Published in final edited form as:

J Leukoc Biol. 2019 November ; 106(5): 1007–1019. doi:10.1002/JLB.1HI0219-064R.

Late CD27 stimulation promotes IL-7Rα transcriptional re-expression and memory T cell qualities in effector CD8+ T cells

Hang Dong, **Andrew Buckner**, **Jessica Prince**, **Timothy Bullock**

Department of Pathology and Human Immune Therapy Center, University of Virginia, Charlottesville, VA 22908 USA.

Abstract

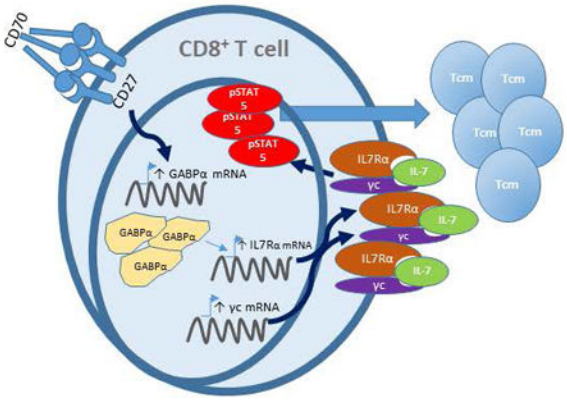
We previously demonstrated that CD27 co-stimulation during a primary CD8+ T cell response was critical for the expression of IL-7Rα on acute effector $CD8^+$ T cells, providing an essential element in the generation of $CD8^+$ T cell memory to infectious pathogens. IL-7 plays a critical role in the generation and maintenance of memory $CD8^+$ T cells, and IL-7Rα has been regarded as a functional marker of long-lived memory precursor effector cells. While IL-7Rα is down-regulated acutely upon TCR stimulation, the regulation of the emergence of IL-7Rα expressing cells around the peak of primary $CD8^+$ responses is less clear. Re-expression could be a default outcome after withdrawal of TCR stimulation. Alternatively, specific stimuli actively antagonize the down-regulation or promote the recovery of IL-7Rα in Ag-activated $CD8^+$ T cells. By utilizing agonistic mAb and transgenic models, here we show: 1) CD27 stimulation acts directly on $CD8^+$ T cells to enhance IL-7Rα-expressing effectors; 2) CD27 stimulation neither alleviates the down-regulation of IL-7Rα upon TCR signaling nor promotes the expansion/survival of IL-7Rα-expressing effectors, but facilitates IL-7Rα re-expression; 3) CD27 stimulation regulates *Il7ra* mRNA abundance but not protein distribution. Importantly, CD27 stimulation promotes not only IL-7Rα, but also the common γ chain of the receptor and the downstream signaling mediated by pSTAT5. Our results demonstrate a previously unappreciated role of CD27 stimulation as a positive regulator of IL-7Rα during CD8 T cell responses, provide insights into the mechanistic basis by which CD27 stimulation influences $CD8^+$ T cell memory differentiation, and highlight the potential of targeting CD27-CD70 axis to enhance IL-7 signaling for antiviral/antitumor immunotherapy.

Summary Sentence:

Late CD27 stimulation promotes CD8+ T cell memory differentiation by increasing transcription of IL7Rα and γc.

Graphical Abstract

Corresponding Author: Dr Timothy N.J. Bullock, University of Virginia, MR6 Building Room G527, 345 Crispell Drive, Charlottesville, VA 22908, 434-982-1932, TB5V@virginia.edu.



Introduction

After encountering pathogens, naïve CD8$^+$ T cells are activated and start rapid proliferation and differentiation into functional cytotoxic T cells (CTL), gaining the ability to secrete cytokines and lytic granules and kill target cells. Following pathogen clearance, the majority of pathogen-specific CTLs, termed short-lived effector cells (SLECs), die via apoptosis during the contraction stage, while a small subset is maintained stably to become long-lived memory cells. Signals provided by interleukin-7 (IL-7) are critical for the long-term maintenance of antigen (Ag)-specific CD8$^+$ T cells during the contraction phase (1, 2). The majority of expanding CD8+ T cells downregulate IL-7R upon TCR stimulation and become KLRG-1 expressing short lived effector cells (SLECs). Towards the peak of the primary response a subset of IL7Rα-expressing T cells emerges, and these cells are enriched for those that have the ability to survive long-term as memory cells, and have been termed memory precursor effector cells (MPECs). Further, IL-7 has been shown to promote CD8+ T cell function in the setting of chronic viral infection and cancer (3, 4) and therefore delineating external stimuli that can promote the expression of the IL-7Rα could be exploited to increase the therapeutic benefit of IL-7 in these contexts.

The regulation of SLEC and MPEC fate choices are thought to be a function of the balance of pro-inflammatory and anti-inflammatory signals. On the one hand, inflammation, primarily in the form of IL-12, drives the expression of T-bet and terminal differentiation and loss of memory cell potential in SLECs(5). Conversely, an IL10/IL-21-STAT3 pathway supports MPEC development, in part by attenuating the influence of inflammatory signals. Arguing for active selection of MPEC, we (6) and others (7) have previously shown that CD27, a member of the TNF receptor superfamily (TNFRSF), influences MPEC frequency during viral infections. Blocking CD27 interaction with its ligand CD70 results in an IL-12-dependent significant loss of IL-7R-expressing MPECs and a concomitant ablation of long-term CD8+ T cell memory. Conversely, stimulating CD27 in mice deficient of CD4+ T cells, which normally fail to develop CD8+ T cell memory, augments MPEC development and rescues protective memory(6). However, it was unclear whether the induction of IL-7R expression was necessary and sufficient for the restoration of MPEC function, and how IL-7R expression was being regulated.

The increase in IL-7Rα expression could result from CD27 signals promoting the survival or expansion of IL-7Rα-retaining cells; resisting the repression of IL-7Rα; or directly promoting the expression of IL-7Rα on effector CD8+ T cells. To address these questions, we used agonistic/antagonistic monoclonal antibodies and transgenic models, utilized *in vitro* and *in vivo* systems, and performed imaging flow cytometry and quantitative PCR to monitor how CD27 stimulation regulates the IL-7Rα profile of activated-$CD8^+$ T cells. Here we show that CD27 stimulation induced *il-7ra* mRNA even on well-differentiated KLRG1-expressing effector T cells, and that co-induction of the common γ chain (CD132) lead to increased IL-7 signaling. Increased expression of IL-7Rα mRNA corresponded with an increase in GABPα, a transcription factor previously described to promote IL-7Rα. Further, the expression of IL-7Rα promoted memory cell function in $KLRG1^+$ SLECs, and that the ability of CD27 to promote memory cell formation was dependent on IL-7/IL-7Rα. Together these data argue that CD27 stimulation provides a signal that promotes the acquisition of memory cell potential in effector T cells by directly supporting IL-7R expression.

