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ORIGINAL RESEARCH

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 P21^{WAF1}, P16^{INK4a}, 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-STATs 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 P21^{WAF1}, P16^{INK4a}, 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

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cell line and primary human lymphomas.[17,18] 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,[19] 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).[20,21] After 14 d, we subsequently transferred the same number of T cells (1×10^7) into the immune-deficient mice, bearing 3 d U266 xenografts.[21] Tumor growth was monitored by U266-specific IgE protein secretion in mouse serum.[22,23] While IL-2-expanded L-chain-specific CD8+ T cells can lyse the tumor cells very well *in vitro*,[17] 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-idiotype-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 adoptively transferred 1×10^7 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-expanded, Id L-chain-specific T cells exhibited delayed cellular senescence

Senescence is a special cell cycle mechanism that living cells become unresponsive to growth stimulation, permanently withdraw from cell cycle and exist with a pattern of specific gene signatures and phenotypes.[24,25] To investigate if the IL-15-expanded T cells have delayed senescence process compared to IL-2-expanded T cells, we performed cell cycle analysis of day 14 IL-2 or IL-15-expanded, L-chain-specific T cells after anti-CD3 antibody (OKT3) stimulation for 72 h, before adoptive transfer. We found that IL-15-expanded, CD8+ central memory (CD8+ Tcm: CD62L+, CD45RA−, $p < 0.01$) and CD8+ effector memory (CD8+ Tem: CD62L−, CD45RA−, $p < 0.01$) L-chain-specific T cells have a significantly higher percentage of cells in S/G2 phase compared with IL-2-expanded T cells after stimulation (Fig. 2A). We also analyzed the expression of cell cycle inhibitors P21[WAF1], P16[INK4a], and P53 in the day 14, L-chain-specific T cells, before the adoptive transfer. We found the expression of P21[WAF1], P16[INK4a], and P53 was significantly lower in IL-15-expanded T cells compared to IL-2-expanded T cells (Fig. 2B). Recent studies found that senescence immune cells can secret a large amount of the senescence-associated proinflammatory cytokines,[26] we performed intracellular cytokines assays and observed that IL-15-expanded day 14 L-chain specific CD8+ Tcm and CD8+ Tem cells expressed lower amounts of IL-8, TNFα, IFNγ, and TGF-

β1 after PMA and ionomycin stimulation, compared with IL-2-expanded T cells (Fig. 2C). We also performed cell surface staining of day 14 T cells just before adoptive transfer, which showed IL-15-expanded L-chain-specific CD8+ T cells have significantly higher expression of CD27 and CD28 compared with IL-2-expanded T cells ($p < 0.05$) (Fig. 2D). Finally, we extracted RNA from IL-2 or IL-15-expanded, L-chain specific T cells before adoptive transfer and reverse transcribed the RNA into cDNA. We performed real-time PCR microarrays with senescence signaling pathway gene-specific primers. We found that the expression of 85% (71 out of 84) cellular senescence biomarker genes was significantly decreased in IL-15-expanded T cells (Fig. 2E). These genes include the following: *53BP1 (TP53BP1), ATM, BMI1, CDK6, ETS1, CDKN1A, CDKN1B, CDKN1C, CDKN2A, CDKN2B, E2F1,MDM2, RB1, RBL2, MDC1, and TWIST1*, which have been reported to play important roles in the regulation of the initiation and progression of cellular senescence and cell cycle inhibition (Table 1 and Fig. S1).[27-29] Taken together, we found IL-15-expanded, L-chain-specific T cells have a higher percentage of S/G2 phase cells after stimulation, lower expression of cell cycle inhibitors, less production of senescence-associated proinflammatory cytokines, higher expression of CD27 and CD28, and downregulation of cellular senescence biomarker genes, suggesting that IL-15-expanded, L-chain-specific T cells exhibit senescence delay.[24,26,30,31]

