of anti-apoptotic Mcl-1, Pim-1, and Pim-2 [39], but not of Bcl-xL and Bcl-2 (Fig. 2A). Notably, while Mcl1 expression was induced by both IL-6 and IL-7 (Fig. 2B), Bcl-2 mRNA expression was only induced by IL-7 and not by IL-6, suggesting that the pro-survival effects of IL-7 and IL-6 are distinct. Collectively, these data establish IL-6 as a pro-survival cytokine for T cells that induces expression of the pro-metabolic kinases Pim-1/2 and upregulates expression of the anti-apoptotic Mcl-1.

#### 3.2 IL-6 promotes peripheral T cell survival *in vivo*

To determine if IL-6 can promote T cell survival *in vivo*, next we analyzed T cell homeostasis in IL-6 transgenic mice (IL-6<sup>Tg</sup>). Importantly, we made IL-6<sup>Tg</sup> mice additionally B cell deficient (IgH<sup>KO</sup>IL6<sup>Tg</sup>) to prevent B cell hyperactivation and plasmacytosis as consistently observed in B cell sufficient IL-6<sup>Tg</sup> mice [36]. Such IgH<sup>KO</sup>IL6<sup>Tg</sup> mice expressed high levels of IL-6 in serum but did not show any signs of inflammation and remained tumor-free for more than 18 months (Fig. 3A and data not shown). Thymocyte development in IgH<sup>KO</sup>IL6<sup>Tg</sup> mice was normal and comparable to control IgH<sup>KO</sup> mice. Specifically, transgenic IL-6 did not affect CD4/CD8 profiles of total and TCR<sup>+</sup>HSA<sup>low</sup> mature thymocytes, and overall thymocyte numbers remained the same (Fig. 3B, C, and D). Thus, IL-6 overexpression did not perturb thymopoiesis or lineage commitment.

In the periphery, however, transgenic IL-6 induced a significant increase of total T cell numbers even as we did not observe increased viability in *ex vivo* isolated cells (Fig. 4A and Suppl. Fig. 1B). Interestingly, we also observed a modest but significant bias toward CD4 T cell survival, suggesting a preferential effect of IL-6 on CD4 lineage T cells (Fig. 4B and C). Moreover, only CD4 T cells and not CD8 T cells accumulated in IgH<sup>KO</sup>IL6<sup>Tg</sup> mice (Fig. 4C and Suppl. Fig. 1C), indicating that transgenic IL-6 mostly promoted survival of CD4 T cells. Finally, in agreement with *in vitro* results (Fig. 1C), we found that *in vivo* IL-6 stimulation also suppressed IL-6R expression on IgH<sup>KO</sup>IL6<sup>Tg</sup> CD4 T cells (Fig. 4D left). Since IL-7R expression was also downregulated *in trans* by *in vivo* IL-6 (Fig. 4D right), these



results suggest an interplay of two pro-survival cytokines that could mutually limit their consumptions and maximizes their bioavailability.

### 3.3 T cell quiescence is maintained in IL-6 transgenic mice

IL-6 can directly induce T cell proliferation under lymphopenic conditions [40], and can indirectly induce T cell expansion by triggering excess IL-7 production in non-hematopoietic cells [41]. In both cases, IL-6 dependent T cell expansion was associated with T cell activation and autoimmunity such as colitis and rheumatoid arthritis. To examine whether IL-6 overexpression in IgH<sup>KO</sup>IL6<sup>Tg</sup> mice induced T cell expansion by T cell activation, next we assessed their T cells for activation markers. Notably, CD25 and CD69 expression were not upregulated and were not different from control IgH<sup>KO</sup> T cells (Fig. 5A). IgH<sup>KO</sup>IL6<sup>Tg</sup> mice also did not show increased accumulation of activated/memory phenotype CD62L<sup>lo</sup>CD44<sup>hi</sup> CD4 T cells or CD122<sup>hi</sup>CD44<sup>hi</sup> CD8 T cells (Fig. 5B). Moreover, intracellular staining for the proliferation-associated nuclear antigen Ki-67 and *in vivo* BrdU labeling showed that IL-6 overexpression did not induce cell cycling and proliferation of peripheral T cells (Fig. 5C, D, and Suppl. Fig. 1D).

Along this line, we found that homeostasis of FoxP3<sup>+</sup> and Helios<sup>+</sup> regulatory CD4 T cells (Tregs) were not affected in IL-6 transgenic mice (Fig. 5E, F) [42, 43]. Previously, IL-6 had been reported to suppress FoxP3<sup>+</sup> Treg cell generation [28], and reduced Treg activity was shown to result in T cell activation and autoimmunity [44]. While lower Treg cell numbers could have been a cause for greater T cell numbers in IgH<sup>KO</sup>IL6<sup>Tg</sup> mice, comparable FoxP3<sup>+</sup>CD4<sup>+</sup> T cell percentages did not favor this possibility. Collectively, these data suggest that increased T cell number in IgH<sup>KO</sup>IL6<sup>Tg</sup> mice is not associated with T cell activation, and that transgenic IL-6 promotes T cell expansion without perturbing quiescence of the peripheral T cell pool.