Materials and Methods

Animals.

C57Bl/6 mice were obtained from National Cancer Institute (Frederick, MD). OT-I mice, expressing TCRs specific for $OVA_{257–264}$ peptide in complex with $H\text{-}2K^b$, were purchased from Taconic (B6.129S7-*Rag1*^{tm1Mom} Tg(TcraTcrb)1100Mjb N9+N1, model # 4175), and crossed on $Thy1.1^+$ (B6.PL-*Thy1*^{a}/CyJ stock # 000406) mice obtained from the Jackson Laboratory. CD27KO mice were provided by Dr. Steven Schoenberger (La Jolla Institute of Immunology and Allergy), with the permission of Dr. Jannie Borst (Netherlands Cancer Institute). Mice were maintained in specific pathogen-free facilities and were treated in accordance with the guidelines established by the Animal Care and Use Committee at the University of Virginia.

Virus and peptides.

Recombinant vaccinia expressing OVA (OVA-vac) was provided by J. Yewdell (National Institute of Allergy and Infectious Diseases), and was propagated on $HuTK^2$ cells. Recombinant adenovirus expressing OVA (OVA-adeno) was either purchased from Gene Transfer Vector Core (University of Iowa) or provided by Dr. Young Hahn (University of Virginia), and was propagated on 293A fibroblasts. Synthetic peptide $OVA_{257–264}$ (SIINFEKL) was purchased from GenScript (Piscataway, NJ). Endotoxin was removed by Detoxi-Gel endotoxin-removal kit (Pierce, Rockford, IL).

Antibodies.

Agonistic AT124.1 anti-CD27 has been described (8) and endotoxin was removed as above. FR70-blocking anti-CD70 has been described before(9). Control Rat IgG was purchased from Sigma (St Louis, MO). GK1.5-depleting anti-CD4 was obtained from hybridomas originally purchased from ATCC. IL-7Rα blocking antibody was purchased from BioX-cell (Hanover, NH). Anti-OX40 and anti-41BB were provided by Drs Hideo Yagita (Juntendo University) and Robert Mitler (Emory University), respectively.

In vitro T cell stimulation.

Splenocytes from naïve Thy1.1$^+$ OT-1 mice were either co-cultured with irradiated, OVA257-pulsed WT C57Bl/6 splenocytes (as Ag-presenting cells), or stimulated with αCD3 (5μg/ml plate bound overnight in dPBS)/CD28 (1μg/ml) antibodies (eBioscience). αCD27 and control IgG were given in some assays as indicated. Cells were cultured in murine T cell culture medium (RPMI-1640 containing: 10% FBS (Gemini Bioproducts); 1.5 μM HEPES; essential and non-essential amino acids; 0.2 mM sodium pyruvate;0.5 μg/ml gentamicin;2 mM L-glutamine (all from Gibco); 8mM NaOH; 0.05 mM β mercaptoethanol (both from Sigma)) for 48h to down-regulate IL-7Rα, and then analyzed with flow cytometry. In some assays, after 48h stimulation cells were labeled with CellTrace Violet proliferation dye according to manufacturer's instructions (Life Technology, Grand Island, NY), and adoptively transferred into OVA-adeno pre-primed C57Bl/6 recipients.

In vivo adoptive transfer and Immunization.

500–1,000 naïve OT-1 cells were transferred into WT or CD27$^{-/-}$ C57Bl/6 mice 1d before 1*10^7 pfu vac-OVA- or 2*10^8 pfu adeno-OVA i.v. priming. CD27, OX40 and 41BB stimulation was performed by injecting 50 μg antibody i.p. on d0, 3 and 6. CD70 blockade was performed by injecting 500 μg FR70 i.p. on d0, 2, 4 and 6. Where indicated, mice were depleted of peripheral CD4$^+$ T cells by i.p. injection of 200 μg GK1.5 7 and 3d before priming and confirmed by tail vain bleed. 7d after priming, spleen, lymph nodes and bleeds were harvested and analyzed by flow cytometry. In the other assays, WT C57Bl/6 mice were primed i.p. with 2*10^8 pfu -adeno-OVA, and 1*10^4 *in vitro*-stimulated, IL-7Rα-downregulated OT1 cells were transferred at d2. Mice were treated with AT124.1 anti-CD27 or control IgG i.p. several hours after the adoptive transfer and then 2d later. 2~4d after the transfer, splenocytes were harvested and analyzed with flow cytometry, or sorted for OT-1 cells for qPCR.

Flow cytometry.

Lymphocytes were isolated from blood or homogenized spleens/lymph nodes and first stained with Aqua vital dye (Life Technologies) for 20 min at 4°C. In some experiments cells were then incubated with $OVA_{257–264}$-specific H2-K^b dextramer-APC (Immudex, Denmark) for 20 min at 4°C to identify endogenous effector CD8$^+$ T cells. After FcR (anti-CD16/32; 2% normal mouse serum) blockade, surface markers were stained for 30 min at 4°C, with antibodies used in various combinations including anti-CD8-ef450 (53–6.7), anti-CD8-PerCP (53–6.7), anti-CD4-PE (GK1.5), anti-Thy1.1-FITC (HIS51), anti-KLRG1-PE (2F1), anti-IL-7Rα-PerCP-Cy5.5 (A7R34), anti-IL-7Rα-PE (A7R34), anti-γc-PE (TUGm2), anti-CD122-PE (5H4), anti-IL-15Rα-PE (DNT15Ra) and anti-IL-21Rα-PE(eBio4A9) all from eBioscience. For Annexin V staining, cells were incubated with anti-Annexin V-Alexa Fluor 647 (Life Technologies) at room temperature in the dark for 15min, and then assessed by flow cytometry within 1h. For pSTAT5 staining, splenocytes were pre-incubated with IL-7 *in vitro* for 20min, and permeabilized with Phosflow Perm Buffer III (BD) on ice for 1h, and incubated with anti-pSTAT5-Alexa Fluor 647 (Cell Signaling) for 30min. Cell counts were assessed by adding counting beads (Life Technologies) at the end

of staining. Staining was assessed by flow cytometry on a FACS Canto II (Becton Dickinson; Franklin Lakes, NJ) and analyzed using FlowJo 7.6.5 Software (Treestar, OR).

