IL-15 enhances the antitumor effect of human antigen-specific CD8⁺ T cells by cellular senescence delay

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ABSTRACT
Optimal expansion protocols for adoptive human T-cell therapy often include interleukin (IL)-15; however, the mechanism by which IL-15 improves the in vivo antitumor effect of T cells remains to be elucidated. Using human T cells generated from HLA-A2+ donors against novel T-cell epitopes derived from the human U266 myeloma cell line Ig light chain V-region (idiotype) as a model, we found that T cells cultured with IL-15 provided superior resistance to tumor growth in vivo, compared with IL-2, after adoptive transfer into immunodeficient hosts. This effect of IL-15 was associated with delayed/reversed senescence in tumor antigen–specific memory CD8⁺ T cells mediated through downregulation of P21WAF1, P16INK4a, and P53 expression. Compared to IL-2, IL-15 stimulation dramatically activated JAK3-STAT5 signaling and inhibited the expression of DNA damage genes. Thus, our study elucidates a new mechanism for IL-15 in the regulation of STAT signaling pathways and CD8⁺ T-cell senescence.

Introduction
In 2016, it is predicted that a total of 1,685,210 new cancer cases and 595,690 cancer deaths will occur in the United States.¹ One promising strategy to improve the survival of cancer patients is adoptive transfer with tumor antigen–specific T cells. In a variety of clinical trials, with both solid and hematologic cancers, adoptive T-cell transfer has emerged as one of the most effective immunotherapies.² Early clinical studies have demonstrated a 50–70% clinical response in patients.³⁻⁵ However, the optimal protocols for expansion of T cells, especially antigen-specific CD8⁺ T cells, remain to be determined.

Cytokines have substantial effects on T-cell phenotype and function.⁶ For example, IL-2 is widely used for T-cell growth because it can actively drive the expansion of T cells and the contraction phase of immune response.⁷⁻⁸ IL-7 and IL-15 are required for the initiation of immune response and the survival of T cells.⁹⁻¹¹ IL-21 can promote the development of both Th17 and Tfh T cells that play roles in antitumour and antiviral responses.¹² Detailed studies revealed these cytokines have a distinguished effect on different T-cells subsets. For example, IL-2 is required for the in vitro growth of CD4⁺ T cells, but is not required for normal clonal expansion of antigen-specific CD8⁺ T cells.¹³ In vivo studies revealed IL-2 induces the apoptosis of effector memory CD4⁺ T cells and IL-15 can enhance the in vivo function of CD8⁺ T cells.¹⁴⁻¹⁶ Interestingly, it is known that most cytokines like IL-2, IL-15, IL-21, and IL-7 can activate the JAK-STAT5s signaling pathway; however, it is not yet clear how these cytokines exert their individual functions through one common signaling pathway.

In this study, we used T cells generated against the Ig light chain V-region epitopes (Idiotype, Id) of the human myeloma U266 cell line as a model to test the effect of cytokines on the generation of T-cells for adoptive therapy. We found that IL-15-expanded, Id-specific T cells mediate long-term antitumor effects in vivo, which were associated with delayed/reversed memory CD8⁺ T-cell senescence. The effect of IL-15 in memory CD8⁺ T-cell senescence delay is through the downregulation of P21WAF1, P16INK4a, and P53. Specifically, we found that IL-15 strongly activated the JAK3-STAT5 signaling pathway and inhibited the expression of DNA damage genes. Our study provides a new mechanism for IL-15 regulation in the CD8⁺ T-cells senescence process.