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

To determine the molecular mechanism underlining IL-15 regulation of 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+ Tcm, 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 STATs 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
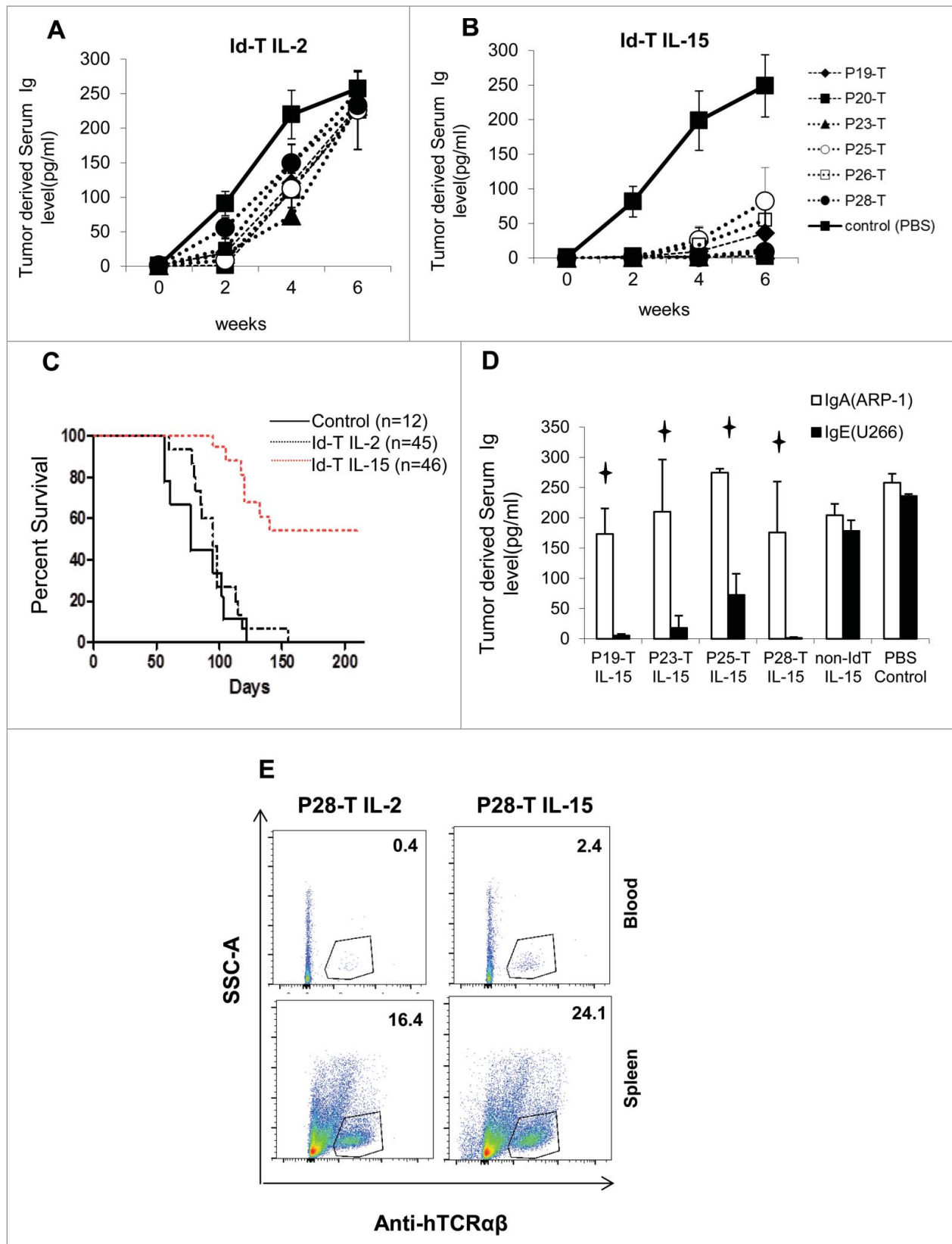
Figure 1. Specific *in vivo* tumor inhibition by adoptively transferred Ig L-chain, V-region (Idiotype, Id)-peptide-specific T cells against U266 xenografts. (A) IL-2-expanded, or (B) IL-15-expanded, L-chain peptide-specific (P19, 20, 23, 25, 26, 28) T cells (1×10^7) were transferred to SCID γc chain 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 (1×10^7) 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 1×10^7 L-chain peptide-specific (P28) T cells 7 d earlier. Panels A, B, and D shown are indicated as mean $\pm$ SD of 5–7 mice per group. $p < 0.05$.
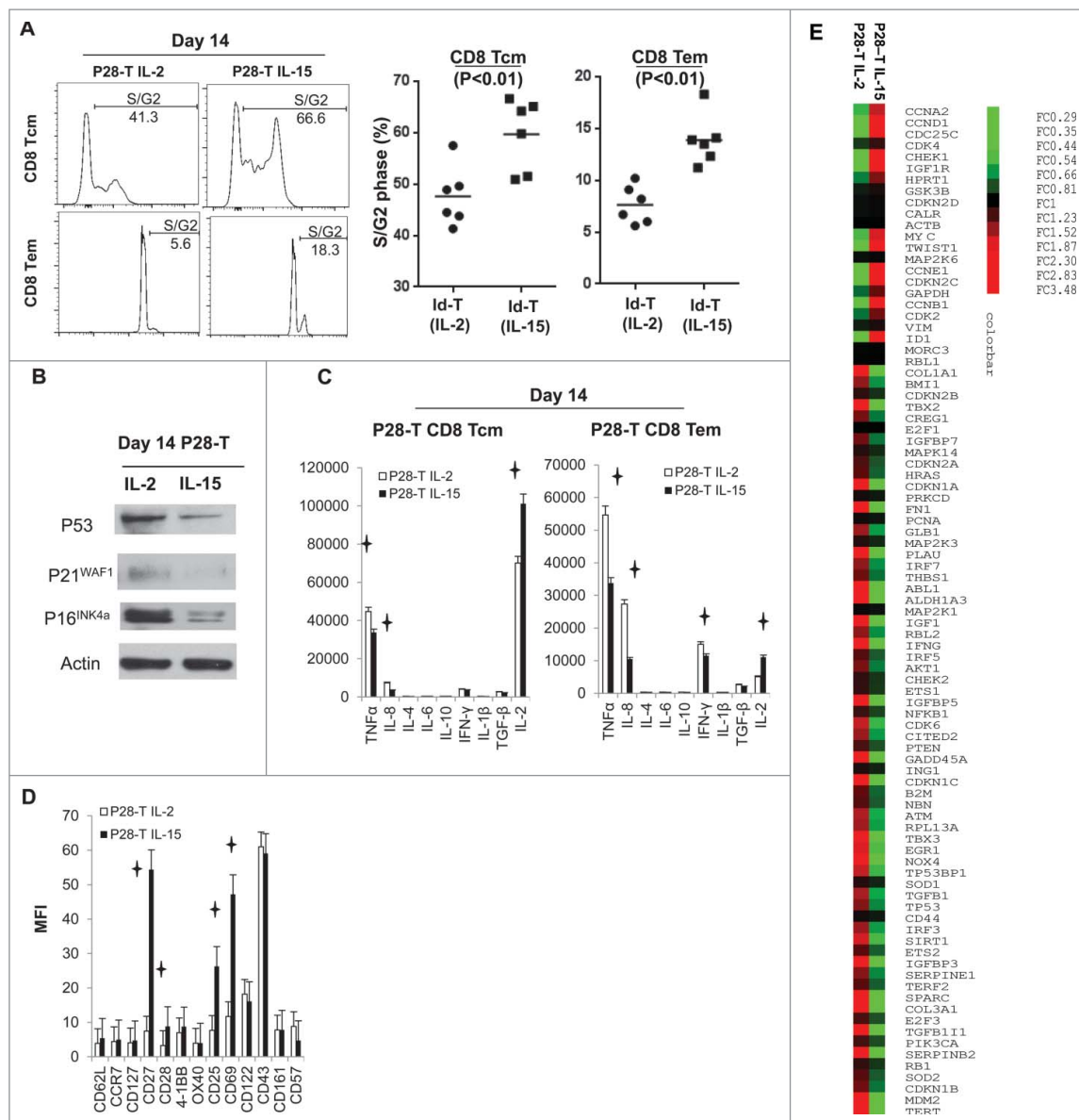
Figure 2. IL-15-expanded, Id L-chain-specific human Tcells exhibit delayed cellular senescence. (A) Cell cycle analysis of IL-2- or IL-15-expanded idiotype-specific memory CD8+ T cells after stimulation with OKT3 (1 μg/mL, plate-bound) for 72 h. CD8+ Tcm (CD8+, CD62L+, CD45RA−); CD8+ Tem (CD8+, CD62L−, CD45RA−). (B) Western blot analysis for P53, P21[WAF1], and P16[INK4a] expression of idiotype-specific memory CD8+ T cells expanded by IL-2 or IL-15, before transfer into mice. (C) Intracellular cytokine staining of IL-2- or IL-15-expanded idiotype-specific (P28) memory CD8+ T cells after 5 h of stimulation with PMA (50 ng/mL) and ionomycin (250 ng/mL) in the presence of 10 ug/mL Brefeldin A. (D) Flow cytometry analysis of cell surface markers of IL-2- or IL-15-expanded, L-chain peptide-specific (P28) day 14 T cells, before transfer into mice. (E) Heat map showing the expression of 84 cellular senescence biomarkers by real-time RT-PCR array assays in IL-15- or IL-2-expanded Id L-chain-specific (P28) T cells, before transfer into mice (List of genes is shown in Table 1). MFI: Mean fluorescence intensity. $p < 0.05$. Tcm = central memory T cells. Tem = effector memory T cells.