Imaging flow cytometry.

The staining followed the same protocol as for regular flow cytometry, except cells were gently permeabilized with 1% saponin (Sigma) for 20min before stained for IL7Rα or the isotype control. DAPI was applied at the end of the staining to identify nuclear compartment. Staining was assessed by ImageStreamX Mark II (Amnis, Seattle) and analyzed using IDEAS software.

qPCR.

Transferred stimulated-OT1 cells were sorted from recipient spleens 3~4d after the transfer (therefore 5~6d after priming the recipient with OVA-adeno). Total mRNA was extracted and subjected to reverse transcription, and the resulting cDNA was analyzed by CFX Connect real-time PCR detect system (BioRad) using Sso SYBR Green supermix. Primers were synthesized by Life Technologies:

Target Gene	sequence forward	sequence reverse
Il7ra	GGAACAACTATGTAAGAAGCCAAAAACG	AAGATCATTGGGCAGAAAACTTTCC
CD132	GCAACAGAGATCGAAGCTGGA	AGATTGGGTTATAGCGGCTCC
GABPa	GTACCAGATTATTATGCAAGACCG	TAAAGAAGATCGCCTACTGAGC
Gfi-1	AGGAACGCAGCTTTGACTGT	GATGAGCTTTGCACACTGGA

Data were normalized to two validated reference genes RPS18 and Rpl13a from BioRad PrimePCR Reference Genes system.

Statistical analysis.

Statistical analysis was performed with Prism 7 (GraphPad Software, Inc., La Jolla, CA) and data were presented as the Mean ± SD. Comparisons between groups were performed by either unpaired two-tailed Student's *t* test when F-tests were used to confirm the homogeneity of the dataset, or one-way ANOVA. Statistical significance was determined for 95% confidence interval.

Results

CD27 stimulation promotes effector T cell responsiveness to IL-7 in an antigen dependent manner by enhancing expression of IL-7Rα and CD132, the common γ chain.

In agreement with our previous finding in mouse spleens (6), abolishment of CD27 stimulation by CD70-blocking mAb FR70 during primary $CD8^+$ T cell responses to OVA-expressing vaccinia virus (Vac-OVA) led to a substantial reduction in the proportion of IL-7Rα-expressing effector CD8+ T cells at day 7 in secondary lymphoid tissues (spleen and lymph nodes) and peripheral blood (Figure 1A). Conversely, stimulation of CD27 by agonistic mAb AT-124.1 during primary responses in CD4-depleted animals resulted in a

significant increase in the proportion of IL7Rα-expressing effectors (Figure 1B), particularly on KLRG1-expressing SLEC effector cells. Importantly, CD27 stimulation promoted not only the proportion, but also the absolute number of IL-7Rα-expressing effectors in spleen (Figure 1A, B), indicating the formation of an enlarged memory precursor pool. Similar upregulation of IL-7Rα was found in d7 CD8+ T cells in mice treated with anti-CD27 after adeno-OVA immunizations (data not shown). These data are consistent with a mechanistic theme that CD27 stimulation promotes $CD8^+$ T cell memory at least partially via enhancing IL-7Rα-expressing memory precursor effector cells (MPECs).

We sought to directly address whether CD27-driven promotion of IL-7Rα expression has direct functional activity that aligns with the increased propensity to form memory T cells. Interestingly, CD27 stimulation promoted not only the α-chain but also the γ-chain of the IL-7 receptor (Figure 1C). To establish whether the change of IL-7R upon CD27 signals subsequently led to a change in cell responsiveness to IL-7, we measured the levels of pSTAT5 after a short *in vitro* stimulation of IL-7 on d7 CD8+ T cell effectors harvested from mouse spleens. We found elevated levels of pSTAT5 in CD8+ T cell effectors from αCD27-treated mice (Figure 1C) and, conversely, decreased levels of pSTAT5 from αCD70-treated mice (Figure 1D), compared with those from control Ig-treated animals. Thus the change in the downstream signaling mediated by pSTAT5 correlates with the change in their IL-7R expression.

No difference was found in the homeostatic level of IL-7Rα on CD27KO and WT on quiescent T cells (Figure 1E) or after providing agonistic CD27 antibody without concurrent immunization (data not shown). This indicated that the impact of CD27 signaling on IL-7Rα expression was specific for effector but not quiescent $CD8^+$ T cells. Further, CD27 stimulation exhibited a marginal effect on the surface expression of the high-affinity (alpha chain) receptor subunits specific for other common γc cytokine members IL-21 and IL-15 (Figure 1F), and no significant increase in levels of STAT phosphorylation effector CD8+ T cells was observed after in vitro stimulation with the relevant cytokines (data not shown). Thus, while CD27-mediated increase in common γ-chain could be important for the increase in pSTAT5 signaling that occurs after Il-7 exposure, it may not augment cell responsiveness to other cytokines due to a failure to mobilize the expression of the cytokine binding receptors.

Direct CD27 stimulation of CD8+ T cells augments IL7-dependent memory potential even in short lived effectors.

We took three approaches to assessing the importance of IL-7Rα expression for the ability of CD27 stimulation to promote the formation of memory populations. First, we examined the relative ability of KLRG1 single positive (K-SP), IL7Rα single positive (7-SP) and KLRG1+IL7Rα+ double positive (K7-DP) cells to differentiate into memory CD8+ T cells. Each population was sorted from control or anti-CD27 treated, OT-1 bearing, adeno-OVA infected $CD45.2^+$ mice, and transferred to $CD45.1^+$ recipients that had been concurrently infected with an irrelevant recombinant adenovirus (see Figure 2A for schema). Mice were rested for 35d and then challenged with vac-OVA. Notably, the size of the recall response assessed 5d later in mice that had received the K7-DP $CD8^+$ T cells from CD27 stimulated

mice was far superior to those that received K-SP $CD8^+$ T cells, and almost equivalent to those that received 7-SP T cells (Figure 2B, **left plot**). Second, we examined the importance of IL-7 to memory T cell formation from K7-DP cells after CD27 stimulation. Sorted OT-1 K7-DP CD8+ T cells were transferred into WT or IL7-knockout (KO) recipient mice that had been infected with an irrelevant adenovirus, and were treated with either control IgG (cIg) or a non-depleting IL-7Rα blocking antibody (1). After vac-OVA challenge 35d later, compared to WT recipients treated with cIg, minimal recall responses were found in both IL-7 KO mice cohorts, and WT mice treated with anti-IL-7Rα (Figure 2B, **right plot**). Thus, the expression of IL-7Rα on CD27stimulated CD8+ T cells augments their ability to mount secondary responses in an IL-7 dependent manner.