Results
In vivo antitumor effects of adoptively transferred Id L-chain-specific T cells expanded by IL-2 or IL-15

We have previously reported the identification of novel immunogenic CD8⁺ T-cell epitopes in the V-region of the Ig light chain (L-chain, Idiotype antigen) of the U266 human myeloma...
cell line and primary human lymphomas. In order to test the in vivo function of these L-chain-specific T cells, we stimulated HLA A2+ normal donors’ T cells as previously reported, and purified Id L-chain, peptide-specific CD8+ T cells and expanded them with IL-2 (180 IU/mL) or IL-15 (50 ng/mL) using the rapid expansion protocol (REP). After 14 d, we subsequently transferred the same number of T cells (1 x 10^7) into the immune-deficient mice, bearing 3 d U266 xenografts. Tumor growth was monitored by U266 tumor-specific IgE protein secretion in mouse serum. While IL-2-expanded L-chain-specific T-cells did not inhibit U266 tumor growth and the non-U266-idiotypic-specific T-cells exhibited a pattern of specific tumor growth delay in vivo, these T-cells only temporarily inhibited tumor cell growth in vivo (Fig. 1A). By contrast, mice receiving IL-15-expanded, L-chain-specific CD8+ T cells demonstrated significantly lower IgE serum concentrations, compared with IL-2-expanded T cells (Fig. 1B), and about 53% of mice remained alive at the end of observation (Fig. 1C). The inhibition was tumor-specific, as the Id L-chain-specific T-cells expanded by IL-15 did not inhibit IgA-secreting ARP-1 myeloma xenografts and the non-U266-idotype-specific T-cells expanded by IL-15 did not inhibit U266 tumor growth in vivo (Fig. 1D). To determine whether the antitumor effect of IL-15-expanded T cells is associated with increased proliferation and persistence of Id L-chain-specific CD8+ T cells, we adaptively transferred 1 x 10^7 L-chain-specific T cells into tumor-free mice and collected the blood and spleens on day 7. We found that significantly more IL-15-expanded, L-chain-specific CD8+ T cells were detectable in both the blood and spleens of mice, compared with IL-2-expanded L-chain-specific CD8+ T cells, suggesting that IL-15-expanded CD8+ T cells have superior proliferation and persistence in vivo (Fig. 1E).

IL-15-regulated senescence delay in antigen-specific T cells through the JAK3-STAT5 signaling pathway

To determine the molecular mechanism underlying IL-15-regulated senescence delay, we expanded the Id L-chain-specific T cells with IL-15 (50 ng/mL) by REP, and added the candidate signaling pathway inhibitors on day 12. On day 14, we analyzed the expression of CD27 and CD28 in these expanded T cells and observed that JAK3 and STAT5 inhibitors significantly downregulated CD27 and CD28 expression (Fig. 3A). The JAK1 and JAK2 inhibitors also partially downregulated the expression of the CD27 and CD28 of L-chain-specific CD8+ T cells, but not CD8+ Tem cells. The signaling pathway inhibitors MEK1/2, PI3, AKT, IKK, P38, and JNK did not have a significant effect on CD27 or CD28 expression in L-chain-specific T cells. Next, the effect of STAT5 in the regulation of senescence delay was confirmed by ShRNA knockdown. We found that knockdown of STAT5b in IL-15-expanded L-chain-specific CD8+ T cells resulted in significant downregulation of CD27 and CD28 (Fig. 3B). These data indicate that IL-15 regulates the senescence delay of antigen-specific T cells through the JAK3-STAT5 signaling pathway.

IL-15 strongly activates STAT5 and inhibits the expression of DNA damage genes in human CD8+ T-cells