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 higher 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 ($p = 0.38$). In unstimulated idiotype-specific CD8+ T cells, there is significant more pSTAT3 than pSTAT5 binding to the nine STATs sites ($p = 0.04$). 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.

	Gene table				Relative expression (unit)	
Position	Unigene	GeneBank	Symbol	Description	P28 (IL-2)	P28 (IL-15)
A01	Hs.431048	NM_005157	ABL1	C-abl oncogene 1, non-receptor tyrosine kinase	0.009894	0.001658
A02	Hs.525622	NM_005163	AKT1	V-akt murine thymoma viral oncogene homolog 1	0.054965	0.028693
A03	Hs.459538	NM_000693	ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	0.006871	0.000016
A04	Hs.367437	NM_000051	ATM	Ataxia telangiectasia mutated	0.044658	0.017633
A05	Hs.380403	NM_005180	BMI1	BMI1 polycomb ring finger oncogene	0.141755	0.065053
A06	Hs.515162	NM_004343	CALR	Calreticulin	0.253652	0.275238
A07	Hs.58974	NM_001237	CCNA2	Cyclin A2	0.014048	0.042295
A08	Hs.23960	NM_031966	CCNB1	Cyclin B1	0.008914	0.03789
A09	Hs.523852	NM_053056	CCND1	Cyclin D1	0.006871	0.000206
A10	Hs.244723	NM_001238	CCNE1	Cyclin E1	0.006871	0.008538
A11	Hs.502328	NM_000610	CD44	CD44 molecule (Indian blood group)	0.159753	0.147131
A12	Hs.656	NM_001790	CDC25C	Cell division cycle 25 homolog C (S. pombe)	0.006871	0.001533
B01	Hs.19192	NM_001798	CDK2	Cyclin-dependent kinase 2	0.018076	0.034652
B02	Hs.95577	NM_000075	CDK4	Cyclin-dependent kinase 4	0.039731	0.056178
B03	Hs.119882	NM_001259	CDK6	Cyclin-dependent kinase 6	0.191699	0.066696
B04	Hs.370771	NM_000389	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	0.405019	0.075319
B05	Hs.238990	NM_004064	CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	0.092236	0.050659
B06	Hs.106070	NM_000076	CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	0.006871	0.000016
B07	Hs.512599	NM_000077	CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	0.017	0.011094
B08	Hs.72901	NM_004936	CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	0.035951	0.028407
B09	Hs.728783	NM_078626	CDKN2C	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	0.006871	0.017095
B10	Hs.435051	NM_001800	CDKN2D	Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	0.009557	0.01036
B11	Hs.24529	NM_001274	CHEK1	CHK1 checkpoint homolog (S. pombe)	0.006871	0.021533
B12	Hs.291363	NM_007194	CHEK2	CHK2 checkpoint homolog (S. pombe)	0.006871	0.003549
C01	Hs.82071	NM_006079	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	0.049903	0.02201
C02	Hs.172928	NM_000088	COL1A1	Collagen, type I, α 1	0.006871	0.000016
C03	Hs.443625	NM_000090	COL3A1	Collagen, type III, α 1	0.006871	0.000016
C04	Hs.5710	NM_003851	CREG1	Cellular repressor of E1A-stimulated genes 1	0.019568	0.010543
C05	Hs.654393	NM_005225	E2F1	E2F transcription factor 1	0.006871	0.006707
C06	Hs.269408	NM_001949	E2F3	E2F transcription factor 3	0.006871	0.002488
C07	Hs.326035	NM_001964	EGR1	Early growth response 1	0.006871	0.001447
C08	Hs.369438	NM_005238	ETS1	V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	0.406507	0.307562
C09	Hs.644231	NM_005239	ETS2	V-Ets erythroblastosis virus E26 oncogene homolog 2 (avian)	0.010578	0.006555
C10	Hs.203717	NM_002026	FN1	Fibronectin 1	0.007966	0.000016
C11	Hs.80409	NM_001924	GADD45A	Growth arrest and DNA-damage-inducible, α	0.019251	0.004137
C12	Hs.443031	NM_000404	GLB1	Galactosidase, β 1	0.039809	0.017389
D01	Hs.445733	NM_002093	GSK3B	Glycogen synthase kinase 3 β	0.020953	0.024772
D02	Hs.37003	NM_005343	HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	0.030779	0.018645
D03	Hs.504609	NM_002165	ID1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	0.006871	0.00338
D04	Hs.856	NM_000619	IFNG	Interferon, gamma	0.069941	0.012135
D05	Hs.160562	NM_000618	IGF1	Insulin-like growth factor 1 (somatomedin C)	0.007121	0.00072
D06	Hs.643120	NM_000875	IGF1R	Insulin-like growth factor 1 receptor	0.006871	0.003122
D07	Hs.450230	NM_000598	IGFBP3	Insulin-like growth factor binding protein 3	0.008874	0.002167
D08	Hs.607212	NM_000599	IGFBP5	Insulin-like growth factor binding protein 5	0.049616	0.000016
D09	Hs.479808	NM_001553	IGFBP7	Insulin-like growth factor binding protein 7	0.009151	0.00502
D10	Hs.46700	NM_005537	ING1	Inhibitor of growth family, member 1	0.008724	0.007556
D11	Hs.75254	NM_001571	IRF3	Interferon regulatory factor 3	0.094596	0.041639
D12	Hs.521181	NM_001098629	IRF5	Interferon regulatory factor 5	0.006871	0.003375
E01	Hs.166120	NM_001572	IRF7	Interferon regulatory factor 7	0.019498	0.009162
E02	Hs.145442	NM_002755	MAP2K1	Mitogen-activated protein kinase kinase 1	0.137061	0.132296
E03	Hs.514012	NM_002756	MAP2K3	Mitogen-activated protein kinase kinase 3	0.019382	0.016122
E04	Hs.463978	NM_002758	MAP2K6	Mitogen-activated protein kinase kinase 6	0.009738	0.010533
E05	Hs.485233	NM_001315	MAPK14	Mitogen-activated protein kinase 14	0.039407	0.032542
E06	Hs.484551	NM_002392	MDM2	Mdm2 p53 binding protein homolog (mouse)	0.56428	0.137308
E07	Hs.421150	NM_015358	MORC3	MORC family CW-type zinc finger 3	0.061078	0.064593

Table 1. (*Continued*)