To explore the cellular mechanisms by which CD27 signaling impacts MPEC differentiation, we sought to identify the cellular target of CD27 stimulation that enhances IL-7Rα-expressing $CD8^+$ effectors during acute viral infection. CD27 is broadly expressed on T(10), NK (11, 12), and subsets of B cells(13) and stimulation of CD27 contributes to their activation. We hypothesized that it was the direct stimulation of CD27 on $CD8^+$ T cells that drives IL-7Rα expression, given our previous finding that direct CD27 stimulation promoted $CD8^+$ T cell memory and that CD27-driven promotion of IL-7Rα-expressing effectors was independent of both $CD4^+$ T cells and NK cells (6). To test this hypothesis, we adoptively transferred small amount (500) of OT1 $Thy1.1^+$ cells into $Thy1.2^+$ WT or CD27KO recipients 1d prior to adeno-OVA priming and treated the mice with either anti-CD27 or cIg during primary responses. Substantial responses were derived from both transferred OT1 and endogenous $CD8^+$ T cell components in d7 spleen (data not shown). The proportion of IL-7Rα-expressing OT1 was increased 2~4 fold upon CD27 stimulation in both WT and CD27KO recipients, indicating that the expression of CD27 on host populations is dispensable for IL-7Rα expression on OT1 cells (Figure 2C, left). Conversely, in comparison to WT endogenous OVA_{257}-specific CD8+ effector T cells, endogenous effectors in CD27KO mice failed to increase their IL-7Rα frequency in response to anti-CD27 treatment, indicating that the expression of CD27 on $CD8^+$ T cells is necessary (Figure 2C, right). Together these data indicate that while CD27 expression was not absolutely necessary for IL-7Rα expression on effector CD8+ T cells, CD27 stimulation worked intrinsically on the responding $CD8^+$ T cells to enhance IL-7Rα-expressing effector populations.

Kinetics of CD27 stimulation and IL-7Rα expression

IL-7Rα expression is markedly down-regulated by TCR signaling at the early stage of primary responses (e.g. as early as 6h *in vitro*(14) and during the first 4–5 d of LCMV infection (1), and expression is regained in a small subset by the peak of the primary response after Ag clearance (e.g. by d 8 after LCMV infection(1)). At the molecular level, the CD27-driven promotion of IL-7Rα-expressing effectors could have been a consequence of either attenuated IL-7Rα down-regulation, and/or accelerated re-expression. To define the mechanisms by which CD27 signal enhances the proportion of IL-7Rα-expressing effectors, we first examined whether CD27 stimulation prevented the acute down-regulation of IL-7Rα upon TCR stimulation. Splenocytes from OT1 mice were co-cultured in vitro with $OVA_{257–264}$ congenic peptide in the presence of either anti-CD27 or control IgG, and

IL-7Rα expression was assessed at 48h by flow cytometry. In both groups IL-7Rα was dramatically down-regulated, and CD27 stimulation showed no impact in either the proportion or absolute number of IL-7Rα-expressing early effectors (Figure 3A). Thus, CD27 stimulation had no impact on IL-7Rα down-regulation in early effectors.

Unlike *in vivo* infections where the down-regulation of IL-7Rα is sustained in the majority of $CD8^+$ effector T cells (generating SLECs), mouse $CD8^+$ T cells stimulated *in vitro* only transiently (less than 48h) repressed IL-7Rα and then regained it uniformly (IL-7Rα was expressed on more than 90% of in vitro stimulated CD8+ T cells by 120h; data not shown), implying that factors which prevent the universal re-expression of IL-7Rα *in vivo* were absent *in vitro*. Two potential determinants that may account for this discrepancy were antigen and inflammatory signals. Supporting this, we found that the majority of transferred, *in vitro* stimulated OT1 cells regained IL-7Rα expression in naïve C57bl/6 recipients, but significantly less expressed IL-7Rα in adeno-OVA pre-primed recipients (Figure 3B, **left**). Furthermore, strong re-expression of IL-7Rα was achieved in mice pre-primed with a recombinant adenovirus expressing tyrosinase (adeno-Tyr) (Figure 3B, right), indicating antigen encounter was necessary, while bystander inflammation was insufficient, for the sustained down-regulation of IL-7Rα *in vivo*.

We therefore switched to an *in vivo* system for further studies of IL-7Rα re-expression after 48h. After co-culturing OT1 splenocytes with $OVA_{257–264}$ cognate peptide in the presence of either anti-CD27 (referred to as 'early' CD27 stimulation) or control IgG for 48h, we adoptively transferred these *in vitro* stimulated, IL-7Rα-down-regulated OT1 cells into adeno-OVA pre-primed B6 recipients. We then treated the mice with anti-CD27 (referred to as 'late' CD27 stimulation) or control IgG, and assessed IL-7Rα at 144h. Along with the finding that early CD27 stimulation had no influence in IL-7Rα down-regulation in early effectors (Figure 3A), the early CD27 stimulation did not alter either the proportion or absolute number of IL-7Rα-expressing effectors at the peak of the primary T cell response (Figure 3C). In contrast, late CD27 stimulation dramatically promoted both the proportion (~ 3.8 fold) and absolute number (~ 7.8 fold) of IL-7Rα-expressing effectors by 144h (Figure 3C), indicating that relatively late CD27 stimulation impacted IL-7Rα re-expression by effector $CD8^+$ T cells.

CD27 stimulation has minimal impact on survival or proliferation of IL-7Rα-expressing effector $CD8^+$ T cells

The change in frequency and absolute number of IL-7Rα-expressing cells induced by CD27 stimulation could be explained by two competing yet not mutually exclusive mechanisms. On the one hand, CD27 stimulation has been regarded as a proliferation and anti-apoptotic signal that contributes to effector expansion and survival in multiple immunization and infection models. Thus CD27 signals might have differentially promoted survival and/or proliferation of CD8+ T cells that re-expressed IL-7Rα, leading to an enrichment in this subset. On the other hand, CD27 signal might have directly promoted IL-7Rα re-expression on T cells that had downregulated it.