In order to see how IL-15 activates the STAT5 signaling pathway, we treated the antigen-specific (Id, L-chain) CD8+ T-cell line with IL-15 or IL-2 at different concentrations for multiple time points, and analyzed the cell extracts for pSTAT5 activity through Western blotting. We observed that IL-15 treatment led to a dramatic increase of pSTAT5 signaling, compared with
Figure 1. Specific in vivo tumor inhibition by adoptively transferred Ig L-chain, V-region (Idiotypic, Id)-peptide-specific T cells against U266 xenografts. (A) IL-2-expanded, or (B) IL-15-expanded, L-chain peptide-specific (P19, 20, 23, 25, 26, 28) T cells (1 x 10^7) were transferred to SCID γc chain knockout (NSG) mice bearing day 3 U266 (10^5) xenografts. U266-derived IgE was monitored as a serum marker of tumor growth by ELISA. (C) Kaplan–Meier survival curves of 103 experimental mice-bearing U266 xenografts treated with either IL-2- or IL-15-expanded, L-chain-specific T cells. (D) Inhibition of tumor growth by IL-15-expanded, L-chain peptide-specific (P19, 23, 25, 28) T cells (1 x 10^7) against day-3 U266 (IgE secreting) or ARP-1 (IgA secreting) (10^5) xenografts, which were injected simultaneously into the same mice. (E) Flow cytometry detection of Id L-chain-specific CD8+ T cells (P28, hCD3+) in the blood and spleens of non-tumor bearing NSG mice that had received 1 x 10^7 L-chain peptide-specific (P28) T cells 7 d earlier. Panels A, B, and D shown are indicated as mean ± SD of 5–7 mice per group. p < 0.05.
IL-2 treatment, in idiotype-specific CD8^+ T-cell populations at all conditions, indicating that IL-15 treatment strongly activates pSTAT5 signaling in CD8^+ T cells (Fig. 4A). By contrast, we found that treatment with IL-15 or IL-2 has little effect on the activation of pSTAT3 in the CD8^+ T-cell population (Fig. 4A and Fig. S2A). As previous studies reported,^32-34 we also found that IL-15 treatment activated pAKT signaling and resulted in higher perforin expression in CD8^+ T cells (Fig. S2B and D). IL-2 treatment led to high Phospho-S6 Ribosomal Protein expression and low pAKT activation in CD8^+ T cells (Fig. S2C).

In our previous data, we found 85% of senescence biomarker genes are downregulated in IL-15-expanded T cells. To confirm that the expression of these genes was regulated by IL-15, we used an online TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html) and identified nine STAT consensus binding sites on the promoters of ATM, 53BP1, and MDC1 genes located between position -11477 and -124 (Fig. 4B). Through ChIP-PCR assay, we observed that there was significantly more pSTAT5 than pSTAT3 binding to the nine STAT sites in IL-15-expanded T cells (Fig. 4C, p < 0.01, Paired t-test). The binding ratio of pSTAT5/pSTAT3 to the sites is not significant in IL-2-expanded T cells (pD0.38). In unstimulated idiotype-specific CD8^+ T cells, there is significantly more pSTAT3 than pSTAT5 binding to the nine STAT sites (p < 0.01). Moreover, we found significantly more binding of transcriptionally repressive histones [H3K27: tri-methy-H3 (Lys27), p = 0.043] and less binding of transcriptionally active...
Table 1. Relative expression levels of cellular senescence biomarker genes in IL-2/IL-15-expanded Id L-chain-specific T cells.

| Position | Unigene | GeneBank | Symbol | Description | Relative expression (unit) |
|----------|---------|----------|--------|-------------|---------------------------|
| A01      | Hs.431048 | NM_005157 | ABL1   | C-abl oncogene 1, non-receptor tyrosine kinase | P28 (IL-2) 0.009894, P28 (IL-15) 0.001658 |
| A02      | Hs.525622 | NM_005163 | AKT1   | V-akt murine thymoma viral oncogene homolog 1 | P28 (IL-2) 0.054965, P28 (IL-15) 0.028693 |
| A03      | Hs.459538 | NM_000693 | ALDH1A3 | Aldehyde dehydrogenase 1 family, member A3 | P28 (IL-2) 0.006871, P28 (IL-15) 0.000016 |
| A04      | Hs.367437 | NM_000051 | ATM     | Ataxia telangiectasia mutated | P28 (IL-2) 0.044658, P28 (IL-15) 0.017633 |
| A05      | Hs.380403 | NM_004343 | CALR    | Calreticulin | P28 (IL-2) 0.141755, P28 (IL-15) 0.065053 |
| A06      | Hs.515162 | NM_005180 | BMI1    | BMI1 polycomb ring finger oncogene | P28 (IL-2) 0.253652, P28 (IL-15) 0.275238 |
| A07      | Hs.58974  | NM_001237 | CCNA2   | Cyclin A2 | P28 (IL-2) 0.054965, P28 (IL-15) 0.028693 |
| A08      | Hs.23960  | NM_001238 | CCNB1   | Cyclin B1 | P28 (IL-2) 0.006871, P28 (IL-15) 0.000016 |
| A09      | Hs.523852 | NM_035056 | CCND1   | Cyclin D1 | P28 (IL-2) 0.006871, P28 (IL-15) 0.000206 |
| A10      | Hs.244723 | NM_001239 | CCNE1   | Cyclin E1 | P28 (IL-2) 0.006871, P28 (IL-15) 0.008538 |

(Continued on next page)
Data is representative of three independent experiments with three Id-specific T-cell lines.