Gene table					Relative expression (unit)	
Position	Unigene	GeneBank	Symbol	Description	P28 (IL-2)	P28 (IL-15)
E08	Hs.202453	NM_002467	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	0.006871	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	0.141479	0.101153
E11	Hs.371036	NM_016931	NOX4	NADPH oxidase 4	0.006871	0.000016
E12	Hs.728886	NM_182649	PCNA	Proliferating cell nuclear antigen	0.195372	0.184122
F01	Hs.553498	NM_006218	PIK3CA	Phosphoinositide-3-kinase, catalytic, α polypeptide	0.073685	0.050435
F02	Hs.77274	NM_002658	PLAU	Plasminogen activator, urokinase	0.006871	0.000414
F03	Hs.155342	NM_006254	PRKCD	Protein kinase C, delta	0.015161	0.014037
F04	Hs.500466	NM_000314	PTEN	Phosphatase and tensin homolog	0.16335	0.10826
F05	Hs.408528	NM_000321	RB1	Retinoblastoma 1	0.121739	0.09276
F06	Hs.207745	NM_002895	RBL1	Retinoblastoma-like 1 (p107)	0.036618	0.038316
F07	Hs.513609	NM_005611	RBL2	Retinoblastoma-like 2 (p130)	0.473698	0.222004
F08	Hs.594481	NM_002575	SERPINB2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2	0.006871	0.000016
F09	Hs.414795	NM_000602	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	0.006871	0.000167
F10	Hs.369779	NM_012238	SIRT1	Sirtuin 1	0.066963	0.021391
F11	Hs.443914	NM_000454	SOD1	Superoxide dismutase 1, soluble	0.207349	0.174491
F12	Hs.487046	NM_000636	SOD2	Superoxide dismutase 2, mitochondrial	0.055267	0.034729
G01	Hs.111779	NM_003118	SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	0.006871	0.000024
G02	Hs.531085	NM_005994	TBX2	T-box 2	0.006871	0.000016
G03	Hs.714737	NM_016569	TBX3	T-box 3	0.006871	0.000016
G04	Hs.63335	NM_005652	TERF2	Telomeric repeat binding factor 2	0.053443	0.033128
G05	Hs.492203	NM_198253	TERT	Telomerase reverse transcriptase	0.006871	0.000016
G06	Hs.645227	NM_000660	TGFB1	Transforming growth factor, β 1	0.312842	0.126748
G07	Hs.513530	NM_015927	TGFB1I1	Transforming growth factor β 1 induced transcript 1	0.006871	0.000016
G08	Hs.164226	NM_003246	THBS1	Thrombospondin 1	0.006871	0.001149
G09	Hs.654481	NM_000546	TP53	Tumor protein p53	0.105234	0.051741
G10	Hs.440968	NM_005657	TP53BP1	Tumor protein p53 binding protein 1	0.064137	0.022034
G11	Hs.66744	NM_000474	TWIST1	Twist homolog 1 (Drosophila)	0.006871	0.002333

Data is representative of three independent experiments with three Id-specific T-cell lines.