To formally identify whether CD27 stimulation accelerated the expansion of IL-7Rα-re-expressing cells, we stimulated OT1 *in vitro* for 48h to down-regulate IL-7Rα, labeled them

with proliferation dye and transferred these OT1 into adeno-OVA pre-primed recipients, and then assessed IL-7Rα expression and cell division in OT1 at 96 and 120h (See Figure 4A for schema). Both the frequency and the absolute number of IL-7Rα-expressing cells were markedly enhanced in the presence of CD27 stimulation as early as 96h (48h post-adoptive transfer) compared to controls, and were further enhanced at 120h (Figure 4A). This occurred without a detectable increase in cell proliferation (as seen by dilution of CTV) of IL-7Rα-expressing (IL-7Rα^{Hi}) effector CD8^{+} T cells at either time point (Figure 4B, upper), indicating that the increased number of IL-7Rα-expressing cells induced by CD27 stimulation was not a consequence of accelerated cell division in this subpopulation. Further, cIg treated effector CD8^{+} T cells with low IL-7Rα (IL-7Rα^{Lo}) had no significant difference in CTV levels than their anti-CD27 stimulated counterparts at 120h; thus, CD27 stimulation did not hinder the proliferation of IL-7Rα^{Lo} T cells (Figure 4B, **bottom**). Together these data indicated that the increased frequency of IL-7Rα was not a secondary consequence of selective expansion of IL7Rα^{Hi}, or hindrance of IL-7Rα^{Lo} T cells.

To determine whether CD27 stimulation makes IL-7Rα-re-expressing cells less prone to activation-induced cell death, we assessed cell apoptosis by Annexin V staining at 96h and 120h. Interestingly, although IL-7 signaling is well known for its anti-apoptotic effects, we did not observe a survival advantage in the IL-7Rα^{Hi} subset of effector CD8^{+} T cells compared to their IL-7Rα^{Lo} counterparts. Rather, at 96h IL-7Rα^{Hi} CD8^{+} T cells were modestly more apoptotic than IL-7Rα^{Hi} CD8^{+} T cells (though this equalized by 120h) (Figure 4C). Moreover, Annexin V staining was nearly identical regardless of the presence or absence of anti-CD27, indicating that the increased number and frequency of IL-7Rα-expressing cells induced by CD27 stimulation was not a consequence of enhanced cell survival. Combined, these data demonstrate that the increased representation of IL-7Rα-expressing CD8+ T cells was not a secondary consequence of selective subset expansion/survival.

To directly establish that CD27 stimulation enhanced IL-7Rα expression on effector CD8+ T cells that had previously down-regulated expression, we stringently sorted for OT1 with minimal IL-7Rα expression after 48h *in vitro* stimulation with OVA$_{257–264}$ peptide (Figure 5A), transferred them into adeno-OVA pre-primed B6 recipients and assessed IL-7Rα re-expression by 144h. In contrast to the control treated group that showed limited increase in IL-7Rα frequency (~5%) compared to the expression on the input cells (~2%), the majority (~70%) of OT1 from antiCD27-treated recipients re-expressed IL-7Rα (Figure 5B). Furthermore, within those late effectors that had re-expressed IL-7Rα in either control or anti-CD27 treated mice, CD27 stimulation induced 30% higher levels of IL-7Rα on a per cell base (Figure 5B). These data, together with our previous finding that CD27 stimulation profoundly upregulated the expression of IL-7Rα in highly-differentiated KLRG1-expressing cells (Figure 1 and (6)), indicated that additive CD27 stimulation directly promoted IL-7Rα re-expression.

CD27 stimulation has minimal impact on IL-7Rα trafficking

We then sought to identify the underlying mechanisms by which CD27 stimulation promotes IL-7Rα. To this point, we had measured the surface expression of IL-7Rα, while several

recent studies have revealed a considerable intracellular pool of IL-7Rα in $CD8^+$ T cells. More intriguingly, it has been reported that the surface expression of IL-7Rα could be regulated post-translationally by targeting protein trafficking by internalization/recycling (15, 16) or degradation(16, 17). We therefore determined the impact of CD27 stimulation on IL-7Rα protein distribution. FACS staining of permeabilized cells showed that CD27 stimulation led to 2~3 fold increase of total (surface plus intracellular) IL-7Rα protein in late effectors, implying CD27 regulation of IL-7Rα is not restricted to surface expression of the protein but rather overall IL7Rα levels (Figure 6A). To visually confirm the result and directly identify whether CD27 stimulation influences the cellular distribution of IL-7Rα, we performed imaging flow cytometry analysis. Imaging revealed that CD8+ T cells from cIg-treated mice had minimal IL-7Rα staining either on the surface or intracellularly. In the few cells where IL-7Rα was visible, the staining was distributed both on the cell surface and in cytoplasm (Figure 6B, **bottom row**). Consistent with regular flow cytometry analysis, we found that CD27 stimulation substantially enhanced the expression of total (surface plus intracellular) IL-7Rα (Figure 6B, **second row**). Subcellular analysis (Figure 6C) revealed that the enhancement occurred equally in both surface and intracellular compartments. Thus, CD27 stimulation promoted the surface expression of IL-7Rα by enhancing its total protein expression rather than by regulation of IL-7Rα trafficking to the cell surface.

CD27 stimulation regulates IL-7Rα re-expression by promoting its mRNA

The preceding studies suggested that CD27 stimulation increases the proportion of IL-7Rα-expressing T cells by directly promoting its transcription. To identify whether CD27 regulates *il7ra* mRNA, we sorted late effectors from control Ig or anti-CD27-treated mice and compared their *il7ra* mRNA level by qt-PCR. Consistent with the 3~4 fold higher levels of IL-7Rα protein expression (Figure 2C), CD27 stimulation led to a ~3.5 fold increase in *il7ra* mRNA. We also detected a similar increase in CD132 mRNA in T cells that had received CD27 (Figure 7). Thus, CD27 signal likely promotes IL-7Rα and γ chains at least partially by enhancing its mRNA abundance In T cells responding to viral infection, the expression of IL-7Rα has previously been reported to be transcriptionally repressed by Gfi-1 and promoted by GABPα (18). Therefore, we asked whether CD27 stimulation differentially affected either of these transcription factors. No difference was found in the expression of Gfi-1 in control vs CD27-stimulated effectors (data not shown). In contrast, we detected a 3-fold higher level of GABPα in CD27-stimulated effectors (Figure 7). Thus CD27 signal likely promotes IL-7Rα at least partially by enhancing its mRNA abundance, potentially by increasing the expression of GABPα.