**Table 1. (Continued)**

| Position | Unigene | GeneBank | Symbol | Description | P28 (IL-2) | P28 (IL-15) |
|----------|---------|----------|--------|-------------|-----------|------------|
| E08      | Hs.202453 | NM_002467 | MYC    | V-myc myelocytomatosis viral oncogene homolog (avian) | 0.006671 | 0.00261 |
| E09      | Hs.492208 | NM_002485 | NBN    | Nibrin | 0.016481 | 0.010458 |
| E10      | Hs.654408 | NM_003998 | NFKB1  | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (P300: Histone acetyltransferase p300, p < 0.01; H3K4: Histone tri methyl lysine 4, p = 0.035; AcyH3: acetyl-Histone H3, p < 0.01) to these nine STAT sites in IL-15, compared to IL-2-expanded T cells, (Fig. 4D). Altogether, these data indicate that IL-15 can strongly activate the STAT5 signaling pathway, which inhibited the expression DNA damage genes in CD8^+^ T cells.

**Discussion**

Adoptive T cell transfer has emerged as an effective immunotherapeutic approach for both solid and hematologic cancers in a variety of clinical trials. Recent studies of adoptive transfer with autologous T cells generated from patients have focused on generation of genetically modified memory CD8^+^ T cells with chimeric antigen receptors or T-cell receptors with a particular focus on improving the proliferation and persistence of T cells after transfer. Traditionally, IL-2 has been a central component of T-cell expansion protocols. However, IL-2-expanded T cells have significant limitations in adoptive therapy, including susceptibility to T-cell activation-induced cell death (AICD), Treg proliferation, and T-cell differentiation. Hence, there is an urgent need to find new cytokines for the growth of T cells. In this study, we found IL-15-expanded T cells mediate superior protection against tumor cells in vivo and mechanism of IL-15 is through the senescence delay/reversal of human CD8^+^ T cells. Specifically, we found IL-15 can strongly activate STAT5 signaling, which changed the ratio of pSTAT5/3 signaling in the CD8^+^ T cells and decreased the expression of DNA damage molecules. Although CD4^+^ T-cell senescence delay/reversal have been reported before, our results are the first to demonstrate senescence delay/reversal in CD8^+^ T cells.