histones (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 immunotherapy for both solid and hematologic cancers in a variety of clinical trials.[4,5], [35] 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.[36-40] Traditionally, IL-2 has been a central component of T-cell expansion protocols.[41-43] 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.[44,45] 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,[46,47] 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.[24] Replicative senescence (telomere-dependent) usually occurs in T cells with shorter telomere length as a process of aging isolated in elderly people.[48-51] Premature senescence (telomere-independent), on the other hand, has many causes, such as DNA damage, oxygen stress, chromatin perturbation, and oncogene perturbation.[52-55] Extended *in vitro* culturing can cause senescence.[56] Human T-cell senescence has been suggested as an important reason for escape from tumor surveillance.[45] 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.[24,52], [57] Other phenotypic changes associated with senescent cells include the following: increased β-galaxidase activity[58], increased expression of cell cycle inhibitors and DNA damage molecules[59], increased expression of senescence-
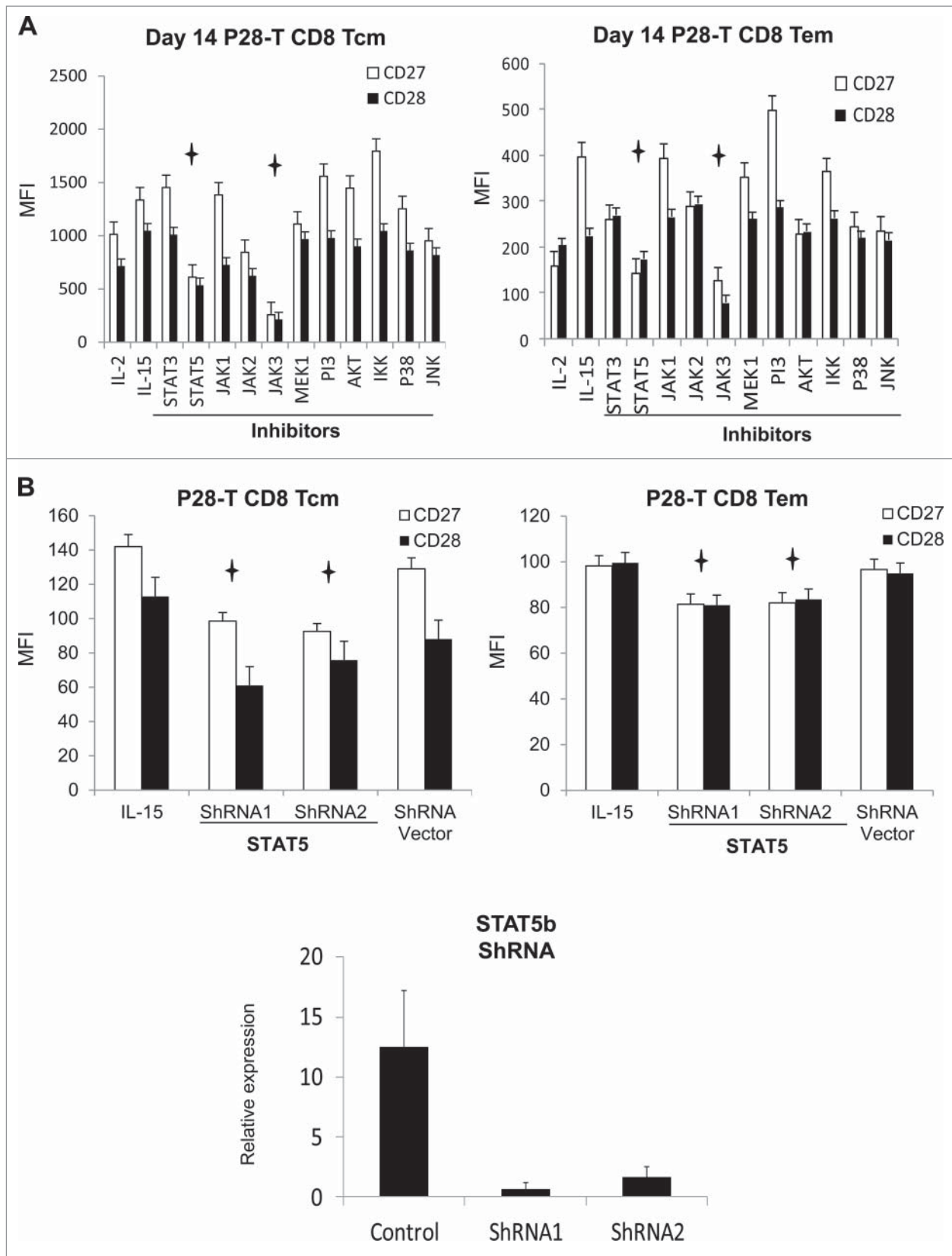
Figure 3. IL-15 regulates senescence delay through the JAK3-STAT5 signaling pathway in Id-specific Tcells. (A) Id L-chain-specific T cells were expanded with IL-15 (50 ng/mL) by rapid expansion protocol (REP) for 12 d before the addition of the signaling pathway inhibitors shown. The effect of signaling 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. $p < 0.05$.
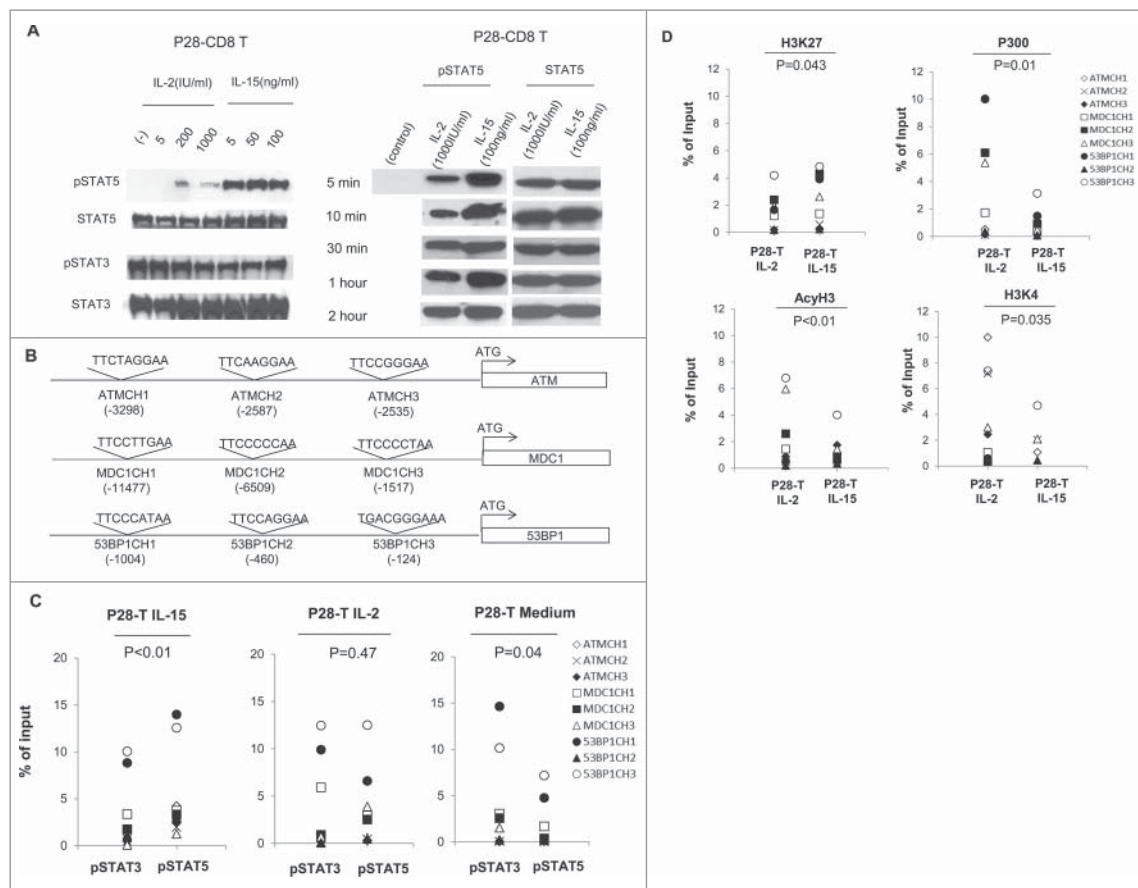
Figure 4. IL-15 strongly activates STAT5 signaling and changes the ratio of pSTAT5/3 signaling in CD8+ T cells. (A) Id-specific T cells starved of cytokines for 24 h were treated with IL-2 or IL-15 at different concentrations for 15 min. Total protein was extracted from the cytokine-treated T cells and 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 idiptype-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-methy-H3 (Lys27); P300: Histone acetyltransferase p300; H3K4: Histone tri methyl lysine 4; AcyH3: acetyL- Histone H3. 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 CD8+ T cells have decreased P53, P21[WAF1], and P16[INK4a] 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 TNFα); 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 STAT sites on the promoter of ATM, 53BP1, MDC1 DNA damage genes. As a consequence of this binding, there are less pSTAT3, more transcriptionally repressive histones (H3K27) and less transcriptionally active histones (H3K4, P300, Acy 300) binding to the promoters. DNA damage molecules are known to play critical roles in the initiation and regulation of senescence and their high expression is a biomarker for cellular senescence.[60,69] 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 pSTAT5/3 signaling in CD8+ T cells. Our results are consistent with recent studies where constitutively

activated STAT5 signaling mediated strong antitumor effects and the inhibition of STAT3 led to an enhanced adoptive therapy effect.[70-73] The mechanisms revealed in this study provide the basis for future rational design of strategies to improve persistence of CD8^{+} 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_2. Flow antibodies for T-cell surface biomarkers and cytokine antibodies were all from BD Biosciences or eBiosciences. The following reagents were used per manufactures' instructions: anti-P53 and anti-P21[WAF1] (Genescript), anti-tubulin and anti-P16[INK4a] (BD biosciences), ChIP grade anti-Histone H3 (tri methyl K4) (Abcam), anti-p300 (Millipore), anti-Histone 3 tri-methy-H3 (Lys27) (Millipore), anti-Histone 3 acetylated (AcyH3) (Millipore), anti-pSTAT3 (Santa Cruz), and anti-pSTAT5 (Santa Cruz), anti-hTCRαβ-PE (eBiosciences), anti-Perforin-PE (eBiosciences), anti-Phospho-S6-FITC (cell signaling), anti-EMOES-APC (eBiosciences).