Discussion

IL-7Rα re-expression is required for the development of memory T cells after initial activation and expansion. The external stimuli that regulate the re-expression of the IL-7R after TCR engagement are poorly understood. Our study demonstrates that CD27 stimulation is a major contributor to the re-expression of IL-7Rα on effector $CD8^+$ T cells *in vivo* after TCR stimulation. Using a vaccinia virus immunization system, we previously reported a positive correlation between CD27 stimulation and IL-7Rα expression during primary $CD8^+$ T cell responses(6), which has also been noted by others in more recent studies using other infectious models(19–21). However, as CD27 stimulation has been

regarded as an anti-apoptotic signal, which contributes to effector survival and expansion, it should be anticipated that CD27 stimulation might promote IL-7Rα-expressing MPECs primarily by facilitating MPEC survival and/or proliferation. In the current study we have formally tested and ruled out this possibility, and rather show that CD27 stimulation directly regulates the expression of the IL-7Rα chain and the common γ chain that together transmit IL-7 signals. Furthermore, adoptive transfer assays using stringently sorted, IL-7Rα-down-regulated, *in vitro*-generated CD8 effectors indicate not only a >8 fold increase in the frequency of IL-7Rα-expressing later effectors induced by CD27 stimulation, but also a striking enhancement in the level of IL-7Rα among those CD8+ T cells that express IL-7Rα. Taken together these data strongly argue for a Signal 3 role of CD27 stimulation that regulates MPEC differentiation, independent of its previously well-established Signal 2 role that promotes overall survival and/or proliferation of effector populations. Importantly, IL-7 plays an essential role in the long-term survival of Ag-specific $CD8^+$ T cells during contraction(1), while tonic CD27 stimulation has been shown to aid the maintenance of quiescent memory $CD8^+$ T cells (22), and we have previously reported the failure in the formation of long-lived memory CD8+ T cells when CD27 stimulation is blocked (6). Our current data suggest that CD27 and IL-7R combine to support the preservation of the T cell's memory potential in an IL-7-dependent manner, and that CD27 stimulation can be considered instructional for memory cell development as stimulation vis CD27 occurs during the primary response. What cells provide this signal, and when, remains to be determined.

We note that *in vivo* the re-expression of IL-7Rα (leading to MPEC formation) during a primary $CD8^+$ T cell response is not a default pathway, which differs from *in vitro* observations. Similarly, KLRG1, which serves as a marker for effectors that have terminally differentiated (SLECs) is also not induced *in vitro* (our unpublished observations), and *in vivo* its expression is driven by inflammatory cytokines including IL-12. Thus, we considered whether the ability of CD27 stimulation to promote IL-7Rα expression and MPEC development occurs via suppressing the impact of pro-inflammatory cytokines. However, these two facets of SLEC/MPEC differentiation are clearly controlled independently as our previous studies and those shown here showed that CD27 stimulation profoundly up-regulates the expression of IL7Rα in differentiated KLRG1-expressing cells (6). Further, we find here that the sustained repression of IL-7Rα *in vivo* is dependent upon exposure to antigen. Importantly, we believe that the enhanced re-expression of IL-7Rα after CD27 stimulation is not simply due to a quicker clearance of antigen as both OT1 and endogenous T cells increased IL-7Rα expression in response to CD27 stimulation in the same wild-type recipient, while only OT1, but not endogenous CD8+ T cells, increased IL-7Rα in CD27KO mice. These T cells were analyzed in the same mice where the antigen persistence is constant.

Based on the imaging flow cytometry analysis that IL-7Rα protein distribution (surface versus intracellular) has not been substantially changed by CD27 stimulation, we speculate CD27 signals are unlikely involved in the mobilization or degradation of IL-7Rα protein. In contrast, the increase of surface expression of IL-7Rα correlates well with an increase in *IL7ra* mRNA, a consequence of either accelerated gene transcription or enhanced mRNA stability. *IL7ra* transcription is controlled by the opposing actions of the transcription factor

pairs GABPα1/Gfi-1(18) and Foxo1(23)(24) /Foxp1(25), suggesting a role for CD27 signaling in the activity of these factors. Induction of IL-7Rα by GABPα plays a key role in naïve T cell homeostasis(26) and has been shown to be required for IL-7Rα expression in MPECs during acute viral infection(18). GABP has been indicated as a downstream target of JNK (c-Jun Nterminal kinase) – which is a main mediator of downstream signaling pathways induced by CD27 stimulation (13, 27, 28), and JNK phosphorylates GABPα and GABPβ in *vitro* (26). Consistent with the notion that CD27 stimulation operates via inducing IL-7Rα transcription, we found that CD27 stimulated CD8+ T cells express higher levels of *gapba* transcripts. Whether CD27 also phosphorylates this transcription factor remains to be established. It should be noted however, that we have not directly determined a causal relationship between CD27, GABPα and IL7Rα expression and their coordinated increase may be coincidental. Thus, it remains possible that CD27 signals may restrict Gfi-1, a transcription factor induced by IL-7 or TCR engagement and required for stable IL-7Rα repression in effector $CD8^+$ T cells by antagonizing GABPα binding(18), although we find no evidence for decreased Gfi-1 expression in CD27stimulated T cells. Future studies are expected to elucidate whether there is a transcriptional cassette that is elicited by CD27 stimulation in effector CD8+ T cells that directly accounts for IL-7Rα re-expression (for example, we have previously reported that CD27 stimulation promotes the expression of Eomesodermin (6)), and how the timing of CD27 stimulation in naïve vs effector/memory T cells influences transcriptional control of IL-7Rα expression.

The finding that CD27 stimulation augments expression of γ-chain, also by increasing message levels, is novel and implicates CD27 co-stimulation may facilitate IL-7 signaling by regulating multiple components of the signaling complex. Increasing γ chain expression is critical for increased responsiveness to cytokines, and possibly explains why simply transfecting effector cells with IL-7Rα is insufficient to impart memory cell characteristics to effector CD8+ T cells (29). It is tempting to infer that the increased STAT5 phosphorylation in response to *in vitro* IL-7 stimulation in effectors from anti-CD27-treated mice is a consequence of enhanced IL-7R (α and γ) expression on the cell surface. However, it could also be a consequence of increased Jak1/3 activation or alternatively higher abundance of STAT5 in the cytosol.