### 3.4 IL-6 expands T cell space by increasing T cell survival and extending life span

To further establish that increased T cell numbers are mediated by increased T cell survival and independent of thymic output, we examined T cells in aged IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> mice (37 to 52 weeks). In aged mice, the thymus begins to atrophy and thymic output is significantly reduced [45]. Transgenic IL-6 did not reverse this phenotype, and overall thymocyte numbers and CD4/CD8 lineage choice remained comparable to control IgH<sup>KO</sup> mice (Suppl. Fig. 2A, B). Nevertheless, LN T cell numbers in aged IgH<sup>KO</sup>IL6<sup>Tg</sup> mice were still significantly increased and T cells displayed a similar phenotype as in young IgH<sup>KO</sup>IL6<sup>Tg</sup> mice (Suppl. Fig. 2C, D, E). Importantly, such increase in cell numbers was not because of preferential T cell migration to LN or tissue re-distribution of T cells since T cell numbers in the spleen was not reduced but rather increased (Fig. 6A), and it was specific to T cells because spleen NKT cell numbers were unaffected (Fig. 6B).

Moreover, T cells populating the periphery of IgH<sup>KO</sup>IL6<sup>Tg</sup> mice contained decreased copy numbers of TRECs (T cell receptor excision circle) when compared to T cells from age matched IgH<sup>KO</sup>IL6<sup>Tg</sup> mice (Fig. 6C). TRECs are generated only in the thymus during T cell receptor recombination and are not degraded in the periphery. Thus, dilution of TREC numbers suggests that cells have undergone increased rounds of proliferations [46]. Upon acute proliferation, however, T cells acquire an activated/memory phenotype which is marked by increased expression of CD44 [47, 48]. Interestingly, even in aged mice (37–52 weeks), IgH<sup>KO</sup>IL6<sup>Tg</sup> T cells contained normal levels of activated/memory phenotype cells (Fig. 6D), which suggest that increased T cell numbers were possibly induced by prolonged survival and slow proliferation. In fact, *in vivo* IL-6 strongly induced expression of a series of pro-survival factors similar to those observed *in vitro*, which included Pim-1, Pim-2, and Mcl-1 (Fig. 6E). Notably, Bcl-xL expression, which was not induced by *in vitro* IL-6

stimulation, was now significantly induced by *in vivo* IL-6, suggesting distinct effects of IL-6 under *in vitro* and *in vivo* conditions (Fig. 6E). Bcl-2 levels, however, remained unaffected by both *in vitro* and *in vivo* IL-6, which documents a pro-survival pathway of IL-6 that is distinct from common  $\gamma$ -chain cytokines. Altogether, these data propose a role for IL-6 as a pro-survival homeostatic cytokine for naïve T cells *in vivo*.

#### 4. Discussion

*In vivo* availability of homeostatic cytokines determines the size of the peripheral T cell pool. In this study, we identified the pro-inflammatory cytokine IL-6 as a novel homeostatic cytokine that expands T cell space by inducing expression of anti-apoptotic genes, such as Mcl-1, and by upregulating expression of pro-metabolic genes, such as Pim-1 and Pim-2. Interestingly, IL-6 overexpression did not promote thymopoiesis or induce T cell activation, but it acted specifically on resting T cells to increase T cell space. Since IL-6 expression is highly upregulated during immune activation and inflammation, IL-6 mediated T cell survival could represent a mechanism to maintain the T cell pool when signaling by other homeostatic cytokines becomes limiting.

Naïve T cell survival is dependent on IL-7 [2, 4, 49]. T cell activation, however, induces rapid downregulation of IL-7 receptor expression so that factors other than IL-7 must mediate T cell survival during immune activation [18, 50]. Previously, a set of cytokines have been identified to promote T cell survival, which includes the  $\gamma$ -chain cytokines IL-2, IL-4, IL-7, IL-15, and the gp130 family cytokine, IL-6 [51, 52]. These cytokines not only prevented apoptosis but also provided trophic effects to maintain cell size and induce cell metabolism. Notably, all these pro-survival cytokines share the common signaling pathways of JAK-STAT and PI3-Kinase/Akt activation [12, 33, 53]. This observation suggests that pro-survival cytokines could be potentially interchangeable and redundant in their effects to provide T cell survival and homeostasis. Whether such is the case, and if so, which cytokine could possibly function redundant to IL-7 has not been clear. However, T cell adoptive transfer experiments into IL-7 deficient mice documented that, at least under resting conditions, IL-7 is a non-redundant factor in T cell survival [2, 4]. Thus, IL-7 is an essential requirement for T cell survival, even in an environment sufficient in other pro-survival cytokines [49]. Our current data now demonstrate that IL-6 can have an additive survival effect to endogenous IL-7, as IL-6 overexpression further expanded peripheral T cell space under IL-7 sufficient conditions. Importantly, IL-6 increased T cell numbers independent of T cell differentiation status, *i.e.* being naïve or activated/memory phenotype cells. Thus, we identified IL-6 as a *bona fide* homeostatic cytokine in T cells, with a potential role in providing T cell survival under inflammatory conditions.