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.[17] Briefly, PBMCs (1 × 10^{5} 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^{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.[20,21]

Adoptive T-cell therapy

Six-to-twelve-week-old NOD SCID IL-2 receptor γc chain knock-out mice (Jackson Laboratory, Stock# 005557), were injected by IV with 0.2 × 10^{6} U266 or ARP-1 human myeloma cells on day 0. Mice were irradiated (200 Cy) on day 2 and received 1 × 10^{7} Id-specific T cells on day 3, followed by rhIL-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^{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^{+}, 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^{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^{+}, 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.[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/TRCN0000232137. ShRNA2: NM_012448/TRCN0000232140, sigma) in the presence of 8 ug/mL of polybrene for 12 h. The cells were then washed with 1 X PBS, and incubated in cytokine-free T-cell complete medium for another 48 h. 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 $= 100 \times 2$^ (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 Mitenyi 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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References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA: A Cancer Journal for Clinicians 2016; 66(1):7-30; PMID:26742998; http://dx.doi.org/10.3322/caac.21332
2. June CH. Adoptive T cell therapy for cancer in the clinic. J Clin Investig 2007; 117(6):1466-76; PMID:17549249; http://dx.doi.org/10.1172/JCI32446
3. June CH. Principles of adoptive T cell cancer therapy. J Clin Investig 2007; 117(5):1204-12; PMID:17476350; http://dx.doi.org/10.1172/JCI31446
4. Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer 2008; 8(4):299-308; PMID:18354418; http://dx.doi.org/10.1038/nrc2355
5. Dudley ME, Rosenberg SA. Adoptive-cell-transfer therapy for the treatment of patients with cancer. Nat Rev Cancer 2003; 3(9):666-75; PMID:12951585; http://dx.doi.org/10.1038/nrc1167
6. Schluns KS, Lefrancois L. Cytokine control of memory T-cell development and survival. Nat Rev Immunol 2003; 3(4):269-79; PMID:12669018; http://dx.doi.org/10.1038/nri1052
7. Badovinac VP, Tvinnereim AR, Harty JT. Regulation of antigen-specific CD8+ T cell homeostasis by perforin and interferon-γ. Science 2000; 290(5495):1354-7; PMID:11082062; http://dx.doi.org/10.1126/science.290.5495.1354
8. Refaeli Y, Van Parijs L, London CA, Tschopp J, Abbas AK. Biochemical mechanisms of IL-2–regulated fas-mediated T cell apoptosis. Immunity 1998; 8(5):615-23; PMID:9620682; http://dx.doi.org/10.1016/S1074-7613(00)80566-X
9. Lodolce JP, Boone DL, Chai S, Swain RE, Dassopoulos T, Trettin S, Ma A. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. Immunity 1998; 9(5):669-76; PMID:9846488; http://dx.doi.org/10.1016/S1074-7613(00)80664-0
10. Rathmell JC, Farkash EA, Gao W, Thompson CB. IL-7 enhances the survival and maintains the size of naive T cells. J Immunol 2001; 167(12):6869-76; PMID:11739504; http://dx.doi.org/10.4049/jimmunol.167.12.6869
11. Tan JT, Dudl E, LeRoy E, Murray R, Sprent J, Weinberg KI, Surh CD. IL-7 is critical for homeostatic proliferation and survival of naïve T cells. Proc Natl Acad Sci 2001; 98(15):8732-7; PMID:11447288; http://dx.doi.org/10.1073/pnas.161126098
12. Spolski R, Leonard WJ. Interleukin-21: a double-edged sword with therapeutic potential. Nat Rev Drug Discov 2014; 13(5):379-95; PMID:24751819; http://dx.doi.org/10.1038/nrd4296
13. Cousens LP, Orange JS, Biron CA. Endogenous IL-2 contributes to T cell expansion and IFN-gamma production during lymphocytic choriomeningitis virus infection. J Immunol 1995; 155(12):5690-9; PMID:7499855
14. Khoruts A, Mondino A, Pape KA, Reiner SL, Jenkins MK. A natural immunological adjuvant enhances T cell clonal expansion through a CD28-dependent, interleukin (IL)-2-independent mechanism. J Exp Med 1998; 187(2):225-36; PMID:9432980; http://dx.doi.org/10.1084/jem.187.2.225
15. Li XC, Demirci G, Ferrari-Lacraz S, Groves C, Coyle A, Malek TR, Strom TB. IL-15 and IL-2: a matter of life and death for T cells in vivo. Nat Med 2001; 7(1):114-8; PMID:11135625; http://dx.doi.org/10.1038/83253
16. Klebanoff CA, Finkelstein SE, Surman DR, Lichtman MK, Gattinoni L, Theoret MR, Grewal N, Spiess PJ, Antony PA, Palmer DC et al. IL-15 enhances the in vivo antitumor activity of tumor-reactive CD8+ T

cells. Proc Natl Acad Sci U S A 2004; 101(7):1969-74; PMID:14762166; http://dx.doi.org/10.1073/pnas.0307298101

17. Weng J, Cha SC, Matsueda S, Alatrash G, Popescu MS, Yi Q, Molldrem JJ, Wang M, Neelapu SS, Kwak LW. Targeting human B-cell malignancies through Ig light chain-specific cytotoxic T lymphocytes. Clin Cancer Res 2011; 17(18):5945-52; PMID:21813633; http://dx.doi.org/10.1158/1078-0432.CCR-11-0970

18. Weng J, Neelapu SS, Woo AF, Kwak LW. Identification of human idiotype-specific T cells in lymphoma and myeloma. Curr Top Microbiol Immunol 2011; 344:193-210; PMID:20549471; http://dx.doi.org/10.1007/82_2010_70

19. Weng J, Rawal S, Chu F, Park HJ, Sharma R, Delgado DA, Fayad L, Fanale M, Romaguera J, Luong A et al. TCL1: a shared tumor-associated antigen for immunotherapy against B-cell lymphomas. Blood 2012; 120(8):1613-23; PMID:22645177; http://dx.doi.org/10.1182/blood-2011-09-382838

20. Topalian SL, Muul LM, Solomon D, Rosenberg SA. Expansion of human tumor infiltrating lymphocytes for use in immunotherapy trials. J Immunol Methods 1987; 102(1):127-41; PMID:3305708; http://dx.doi.org/10.1016/S0022-1759(87)80018-2

21. Riddell SR, Greenberg PD. The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. J Immunol Methods 1990; 128(2):189-201; PMID:1691237; http://dx.doi.org/10.1016/0022-1759(90)90210-M

22. Miyakawa Y, Ohnishi Y, Tomisawa M, Monnai M, Kohmura K, Ueyama Y, Ito M, Ikeda Y, Kizaki M, Nakamura M. Establishment of a new model of human multiple myeloma using NOD/SCID/gammac (null) (NOG) mice. Biochem Biophys Res Commun 2004; 313(2):258-62; PMID:14684154; http://dx.doi.org/10.1016/j.bbrc.2003.11.120

23. Mirandola L, Yu Y, Chui K, Jenkins MR, Cobos E, John CM, Chiriva-Internati M. Galectin-3C inhibits tumor growth and increases the anticancer activity of bortezomib in a murine model of human multiple myeloma. PLoS One 2011; 6(7):e21811; PMID:21765917; http://dx.doi.org/10.1371/journal.pone.0021811