Cellular senescence is a specific cell cycle status in which the cells permanently withdraw from the cell cycle. Replicative senescence (telomere-dependent) usually occurs in T cells with shorter telomere length as a process of aging isolated in elderly people. Premature senescence (telomere-independent), on the other hand, has many causes, such as DNA damage, oxygen stress, chromatin perturbation, and oncogene perturbation. Extended in vitro culturing can cause senescence. Human T-cell senescence has been suggested as an important reason for escape from tumor surveillance. Unlike phenotypic biomarkers for memory T cells, there is no defined biomarker for senescent cells and the most consistent feature of senescent cells is their resistance to enter the S/G2 cell cycle stage after proliferative stimulation. Other phenotypic changes associated with senescent cells include the following: increased β-galactosidase activity, increased expression of cell cycle inhibitors and DNA damage molecules, increased expression of senescence-
IL-15 regulates senescence delay through the JAK3-STAT5 signaling pathway in Id-specific T cells. (A) Id L-chain-specific T cells were expanded with IL-15 (50 ng/mL) by rapid expansion protocol (REP) for 12 d before the addition of the signaling pathway inhibitors shown. The effect of signaling pathway inhibitors on CD27 and CD28 expression in Id L-chain-specific (P28) memory CD8+ T cells were analyzed by flow cytometry on day 14. (Detailed information on signaling pathway inhibitors is listed in Table S1.) (B) IL-15-expanded Id L-chain-specific (P28) T cells on day 14 were activated by plate-bound anti-CD3 antibody for 72 h and transfected with one of two (ShRNA1 or 2) STAT5b ShRNA-containing a lentivirus or vector alone for 12 h. 48 h later, the expression of STAT5, CD27, and CD28 was analyzed by real-time PCR or flow cytometry. MFI: Mean fluorescence intensity. \( p < 0.05 \).
associated pro-inflammatory cytokines, \(^54,55,60\) and decreased expression of CD27, CD28 biomarkers on the cell surface.\(^31,46\) Senescent human CD8\(^+\) T cells have poor proliferation capacity, defective killing abilities, and defective granule exocytosis.\(^61,62\) Thus, strategies to delay/reverse the senescence of tumor antigen-specific CD8\(^+\) T cells may improve the effectiveness of adoptive T-cell therapy. In this study, we found that IL-15-expanded idiotype L-chain-specific (P28) T cells have decreased P53, P21WAF1, and P16INK4a expression. They also have a higher percentage of cells in the S/G2 phase after proliferative stimulation; decreased senescence-associated pro-inflammatory cytokine expression (IL-8 and TNF\(\alpha\)); decreased senescence biomarkers expression; and higher CD27 and CD28 expression, compared to IL-2-cultured T cells. All of these changes indicate that IL-15-expanded antigen-specific memory CD8\(^+\) T cells have delayed/reverse senescence.

The Signal Transducer and Activator of Transcription (STAT) family of proteins consist of seven members that play important roles in immune system regulation.\(^63,64\) STAT proteins are highly homologous in several domains, including SH2, DNA-binding, and transactivation and they can mediate their function through the mechanism of homodimers or heterodimers.\(^64\) Recent studies found that cross-regulation among the STAT family members has an important role in the maintenance of cytokine signaling specificity.\(^63\) For example, IL-6 stimulation can form three distinct dimers: STAT1–STAT1, STAT1–STAT3, and STAT3–STAT3, which can play dramatically different functions in the cells.\(^65\) The binding ratio of different STAT members to the same STAT sites can affect gene expression and cell differentiation dramatically.\(^66-68\)

In our study, we found IL-15 stimulation dramatically activated STAT5 signaling and induced more pSTAT5 binding to the nine potential STAT binding sites on the promoters of DNA damage genes. Isotype-matched antibodies were used as negative controls for all experiments (data not shown). The downregulation of these genes in IL-15-expanded T cells confirmed the senescence process was delayed.

In summary, we found that IL-15 can delay the senescence process in memory CD8\(^+\) T cells through the strong activation of STAT5 and the changes of pSTATS3 signaling in CD8\(^+\) T cells. Our results are consistent with recent studies where constitutively associated pro-inflammatory cytokines, \(^54,55,60\) and decreased expression of CD27, CD28 biomarkers on the cell surface.\(^31,46\)

**Figure 4.** IL-15 strongly activates STAT5 signaling and changes the ratio of pSTAT5/3 signaling in CD8\(^+\) T cells. (A) Id-specific T cells starved of cytokines for 24 h were treated with IL-2 or IL-15 at different concentrations for 15 min. Total protein was extracted from the cytokine-treated T cells and equal amounts of protein were loaded into each lane. Anti-pSTAT3, anti-pSTAT5, anti-total STAT3, and anti-total STAT5 antibodies were used in Western blotting. (B) Schema for the nine potential STAT binding sites in the promoter regions of ATM, MDC1, and 53BP1. The prediction was carried out with TFSEARCH online program, and the potential STAT binding sequences and relative locations are indicated. (C) ChIP-PCR analysis of pSTAT5 and pSTAT3 binding to the STAT sites on the promoters of ATM, MDC1, and 53BP1 genes in cytokine-stimulated, day 14 IL-2- or IL-15-expanded Id L-chain-specific (P28) T cells, or unstimulated idotype-specific CD8\(^+\) T cells. Shown are pooled data for nine STAT binding sites on the promoters of DNA damage genes. Isotype-matched antibodies were used as negative controls for all experiments (data not shown). (D) ChIP-PCR analysis of histones binding to the STAT sites on the promoters of ATM, MDC1, 53BP1 genes in day 14, IL-2- or IL-15-expanded idiotype L-chain specific T cells. H3K27: tri-methyl-H3 (Lys27); P300: Histone acetyltransferase p300; H3K4: Histone tri methyl lysine 4; AcyH3: acetyl-Histone H3. MFI: Mean fluorescence intensity. \(p < 0.05\).
activated STAT5 signaling mediated strong antitumor effects and the inhibition of STAT3 led to an enhanced adoptive therapy effect.\textsuperscript{70–73} The mechanisms revealed in this study provide the basis for future rational design of strategies to improve persistence of CD8\textsuperscript{+} T-cell therapy in the clinical setting.