A role of pro-inflammatory cytokines in T cell survival has been demonstrated in earlier studies. For example, T cell priming to tolerogenic antigens was greatly enhanced by injection of bacterial lipopolysaccharide (LPS) [54]. In bacterial superantigen-stimulated T cells, pro-inflammatory cytokines such as TNF $\alpha$  or IFN $\gamma$  protected T cells from activation induced cell death, presumably by converting them into a Fas-resistant state [55]. Specifically, co-injection of LPS inhibited the deleterious effect of Staphylococcal enterotoxin A (SEA) on V $\beta$ 3<sup>+</sup> CD4 T cells in a TNF $\alpha$  dependent manner [55]. Thus, pro-inflammatory cytokines have been previously appreciated as pro-survival factors during T cell activation. A role for pro-inflammatory IL-6, however, has been less clear. Subsequent studies on SEA stimulated T cells reported that IL-6 failed to prevent cell death in activated cells or in memory phenotype cells (CD44<sup>hi</sup>CD62L<sup>lo</sup>) *in vitro*, and that IL-6 promoted cell survival of bystander resting T cells rather than of activated cells [56]. In contrast to such reports, however, IL-6 was reported to promote survival and expansion of activated T cells *in vivo*. Specifically, IL-6 improved survival and expansion of primed 5CC7 TCR

transgenic CD4 T cells *in vivo* [57], and rescued naïve CD4 T cells from TCR-induced cell death without Th1/Th2 polarization *in vivo* [58]. Along these lines, IL-6 also reduced activation induced cell death in T cells independent of IL-2 production [59]. Thus, an IL-6 pro-survival effect in activated T cells remains controversial and possibly reflects a difference of *in vivo* and *in vitro* experimental systems.

In addition to its role under activating/inflammatory conditions, a role for IL-6 under steady-state conditions also has remained unclear. While peripheral T cell numbers in thymectomized IL-6 deficient mice did not show an accelerated decrease compared to thymectomized wildtype mice [60], a direct analysis of IL-6 deficient mice reported a 20–40% reduction in total number of thymocytes and peripheral T cells [61]. Whether this is a direct effect on T cells or an indirect effect on dendritic cells and stromal cells is not known [62, 63]. Altogether, an IL-6 effect on T cell maintenance *in vivo* has been inconclusive.

Assessing long-term homeostatic effects of IL-6 on T cells has been difficult because of its pleiotropic effect on other immune cells, specifically B cells [36, 37]. As such, IL-6 transgenic mice display enlarged peripheral lymphoid organs and are prone to inflammation and autoimmunity [36]. The major pathology of IL-6 transgenic mice is manifested in massive polyclonal plasmacytosis, a dramatic increase in serum IgG1 levels, and renal failure that leads to premature death [36]. In the T cell compartment of these mice, both CD4 and CD8 T cells contain a large population of activated CD62L<sup>lo</sup> phenotype cells, concomitant to increased IL-17 and IFN $\gamma$  producing CD4 T cells [31]. Thus, IL-6 overexpression induces activation of both B and T cells *in vivo*. Strikingly, we found that T cells remained quiescent in B cell deficient IgH<sup>KO</sup>IL6<sup>Tg</sup> mice, and that it did not result in aberrant accumulation of activated phenotype cells despite an overall increase in T cell numbers. These results indicate that the immunopathology of transgenic IL-6 was B cell dependent and that IL-6 overexpression did not have any direct stimulatory effects on T cells. Why IL-6 signaled B cells impose a pro-inflammatory environment for T cells is an interesting question. Identification of B cell dependent factors that trigger T cell activations and inflammation in B cell sufficient IL-6<sup>Tg</sup> mice is currently under investigation.

In addition to upregulation of survival factors, another striking feature of *in vivo* IL-6 signaling was the downregulation of IL-7R $\alpha$  expression *in trans*. Previously, we showed that pro-survival cytokines, including IL-6, suppressed IL-7R $\alpha$  expression *in vitro* [18]. Thus, IL-7R $\alpha$  downregulation by IL-6 *in vivo* is in agreement with these results. We consider this mechanism important to exclude IL-6 signaled cells from being redundantly signaled by IL-7 so that repetitive pro-survival signaling and unnecessary consumption of IL-7 are prevented. Moreover, IL-7R $\alpha$  downregulation by IL-6 *in trans* will also maximize *in vivo* availability of IL-7 for cells that failed to get signaled by IL-6. Thus, we propose that cross-talk and balancing of IL-6R $\alpha$  and IL-7R $\alpha$  expression act synergistically to optimize pro-survival cytokine consumption and maximize T cell homeostasis.

Finally, while we demonstrated that IL-6 can act as a homeostatic T cell survival factor, our data also indicate that its pro-survival mechanism is distinct from that of IL-7. Both IL-6 and IL-7 activates the JAK/STAT signaling pathway. However, STAT5 is the major signaling molecule for IL-7 whereas STAT3, and to a lesser extent STAT1, is the primary downstream molecule of IL-6 [35, 64, 65]. Consistent with such divergent signaling pathways, we found that Bcl-2 mRNA expression was induced by IL-7 but not by IL-6. Mcl-1 expression, on the other hand, was induced by both IL-7 and IL-6, suggesting that IL-7 exerts a broader and more potent anti-apoptotic effect than IL-6. It would be important to assess to what extent IL-6 overexpression could replace IL-7 deficiency during thymopoiesis and T cell homeostasis. Generation of IL-6 transgenic mice on an IL-7 deficient background could answer these questions, and we are currently in the process of



generating such mice. Together with *in vitro* cytokine co-stimulation studies, we expect that these tools will provide insights on the unique role of IL-7 and synergistic effects of IL-6 in T cell survival.