Finally, our findings of CD27-induced enhanced IL-7R (both α and γc subunits) and augmented pSTAT5-mediated signaling highlight the potential of targeting CD27-CD70 axis to enhance IL-7 signaling for antiviral/antitumor immunotherapy. The pre-clinical and clinical application of rIL-7 has been reported to result in enhanced T cell immunity, raising the prospect that IL-7 signaling could mediate therapeutic benefits in the treatment of acute and chronic infections and cancer(3, 4, 30, 31). Given the published clinical experience with the use of rhIL7 therapy so far is modest(32), enhancing IL-7Rα by targeting CD27 may synergize with rhIL-7 therapy to achieve optimized IL-7 signaling especially in the elderly (33), leading to more favorable outcomes. Moreover, in comparison to rhIL-7 therapy that primarily targets steady-state T cell populations and improves immune reconstitution through increasing thymic output and through Ag-independent homeostatic driven proliferation in the periphery(30), CD27 stimulation promotes IL-7Rα in effector $CD8^+$ T cell population in an Ag-dependent manner and contributes to their long-term maintenance. Therefore, CD27 regulation of IL-7Rα presents a suitable candidate therapeutic strategy to

enhance the effectiveness of adoptive immunotherapy for cancer (34, 35); and probably more importantly, this strategy could be applied to clinical settings where deleterious off-target effects obscure the efficacy of rIL-7 therapy – such as in HIV infection where rhIL-7 has been reported in clinical trials to promote viral persistence during antiretroviral therapy by inducing survival and expansion of HIV-1-latently infected memory CD4(+) T lymphocytes(36, 37). In addition, our finding that CD27 stimulation is capable of inducing IL-7Rα on KLRG1-expressing cells raises the prospect that targeting CD27 could boost the endogenous responses in patients with chronic infection and cancer by altering the exhausted/terminally differentiated characteristics of Ag-specific $CD8^+$ T cells to promote their maintenance and function.

References

1. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, and Ahmed R. 2003 Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat. Immunol. 4: 1191–1198. [PubMed: 14625547]
2. Nanjappa SG, Walent JH, Morre M, and Suresh M. 2008 Effects of IL-7 on memory CD8 T cell homeostasis are influenced by the timing of therapy in mice. J. Clin. Invest. 118: 1027–39. [PubMed: 18246202]
3. Pellegrini M, Calzascia T, Elford AR, Shahinian A, Lin AE, Dissanayake D, Dhanji S, Nguyen LT, Gronski MA, Morre M, Assouline B, Lahl K, Sparwasser T, Ohashi PS, and Mak TW. 2009 Adjuvant IL-7 antagonizes multiple cellular and molecular inhibitory networks to enhance immunotherapies. Nat. Med. 15: 528–536. [PubMed: 19396174]
4. Pellegrini M, Calzascia T, Toe JG, Preston SP, Lin AE, Elford AR, Shahinian A, Lang PA, Lang KS, Morre M, Assouline B, Lahl K, Sparwasser T, Tedder TF, Paik J, DePinho RA, Basta S, Ohashi PS, and Mak TW. 2011 IL-7 Engages Multiple Mechanisms to Overcome Chronic Viral Infection and Limit Organ Pathology. Cell 144: 601–613. [PubMed: 21295337]
5. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, Gapin L, and Kaech SM. 2007 Inflammation Directs Memory Precursor and Short-Lived Effector CD8+ T Cell Fates via the Graded Expression of T-bet Transcription Factor. Immunity 27: 281–295. [PubMed: 17723218]
6. Dong H, Franklin NA, Roberts DJ, Yagita H, Glennie MJ, and Bullock TNJ. 2012 CD27 stimulation promotes the frequency of IL-7 receptor-expressing memory precursors and prevents IL-12mediated loss of CD8(+) T cell memory in the absence of CD4(+) T cell help. J. Immunol. 188: 3829–38. [PubMed: 22422886]
7. McKinstry KK, Strutt TM, Bautista B, Zhang W, Kuang Y, Cooper AM, and Swain SL. 2014 Effector CD4 T-cell transition to memory requires late cognate interactions that induce autocrine IL-2. Nat. Commun. 5: 5377. [PubMed: 25369785]
8. Roberts DJ, Franklin NA, Kingeter LM, Yagita H, Tutt AL, Glennie MJ, and Bullock TNJ. 2010 Control of established melanoma by CD27 stimulation is associated with enhanced effector function and persistence, and reduced PD-1 expression of tumor infiltrating CD8(+) T cells. J. Immunother. 33: 769–779. [PubMed: 20842060]
9. Oshima H, Nakano H, Nohara C, Kobata T, Nakajima A, Jenkins NA, Gilbert DJ, Copeland NG, Muto T, Yagita H, and Okumura K. 1998 Characterization of murine CD70 by molecular cloning and mAb. Int. Immunol. 10: 517–26. [PubMed: 9620608]
10. Hintzen RQ, de Jong R, Lens SMA, and van Lier RW. 1994 CD27: marker and mediator of Tcell activation? Immunol. Today 15: 307–311. [PubMed: 8086099]
11. Takeda K, Oshima H, Hayakawa Y, Akiba H, Atsuta M, Kobata T, Kobayashi K, Ito M, Yagita H, and Okumura K. 2000 CD27-mediated activation of murine NK cells. J. Immunol. 164: 1741–5. [PubMed: 10657619]
12. Sugita K, Robertson MJ, Torimoto Y, Ritz J, Schlossman SF, Morimoto C, Kobayashi K, Ito M, Yagita H, and Okumura K. 1992 Participation of the CD27 antigen in the regulation of IL-2-activated human natural killer cells. J. Immunol. 149: 1199–203. [PubMed: 1354231]