24. Passos JF, Simillion C, Hallinan J, Wipat A, von Zglinicki T. Cellular senescence: unravelling complexity. Age 2009; 31(4):353-63; PMID:19618294; http://dx.doi.org/10.1007/s11357-009-9108-1

25. Campisi J. senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell 2005; 120(4):513-22; PMID:15734683; http://dx.doi.org/10.1016/j.cell.2005.02.003

26. Ye J, Huang X, Hsueh EC, Zhang Q, Ma C, Zhang Y, Varvares MA, Hoft DF, Peng G. Human regulatory T cells induce T-lymphocyte senescence. Blood 2012; 120(10):2021-31; PMID:22723548; http://dx.doi.org/10.1182/blood-2012-03-416040

27. Ben-Porath I, Weinberg RA. The signals and pathways activating cellular senescence. Int J Biochem Cell Biol 2005; 37(5):961-76; PMID:15743671; http://dx.doi.org/10.1016/j.biocel.2004.10.013

28. Zhang H. Molecular signaling and genetic pathways of senescence: Its role in tumorigenesis and aging. J Cell Physiol 2007; 210(3):567-74; PMID:17133363; http://dx.doi.org/10.1002/jcp.20919

29. Fridman AL, Tainsky MA. Critical pathways in cellular senescence and immortalization revealed by gene expression profiling. Oncogene 2008; 27(46):5975-87; PMID:18711403; http://dx.doi.org/10.1038/onc.2008.213

30. Akbar AN, Henson SM. Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? Nat Rev Immunol 11(4):289-95; PMID:21436838; http://dx.doi.org/10.1038/nri2959

31. Montes CL, Chapoval AI, Nelson J, Orhue V, Zhang X, Schulze DH, Strome SE, Gastman BR. Tumor-induced senescent T cells with suppressor function: A potential form of tumor immune evasion. Cancer Research 2008; 68(3):870-9; PMID:18245489; http://dx.doi.org/10.1158/0008-5472.CAN-07-2282

32. Pipkin ME, Sacks JA, Cruz-Guilloty F, Lichtenheld MG, Bevan MJ, Rao A. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. Immunity 2010; 32(1):79-90; PMID:20096607; http://dx.doi.org/10.1016/j.immuni.2009.11.012

33. Malamut G, El Machhour R, Montcuquet N, Martin-Lannerée S, Dusanter-Fourt I, Verkarre V, Mention JJ, Rahmi G, Kiyono H, Butz EA et al. IL-15 triggers an antiapoptotic pathway in human intraepithelial lymphocytes that is a potential new target in celiac disease–

34. associated inflammation and lymphomagenesis. J Clin Investig 120(6):2131-43; PMID:20440074; http://dx.doi.org/10.1172/JCI41344

35. Cornish GH, Sinclair LV, Cantrell DA. Differential regulation of T-cell growth by IL-2 and IL-15. Blood 2006; 108(2):600-8; PMID:16569767; http://dx.doi.org/10.1182/blood-2005-12-4827

35. Gattinoni L, Powell DJ, Rosenberg SA, Restifo NP. Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol 2006; 6(5):383-93; PMID:16622476; http://dx.doi.org/10.1038/nri1842

36. Besser MJ, Shapira-Frommer R, Treves AJ, Zippel D, Itzhaki O, Hershkovitz L, Levy D, Kubi A, Hovav E, Chermoshniuk N et al. Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. Clin Cancer Res 2010; 16(9):2646-55; PMID:20406835; http://dx.doi.org/10.1158/1078-0432.CCR-10-0041

37. Schmitt TM, Ragnarsson GB, Greenberg PD. T cell receptor gene therapy for cancer. Hum Gene Ther 2009; 20(11):1240-8; PMID:19702439; http://dx.doi.org/10.1089/hum.2009.146

38. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 2002; 298(5594):850-4; PMID:12242449; http://dx.doi.org/10.1126/science.1076514

39. Hardy NM, Fellowes V, Rose JJ, Odom J, Pittaluga S, Steinberg SM, Blacklock-Schuver B, Avila DN, Memon S, Kurlander RJ et al. Costimulated tumor-infiltrating lymphocytes are a feasible and safe alternative donor cell therapy for relapse after allogeneic stem cell transplantation. Blood 2012; 119(12):2956-9; PMID:22289893; http://dx.doi.org/10.1182/blood-2011-09-378398

40. Liddy N, Bossi G, Adams KJ, Lissina A, Mahon TM, Hassan NJ, Gavarret J, Bianchi FC, Pumphrey NJ, Ladell K et al. Monoclonal TCR-redirected tumor cell killing. Nat Med 2012; 18(6):980-7; PMID:22561687; http://dx.doi.org/10.1038/nm.2764

41. Cantrell D, Smith K. The interleukin-2 T-cell system: a new cell growth model. Science 1984; 224(4655):1312-6; PMID:6427923; http://dx.doi.org/10.1126/science.6427923

42. Gillis S, Smith KA. Long term culture of tumour-specific cytotoxic T cells. Nature 1977; 268(5616):154-6; PMID:145543; http://dx.doi.org/10.1038/268154a0

43. Morgan D, Ruscetti F, Gallo R. Selective in vitro growth of T lymphocytes from normal human bone marrows. Science 1976; 193(4257):1007-8; PMID:181845; http://dx.doi.org/10.1126/science.181845

44. Crompton JG, Sukumar M, Restifo NP. Uncoupling T-cell expansion from effector differentiation in cell-based immunotherapy. Immunological Rev 2014; 257(1):264-76; PMID:24329803; http://dx.doi.org/10.1111/imr.12135

45. Crespo J, Sun H, Welling TH, Tian Z, Zou W. T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment. Curr Opin Immunol 2013; 25(2):214-21; PMID:23298609; http://dx.doi.org/10.1016/j.coi.2012.12.003

46. Di Mitri D, Azevedo RI, Henson SM, Libri V, Riddell NE, Macaulay R, Kipling D, Soares MV, Battistini L, Akbar AN. Reversible senescence in human CD4+CD45RA+CD27- memory T cells. J Immunol 187(5):2093-100; PMID:21788446; http://dx.doi.org/10.4049/jimmunol.1100978

47. Warrington KJ, Vallejo AN, Weyand CM, Goronzy Jr J. CD28 loss in senescent CD4+ T cells: reversal by interleukin-12 stimulation. Blood 2003; 101(9):3543-9; PMID:12506015; http://dx.doi.org/10.1182/blood-2002-08-2574

48. Herbig U, Jobling WA, Chen BP, Chen DJ, Sedivy JM. Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). Mol Cell 2004; 14(4):501-13; PMID:15149599; http://dx.doi.org/10.1016/S1097-2765(04)00256-4

49. Fagagna FdAd, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, von Zglinicki T, Saretzki G, Carter NP, Jackson SP. A DNA damage checkpoint response in telomere-initiated senescence. Nature 2003; 426(6963):194-8; PMID:14608368; http://dx.doi.org/10.1038/nature02118