Collectively, here we demonstrated a homeostatic effect of IL-6 in T cell survival that was revealed through the genetic depletion of B cells. This system allowed us to assess a direct effect of IL-6 on T cell survival, and we demonstrate that IL-6 promotes survival of resting T cells and expands peripheral T cell space.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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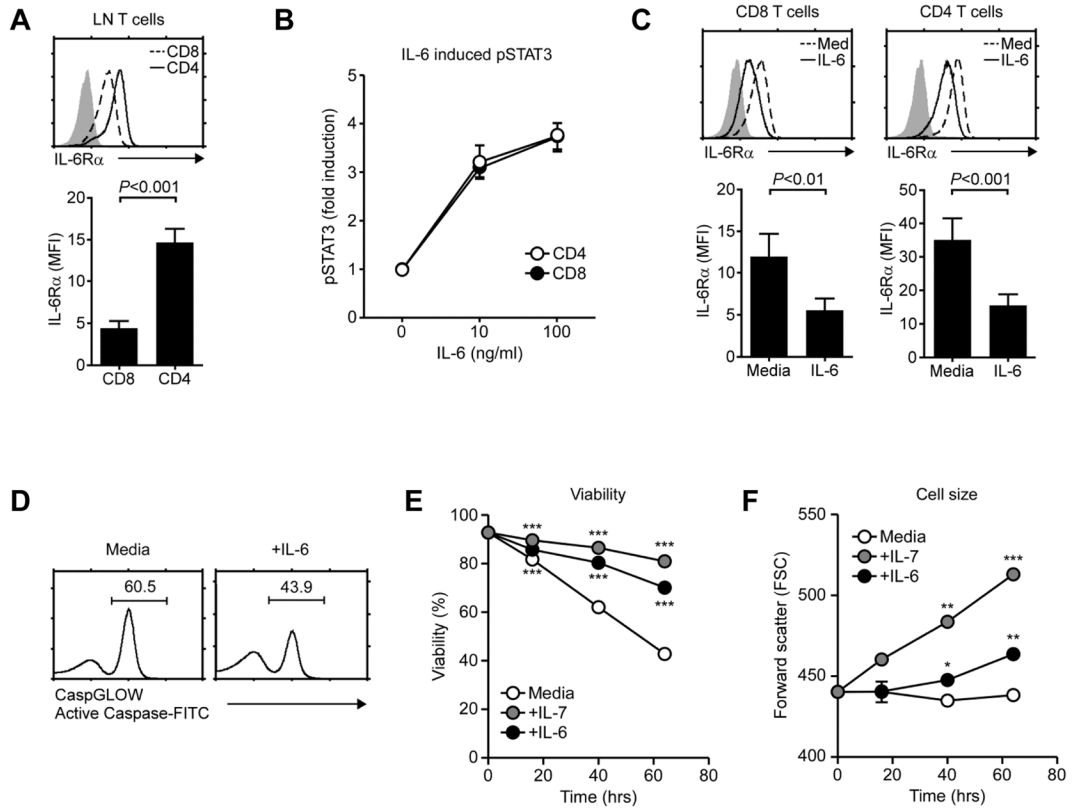
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**Highlights**

- IL-6 signaling downregulates expression of its own receptor
- IL-6 overexpression increases T cell numbers without affecting thymopoiesis
- IL-6 signaling promotes cell survival by inducing expression of Mcl-1 but not Bcl-2
- IL-6 is a novel homeostatic cytokine for naïve T cells





**Fig. 1. IL-6 promotes T cell survival *in vitro***

(A) IL-6R $\alpha$  expression on LN T cells. IL-6R $\alpha$  levels were determined on freshly isolated CD4 and CD8 LN T cells (top) and quantified in mean fluorescence intensities (MFI) (bottom). Results show the mean  $\pm$  SEM of five independent experiments.