Materials and methods

Cell lines, antibodies, and reagents

Human myeloma cell lines U266 and ARP-1 were cultured in RPMI 1640 medium supplemented with 10\% fetal bovine serum and 10 µg/mL gentamicin at 37°C and 5\% CO\textsubscript{2}. Flow antibodies for T-cell surface biomarkers and cytokine antibodies were all from BD Biosciences or eBiosciences. The following reagents were used per manufacturers’ instructions: anti-P53 and anti-P21\textsuperscript{WAF1} (Genescript), anti-tubulin and anti-P16\textsuperscript{INK4a} (BD biosciences), ChIP grade anti-Histone H3 (tri methyl K4) (Abcam), anti-p300 (Millipore), anti-Histone 3 tri-methy-H3 (Lys27) (Millipore), anti-P21WAF1 (Genescript), anti-tubulin and anti-P16INK4a (BD biosciences), and a standard Western blot assay protocol was followed.\textsuperscript{19}

Expansion of U266 myeloma Id-specific T cells

Peptide-specific T cells (P20-T, P23-T, P25-T, P26-T, P28-T) were generated from HLA-A2+ normal donors as previously reported.\textsuperscript{17} Briefly, PBMCs (1 × 10\textsuperscript{6} cells/well) were incubated with 10 µg/mL Id-specific peptide (P20, P23, P25, P26, P28) in quadruplicate in 96-well U-bottom microculture plates in 200 µL of culture medium (50\% AIM-V, 50\% RPMI-1640, 10\% human AB serum, 100 IU/mL of IL-2) and restimulated with peptide every 3 d. After five stimulations, T cells were cultured with peptide-pulsed T2 cells and interferon (IFN)-γ production was determined from the supernatants by ELISA. The IFNγ-producing T cells were purified by an IFNγ-secreting Cell Enrichment and Detection Kit and further expanded in the presence of 30 × 10\textsuperscript{6} allogeneic feeder cells and 30 ng/mL anti-CD3 antibody in a T25 flask with AIM-V media including 10\% human AB serum. Cytokines (IL-2 180 IU/mL or IL-15 50 ng/mL) were added the next day. The culture medium was changed with same cytokine conditions on day 5 and every 3 d subsequently for 14–18 days, as described in the REP.\textsuperscript{20,21}

Adaptive T-cell therapy

Six-to-twelve-week-old NOD SCID IL-2 receptor γc chain knockout mice (Jackson Laboratory, Stock# 005557), were injected by IV with 0.2 × 10\textsuperscript{6} U266 or ARP-1 human myeloma cells on day 0. Mice were irradiated (200 Gy) on day 2 and received 1 × 10\textsuperscript{6} Id-specific T cells on day 3, followed by rIL-2 at 10,000 IU with IP injection twice daily for a total of six doses. Tumor growth was monitored by an ELISA assay of tumor-specific serum-secreted Ig protein (IgE for U266 and IgA for ARP-1, Bethyl laboratories) and the survival time of the mice was recorded.