50. Adams PD. Healing and hurting: molecular mechanisms, functions, and pathologies of cellular senescence. Mol Cell 2009; 36(1):2-14; PMID:19818705; http://dx.doi.org/10.1016/j.molcel.2009.09.021

51. Chen WH, Kozlovsky BF, Effros RB, Grubeck-Loebenstein B, Edelman R, Sztein MB. Vaccination in the elderly: an immunological perspective. Trends Immunol 2009; 30(7):351-9; PMID:19540808; http://dx.doi.org/10.1016/j.it.2009.05.002

52. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 2007; 8(9):729-40; PMID:17667954; http://dx.doi.org/10.1038/nrm2233

53. Lleonart ME, Artero-Castro A, Kondoh H. Senescence induction; a possible cancer therapy. Mol Cancer 2009; 8:3; PMID:19133111; http://dx.doi.org/10.1186/1476-4598-8-3

54. Acosta JC, O'Loghlen A, Banito A, Guijarro MV, Augert A, Raguz S, Fumagalli M, Da Costa M, Brown C, Popov N et al. Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell 2008; 133(6):1006-18; PMID:18555777; http://dx.doi.org/10.1016/j.cell.2008.03.038

55. Kuilman T, Michaloglou C, Vredeveld LCW, Douma S, van Doorn R, Desmet CJ et al. Oncogene-Induced Senescence Relayed by an Interleukin-Dependent Inflammatory Network. Cell 2008; 133(6):1019-31; PMID:18555778; http://dx.doi.org/10.1016/j.cell.2008.03.039

56. Kassem M, Ankersen L, Eriksen EF, Clark BF, Rattan SI. Demonstration of cellular aging and senescence in serially passaged long-term cultures of human trabecular osteoblasts. Osteoporos Int 1997; 7 (6):514-24; PMID:9604046; http://dx.doi.org/10.1007/BF02652556

57. d'Adda di Fagagna F. Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer 2008; 8(7):512-22; PMID:18574463; http://dx.doi.org/10.1038/nrc2440

58. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci 1995; 92(20):9363-7; PMID:7568133; http://dx.doi.org/10.1073/pnas.92.20.9363

59. Jackson JG, Pant V, Li Q, Chang LL, Quintas-Cardama A, Garza D, Tavana O, Yang P, Manshouri T, Li Y et al. p53-mediated senescence impairs the apoptotic response to chemotherapy and clinical outcome in breast cancer. Cancer Cell 2012; 21(6):793-806; PMID:22698404; http://dx.doi.org/10.1016/j.ccr.2012.04.027

60. Rodier F, Coppe J-P, Patil CK, Hoeijmakers WAM, Munoz DP, Raza SR, Freund A, Campeau E, Davalos AR, Campisi J. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. Nat Cell Biol 2009; 11(8):973-9; PMID:19597488; http://dx.doi.org/10.1038/ncb1909

61. Appay V, Nixon DF, Donahoe SM, Gillespie GM, Dong T, King A, Ogg GS, Spiegel HM, Conlon C, Spina CA et al. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. J Exp Med 2000; 192(1):63-75; PMID:10880527; http://dx.doi.org/10.1084/jem.192.1.63

62. Vallejo AN. CD28 extinction in human T cells: altered functions and the program of T-cell senescence. Immunol Rev 2005; 205:158-69; PMID:15882352; http://dx.doi.org/10.1111/j.0105-2896.2005.00256.x

63. Darnell JE. STATs and Gene Regulation. Science 1997; 277(5332):1630-5; PMID:9287210; http://dx.doi.org/10.1126/science.277.5332.1630

64. Shuai K, Liu B. Regulation of JAK-STAT signalling in the immune system. Nat Rev Immunol 2003; 3(11):900-11; PMID:14668806; http://dx.doi.org/10.1038/nri1226

65. Costa-Pereira AP, Tininini S, Strobl B, Alonzi T, Schlaak JF, Is'harc H, Gesualdo I, Newman SJ, Kerr IM, Poli V. Mutational switch of an IL-6 response to an interferon-γ-like response. Proc Natl Acad Sci 2002; 99(12):8043-7; PMID:12060750; http://dx.doi.org/10.1073/pnas.122236099

66. Avalle L, Pensa S, Regis G, Novelli F, Poli V. STAT1 and STAT3 in tumorigenesis: A matter of balance. JAKSTAT 2012; 1(2):65-72; PMID:24058752; http://dx.doi.org/10.4161/jkst.20045

67. Cohen PA, Koski GK, Czerniecki BJ, Bunting KD, Fu X-Y, Wang Z, Zhang WJ, Carter CS, Awad M, Distel CA et al. STAT3- and STAT5-dependent pathways competitively regulate the pan-differentiation of CD34pos cells into tumor-competent dendritic cells. Blood 2008; 112 (5):1832-43; PMID:18577706; http://dx.doi.org/10.1182/blood-2007-12-130138

68. Yang X-P, Ghoreschi K, Steward-Tharp SM, Rodriguez-Canales J, Zhu J, Grainger JR, Hirahara K, Sun HW, Wei L, Vahedi G et al. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. Nat Immunol 2011; 12(3):247-54; PMID:21278738; http://dx.doi.org/10.1038/ni.1995

69. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T, Saretzki G, Carter NP, Jackson SP. A DNA damage checkpoint response in telomere-initiated senescence. Nature 2003; 426(6963):194-8; PMID:14608368; http://dx.doi.org/10.1038/nature02118

70. Grange M, Buferne M, Verdeil G, Leserman L, Schmitt-Verhulst AM, Auphan-Anezin N. Activated STAT5 promotes long-lived cytotoxic CD8+ T cells that induce regression of autochthonous melanoma. Cancer Res 2012; 72(1):76-87; PMID:22065720; http://dx.doi.org/10.1158/0008-5472.CAN-11-2187

71. Kujawski M, Zhang C, Herrmann A, Reckamp K, Scuto A, Jensen M, Deng J, Forman S, Figlin R, Yu H. Targeting STAT3 in Adoptively Transferred T Cells Promotes Their In Vivo Expansion and Antitumor Effects. Cancer Res 2010; 70(23):9599-610; PMID:21118964; http://dx.doi.org/10.1158/0008-5472.CAN-10-1293

72. Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, Niu G, Kay H, Mulé J, Kerr WG et al. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. Nat Med 2005; 11(12):1314-21; PMID:16288283; http://dx.doi.org/10.1038/nm1325

73. Yang H, Yamazaki T, Pietrocola F, Zhou H, Zitvogel L, Ma Y, Kroemer G. Improvement of immunogenic chemotherapy by STAT3 inhibition. OncoImmunology 2016; 5(2):e1078061; PMID:27057456; http://dx.doi.org/10.1080/2162402X.2015.1078061