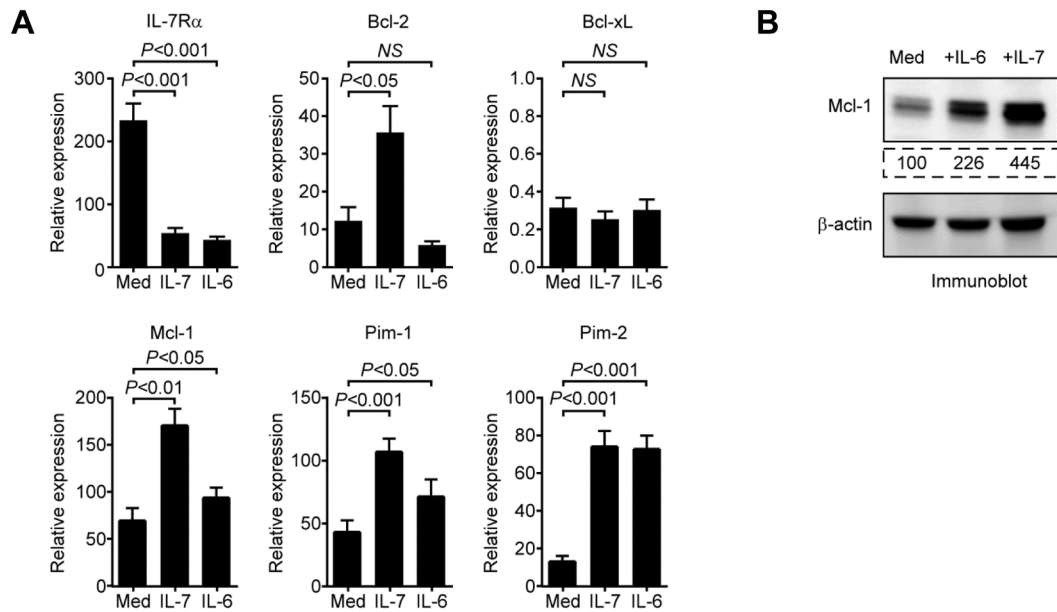
(B) IL-6 signaling in CD4 and CD8 T cells. Phospho-STAT3 (pSTAT3) contents upon IL-6 signaling were assessed in wildtype CD4 and CD8 T cells by intracellular staining. Data show the summary of eight independent experiments.

(C) IL-6R $\alpha$  levels on IL-6 stimulated LN T cells. LN T cells were incubated overnight with IL-6 or medium alone. Next day, surface IL-6R $\alpha$  levels were assessed on CD4 and CD8 T cells (top). Bar graphs show the mean  $\pm$  SEM of IL-6R $\alpha$  levels from seven independent experiments.

(D) Total caspase activity upon IL-6 stimulation. Purified LN T cells were cultured for 48 hours with IL-6 or medium alone. Caspase activities were assessed by incubation with FITC-conjugated caspase inhibitor peptides. Histograms show representative results from three independent experiments.

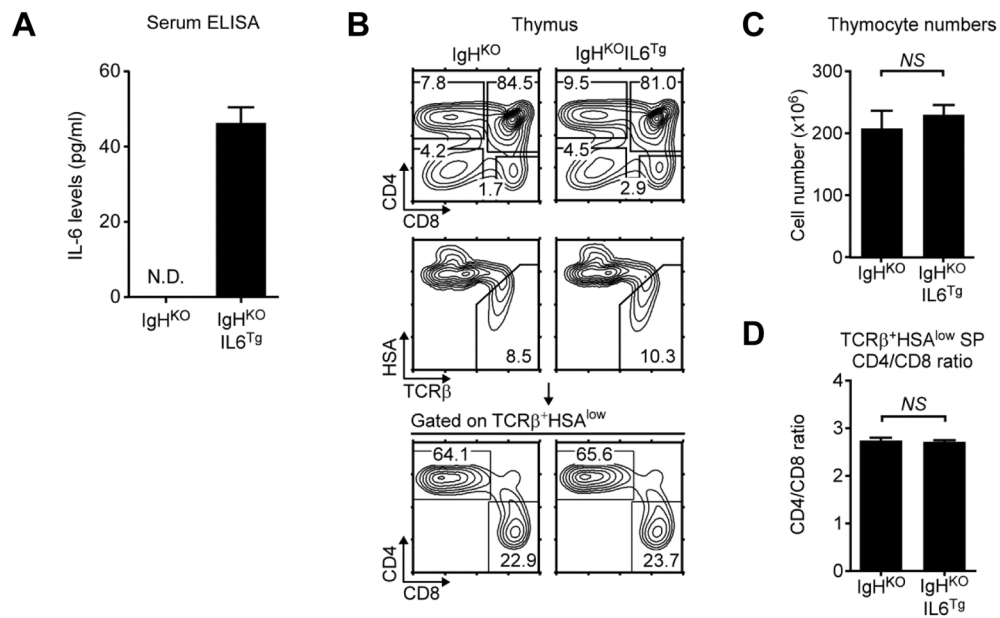
(E) Survival curve of IL-6 treated T cells. Purified LN T cells were cultured in medium, IL-7 or IL-6 for the indicated time. Cell viability was determined by propidium iodide exclusion. Data are the summary of four independent experiments. \*\*\* $P < 0.001$ , two-tailed Student's *t*-test.

(F) Cell size assessment of IL-6 treated T cells. LN T cells were cultured in media, IL-7 or IL-6 for the indicated time. Cell sizes were determined by Forward Scatter (FSC) analysis. Data are the results of three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , two-tailed Student's *t*-test.



**Fig. 2. IL-6 signaling induces expression pro-survival and pro-metabolic factors**

A) Pro-survival factor expression in IL-6 stimulated T cells. Purified LN T cells were incubated overnight in medium, IL-7 or IL-6. mRNA expression of the indicated genes were assessed by qRT-PCR. Data show the mean  $\pm$  SEM of three independent experiments. (B) Immunoblot analysis of Mcl-1 expression in cytokine stimulated cells. Purified LN T cells were incubated with IL-6, IL-7 or in medium alone for 24 hours, and total cell lysates were probed for Mcl-1 and then reprobbed for  $\beta$ -actin as loading control. Numbers in box indicate relative Mcl-1 expression as determined by densitometry of the bands. Blot is representative of two independent experiments.