Cell cycle assay

Id-specific T cells (1 × 10\textsuperscript{6}) expanded with IL-2 or IL-15 for 14 d were put in a complete T-cell medium in a 24-well plate which was coated with 1 µg/mL of OKT3 antibody. Seventy-two hours later, the T cells were stained with anti-human CD8\textsuperscript{+}, CD62L, and CD45RA for 30 min, washed in 1XPBS, and fixed with 70\% ethanol for overnight. The next day, 5 µg/mL Propidium iodide (PI) was added for 15 min at 37°C to stain the cells. After washing, the T cells were analyzed by cytometry. The fluorescence intensity of the stained cells was used to determine the G0/G1 and S/G2 phase of T cells.

Intracellular staining of pro-inflammatory cytokines

2 × 10\textsuperscript{6} idiotype-specific T cells expanded with IL-2 or IL-15 for 14 d were washed and stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 250 ng/mL ionomycin; after 2 h, 5 µg/mL brefeldin A was added. Five hours later the cells were stained with anti-human CD8\textsuperscript{+}, CD62L, and CD45RA for 30 min, washed in 1XPBS, fixed and permeabilized (BD Cytofix/Cytoperm Plus kit). Following this procedure the cells were stained with cytokine-specific antibodies and analyzed by flow cytometry.

Western blotting

Approximately 20 µg of total cell protein was extracted from Id-specific T cells and a standard Western blot assay protocol was followed.\textsuperscript{19}

Signaling pathway inhibition assay

Idiotype-specific T cells expanded with 50 ng/mL IL-15 and allogeneic feeder cells for 12 d were cultured with signaling pathway inhibitors in the presence of 50 ng/mL IL-15 in complete T cell medium. The concentrations of signaling pathway inhibitors used are listed in Table S1. The expression of CD27 and CD28 were analyzed on day 14 by flow cytometer.

STAT5 ShRNA knockdown

IL-15-expanded, day 14 idiotype-specific T cells were activated with a plate-coated in OKT3 antibody for 72 h, washed, and transfected with lentivirus containing STAT5b-ShRNA (ShRNA 1: NM_012448/TRCN0000232140, sigma) in the presence of 8 µg/mL of polybrene for 12 h. The cells were then washed with 1 X PBS, and incubated in cytokine-free T-cell complete medium. The expression of STAT5b was analyzed by real-time RT-PCR normalized with GAPDH expression and the surface expression of CD27 and CD28 were analyzed by flow cytometry.

Real-time PCR array assay

3 µg of RNA was extracted from IL-2 or IL-15-expanded idiotype-specific T cells and reverse transcribed into cDNA with the Superscript III kit (Invitrogen). The expression of 84 cellular senescence genes and MDC1 gen was analyzed with primers pre-located inside the real-time PCR array (Qiagen, Cat#
PAHS-050ZC), using the Applied Biosystems StepOne™/Real-Time PCR System. The real-time PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The results were analyzed by Qiagen on-line software and the list of genes are in Table 1.

**ChIP-qPCR assays**

IL-2 or IL-15-expanded idiotype-specific T cells were cross-linked and lysed with the ChIP assay kit (Cat# 26156, Thermo scientific). The digested chromatin was then immune-precipitated with 2 µg of anti-human pSTAT3, pSTAT5, Histone 3 tri-methy-H3 (Lys27), Histone H3 (tri methyl K4), Histone 3 acetylated, or p300 antibodies. The recovered DNA was purified through a column and amplified by real-time PCR at the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min. The ChIP primers used are listed in Table S2. The percentage of input was calculated as: % Input = 100 × 2\(^{-}\text{Average Ct}\) – Adjusted Input Ct. In all assays, only living cells were analyzed and the dead cells were removed with a dead cell removal kit (Cat# 130-090-101) from Miltenyi Biotec. Isotype-matched antibodies were used as negative control for all experiments (data not shown).

**Statistical analysis**

The Student t-test was used to compare various experimental groups; p values <0.05 were considered statistically significant. Unless otherwise indicated, means and standard deviations (SD) are shown.

**Study approval**

Animal studies were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Author Contributions**

J. W. and L. W. K. designed experiments. J. W., K. M., F. C., S. K., Z. J., X. X., and B. F. performed experiments. H. J., J. Q., L. Z., J. Y., S. N., and Q. Y. provided critical reagents or suggestions. J. W. and L. W. K. analyzed data and wrote the paper.

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