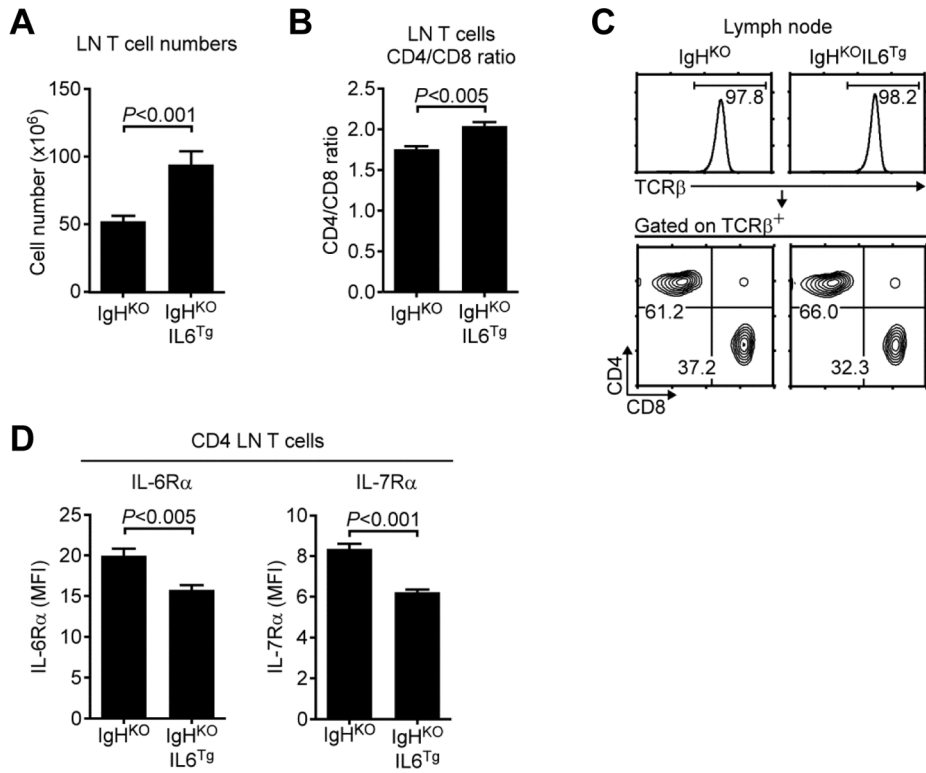
**Fig. 3. Thymocyte development in IL-6 transgenic mice**

(A) Transgenic IL-6 expression in IgH<sup>KO</sup>IL6<sup>Tg</sup> mice. Serum levels of transgenic human IL-6 were determined by ELISA. Results show the mean  $\pm$  SEM of two independent experiments with total six IgH<sup>KO</sup> and seven IgH<sup>KO</sup>IL6<sup>Tg</sup> mice.

(B) Thymocyte development in IgH<sup>KO</sup>IL6<sup>Tg</sup> mice. Contour plots show CD4/CD8 profiles of total thymocytes (top) and of TCR<sup>+</sup>HSA<sup>low</sup> mature thymocytes (bottom).

(C) Thymocyte numbers in IgH<sup>KO</sup>IL6<sup>Tg</sup> mice. Total thymocyte numbers were determined in five separate experiments from seven IgH<sup>KO</sup> and nine IgH<sup>KO</sup>IL6<sup>Tg</sup> mice.

(D) CD4/CD8 lineage differentiation in IgH<sup>KO</sup>IL6<sup>Tg</sup> mice. CD4/CD8 ratio was determined from TCR<sup>+</sup>HSA<sup>low</sup> mature thymocytes. Data show mean  $\pm$  SEM of four independent experiments with a total of seven IgH<sup>KO</sup> and six IgH<sup>KO</sup>IL6<sup>Tg</sup> mice.



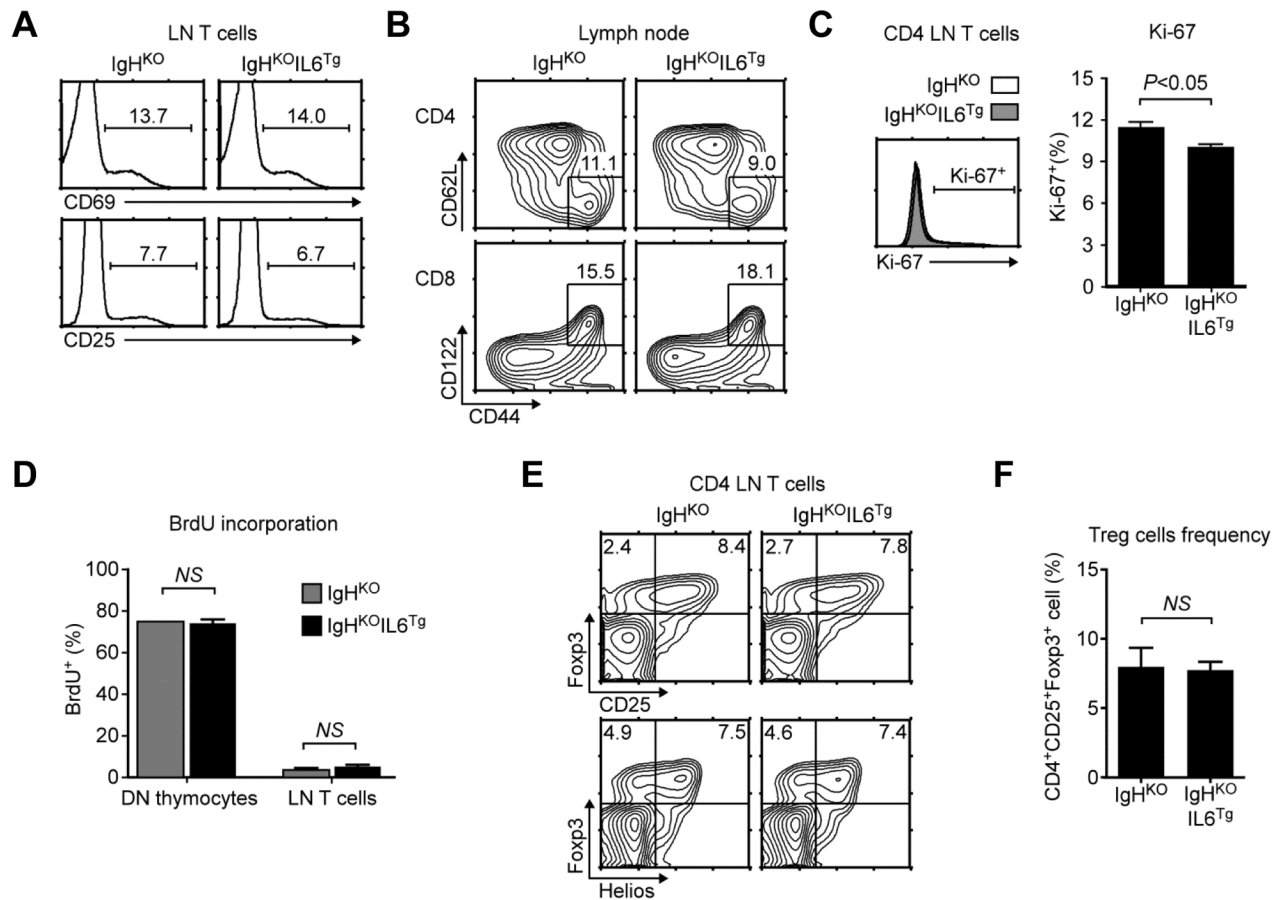
**Fig. 4. IL-6 promotes peripheral T cell homeostasis**

(A) LN T cell numbers in IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> mice. Data show the mean +/- SEM of five independent experiments with a total of seven IgH<sup>KO</sup> and eight IgH<sup>KO</sup>IL6<sup>Tg</sup> mice.

(B) LN cell analysis of IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> mice. Histograms show TCRβ expression of total LN cells (top). Contour plots show CD4/CD8 profiles of TCRβ<sup>+</sup> gated LN T cells. Results are representative of five independent experiments with seven IgH<sup>KO</sup> and eight IgH<sup>KO</sup>IL6<sup>Tg</sup> mice.

(C) CD4/CD8 ratio of LN T cells. Data show mean +/- SEM of four independent experiments with a total of seven IgH<sup>KO</sup> and six IgH<sup>KO</sup>IL6<sup>Tg</sup> mice.

(D) Cytokine receptor expression on IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> T cells. Surface IL-6R and IL-7R levels were quantified in MFI. Bar graphs show the mean +/- SEM of four independent experiments with seven IgH<sup>KO</sup> and six IgH<sup>KO</sup>IL6<sup>Tg</sup> mice.



### Fig. 5. T cell quiescence is maintained in IgH<sup>KO</sup>IL6<sup>Tg</sup> mice

(A) Surface CD69 and CD25 expression on IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> T cells. Histograms are representative of three independent analyses with each one mouse.

(B) Activated/memory phenotype cells percentages in CD4 and CD8 LN T cells. Contour plots of surface CD62L and CD122 versus CD44 expression are representative of three independent analyses with each one mouse.

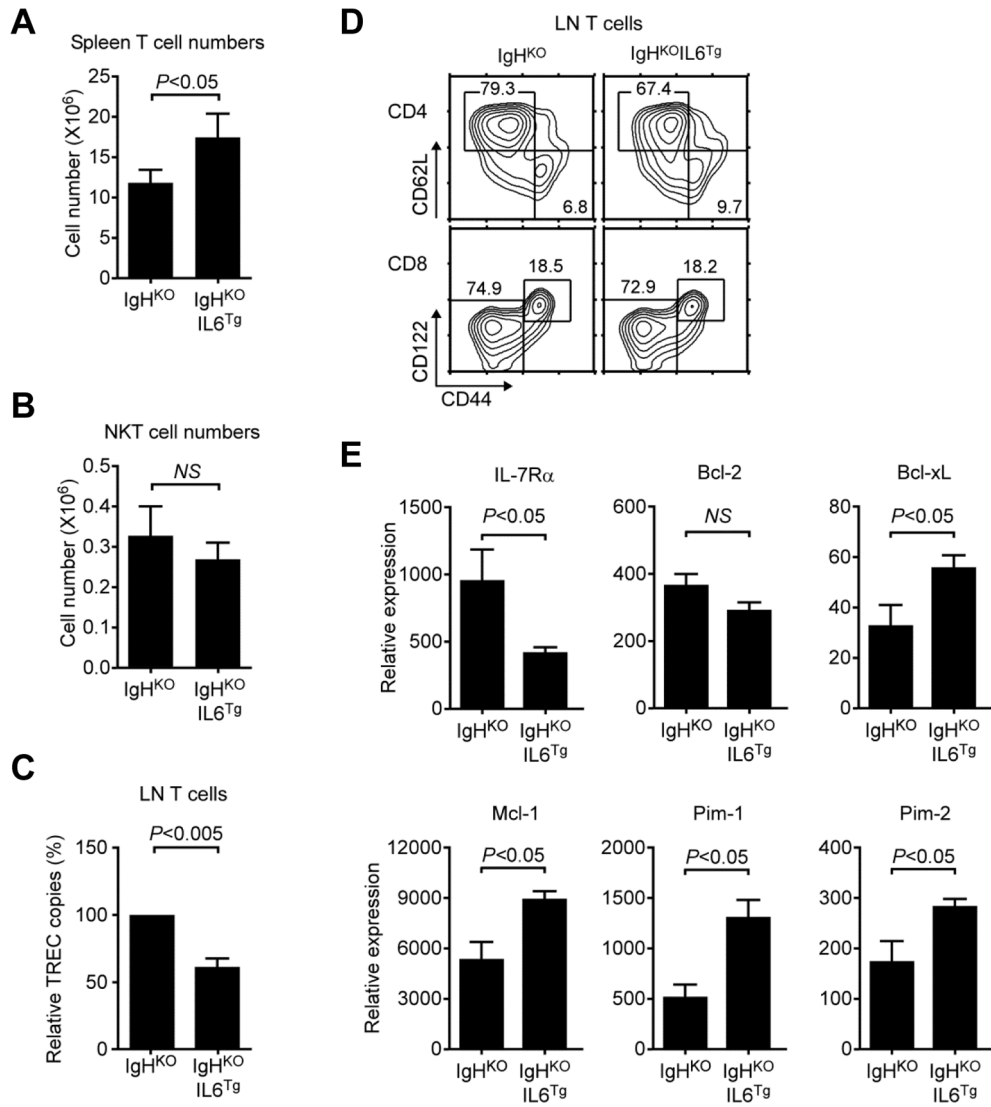
(C) Ki-67 intra-nuclear staining of CD4 LN T cells from IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> mice. Histogram is representative of and bar graph shows the mean  $\pm$  SEM from three independent experiments with each one mouse.

(D) BrdU incorporation in DN thymocytes and LN T cells from IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> mice. After initial i.p. injection with BrdU, mice were supplied with BrdU in drinking water for 3 days. BrdU incorporation was assessed by anti-BrdU intracellular staining. Bar graph shows the mean  $\pm$  SEM for the indicated cells from two independent experiments with two IgH<sup>KO</sup> and five IgH<sup>KO</sup>IL6<sup>Tg</sup> mice.

(E) FoxP3<sup>+</sup> and Helios<sup>+</sup> CD4 T cells in IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> mice. Contour plots are representative of three independent analyses with each one mouse.

(F) FoxP3<sup>+</sup>CD25<sup>+</sup> CD4 Treg cell percentages in IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> LN cells. Data show the mean  $\pm$  SEM percentages of FoxP3<sup>+</sup>CD25<sup>+</sup> cells among CD4 LN T cells from three independent experiments.





**Fig. 6. IL-6 is an *in vivo* T cell survival factor**

(A) Spleen T cell numbers in aged IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> mice. Data show the mean +/- SEM from ten IgH<sup>KO</sup> and nine IgH<sup>KO</sup>IL6<sup>Tg</sup> mice.

(B) Spleen NKT cell numbers in aged IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> mice. Data show the mean +/- SEM from six IgH<sup>KO</sup> and seven IgH<sup>KO</sup>IL6<sup>Tg</sup> mice.

(C) TREC copy numbers in IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> T cells. TREC copy numbers were determined relative to TREC numbers in IgH<sup>KO</sup> T cells, which was set to 100. Results show the mean +/- SEM of three independent experiments.

(D) Naïve and memory phenotype T cells in aged IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> mice. CD62L, CD122 and CD44 expression were determined on gated CD4 and CD8 T cells of aged (37–52 weeks) mice. Data are representative of eight independent experiments with eight IgH<sup>KO</sup> and eleven IgH<sup>KO</sup>IL6<sup>Tg</sup> mice.

(E) Pro-survival factor expression in IgH<sup>KO</sup>IL6<sup>Tg</sup> T cells. mRNA content of the indicated genes were assessed in purified IgH<sup>KO</sup> and IgH<sup>KO</sup>IL6<sup>Tg</sup> CD4 T cells by RT-qPCR. Data show the mean +/- SEM of five independent experiments with a total of six IgH<sup>KO</sup> and six IgH<sup>KO</sup>IL6<sup>Tg</sup> mice.