13. Borst J, Hendriks J, and Xiao Y. 2005 CD27 and CD70 in T cell and B cell activation. Curr. Opin. Immunol. 17: 275–281. [PubMed: 15886117]
14. Alves NL, van Leeuwen EMM, Derks IAM, and van Lier RAW. 2008 Differential regulation of human IL-7 receptor alpha expression by IL-7 and TCR signaling. J. Immunol. 180: 5201–10. [PubMed: 18390701]
15. McLeod IX, Zhou X, Li Q-J, Wang F, and He Y-W. 2011 The class III kinase Vps34 promotes T lymphocyte survival through regulating IL-7Rα surface expression. J. Immunol. 187: 5051–61. [PubMed: 22021616]
16. Henriques CM, Rino J, Nibbs RJ, Graham GJ, and Barata JT. 2010 IL-7 induces rapid clathrinmediated internalization and JAK3-dependent degradation of IL-7Ralpha in T cells. Blood 115: 3269–77. [PubMed: 20190194]
17. Faller EM, Sugden SM, McVey MJ, Kakal JA, and MacPherson PA. 2010 Soluble HIV Tat protein removes the IL-7 receptor alpha-chain from the surface of resting CD8 T cells and targets it for degradation. J. Immunol. 185: 2854–66. [PubMed: 20660706]
18. Chandele A, Joshi NS, Zhu J, Paul WE, Leonard WJ, and Kaech SM. 2008 Formation of IL7Ralphahigh and IL-7Ralphalow CD8 T cells during infection is regulated by the opposing functions of GABPalpha and Gfi-1. J. Immunol. 180: 5309–19. [PubMed: 18390712]
19. Feau S, Garcia Z, Arens R, Yagita H, Borst J, and Schoenberger SP. 2012 The CD4+ T-cell help signal is transmitted from APC to CD8+ T-cells via CD27–CD70 interactions. Nat. Commun. 3: 948. [PubMed: 22781761]
20. Shin HM, Kapoor V, Guan T, Kaech SM, Welsh RM, and Berg LJ. 2013 Epigenetic modifications induced by Blimp-1 Regulate CD8+ T cell memory progression during acute virus infection. Immunity 39: 661–75. [PubMed: 24120360]
21. Welten SPM, Redeker A, Franken KL, Benedict CA, Yagita H, Wensveen FM, Borst J, Melief CJM, van Lier RAW, van Gisbergen KPJM, and Arens R. 2013 CD27-CD70 costimulation controls T cell immunity during acute and persistent cytomegalovirus infection. J. Virol. 87: 6851–65. [PubMed: 23576505]
22. Allam A, Conze DB, Giardino Torchia ML, Munitic I, Yagita H, Sowell RT, Marzo AL, and Ashwell JD. 2009 The CD8+ memory T-cell state of readiness is actively maintained and reversible. Blood 114: 2121–30. [PubMed: 19617575]
23. Kerdiles YM, Beisner DR, Tinoco R, Dejean AS, Castrillon DH, DePinho RA, and Hedrick SM. 2009 Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. Nat. Immunol. 10: 176–184. [PubMed: 19136962]
24. Kerdiles YM, Stone EL, Beisner DL, McGargill MA, Ch'en IL, Stockmann C, Katayama CD, and Hedrick SM. 2010 Foxo Transcription Factors Control Regulatory T Cell Development and Function. Immunity 33: 890–904. [PubMed: 21167754]
25. Feng X, Wang H, Takata H, Day TJ, Willen J, and Hu H. 2011 Transcription factor Foxp1 exerts essential cell-intrinsic regulation of the quiescence of naive T cells. Nat. Immunol. 12: 544–550. [PubMed: 21532575]
26. Rosmarin AG, Resendes KK, Yang Z, McMillan JN, and Fleming SL. 2004 GA-binding protein transcription factor: a review of GABP as an integrator of intracellular signaling and protein–protein interactions. Blood Cells, Mol. Dis. 32: 143–154. [PubMed: 14757430]
27. Denoeud J, and Moser M. 2011 Role of CD27/CD70 pathway of activation in immunity and tolerance. J. Leukoc. Biol. 89: 195–203. [PubMed: 20699361]
28. Nolte MA, van Olffen RW, van Gisbergen KPJM, and van Lier RAW. 2009 Timing and tuning of CD27-CD70 interactions: the impact of signal strength in setting the balance between adaptive responses and immunopathology. Immunol. Rev. 229: 216–231. [PubMed: 19426224]
29. Hand TW, Morre M, and Kaech SM. 2007 Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. Proc. Natl. Acad. Sci. U. S. A. 104: 11730–5. [PubMed: 17609371]
30. Mackall CL, Fry TJ, and Gress RE. 2011 Harnessing the biology of IL-7 for therapeutic application. Nat. Rev. Immunol. 11: 330–342. [PubMed: 21508983]

31. Ding Z-C, Habtetsion T, Cao Y, Li T, Liu C, Kuczma M, Chen T, Hao Z, Bryan L, Munn DH, and Zhou G. 2017 Adjuvant IL-7 potentiates adoptive T cell therapy by amplifying and sustaining polyfunctional antitumor CD4+ T cells. Sci. Rep. 7: 12168. [PubMed: 28939858]

32. ElKassar N, and Gress RE. 2010 An overview of IL-7 biology and its use in immunotherapy. J. Immunotoxicol. 7: 1–7. [PubMed: 20017587]

33. Kim H-R, Hong MS, Dan JM, and Kang I. 2006 Altered IL-7Ralpha expression with aging and the potential implications of IL-7 therapy on CD8+ T-cell immune responses. Blood 107: 2855–62. [PubMed: 16357322]

34. Johnson CB, Riesenberg BP, May BR, Gilreath SC, Li G, Staveley-O'Carroll KF, GarrettMayer E, Mehrotra S, Cole DJ, and Rubinstein MP. 2015 Effector CD8+ T-cell Engraftment and Antitumor Immunity in Lymphodepleted Hosts Is IL7Rα Dependent. Cancer Immunol. Res. 3: 1364–74. [PubMed: 26297711]

35. Chandran SS, Paria BC, Srivastava AK, Rothermel LD, Stephens DJ, and Kammula US. 2015 Tumor-Specific Effector CD8+ T Cells That Can Establish Immunological Memory in Humans after Adoptive Transfer Are Marked by Expression of IL7 Receptor and c-myc. Cancer Res. 75: 3216–26. [PubMed: 26100671]

36. Vandergeeten C, Fromentin R, DaFonseca S, Lawani MB, Sereti I, Lederman MM, Ramgopal M, Routy J-P, Sékaly R-P, and Chomont N. 2013 Interleukin-7 promotes HIV persistence during antiretroviral therapy. Blood 121: 4321–9. [PubMed: 23589672]

37. Yin Y, Zhang S, Luo H, Zhang X, Geng G, Li J, Guo X, Cai W, Li L, Liu C, and Zhang H. 2015 Interleukin 7 up-regulates CD95 protein on CD4+ T cells by affecting mRNA alternative splicing: priming for a synergistic effect on HIV-1 reservoir maintenance. J. Biol. Chem. 290: 35–45. [PubMed: 25411246]

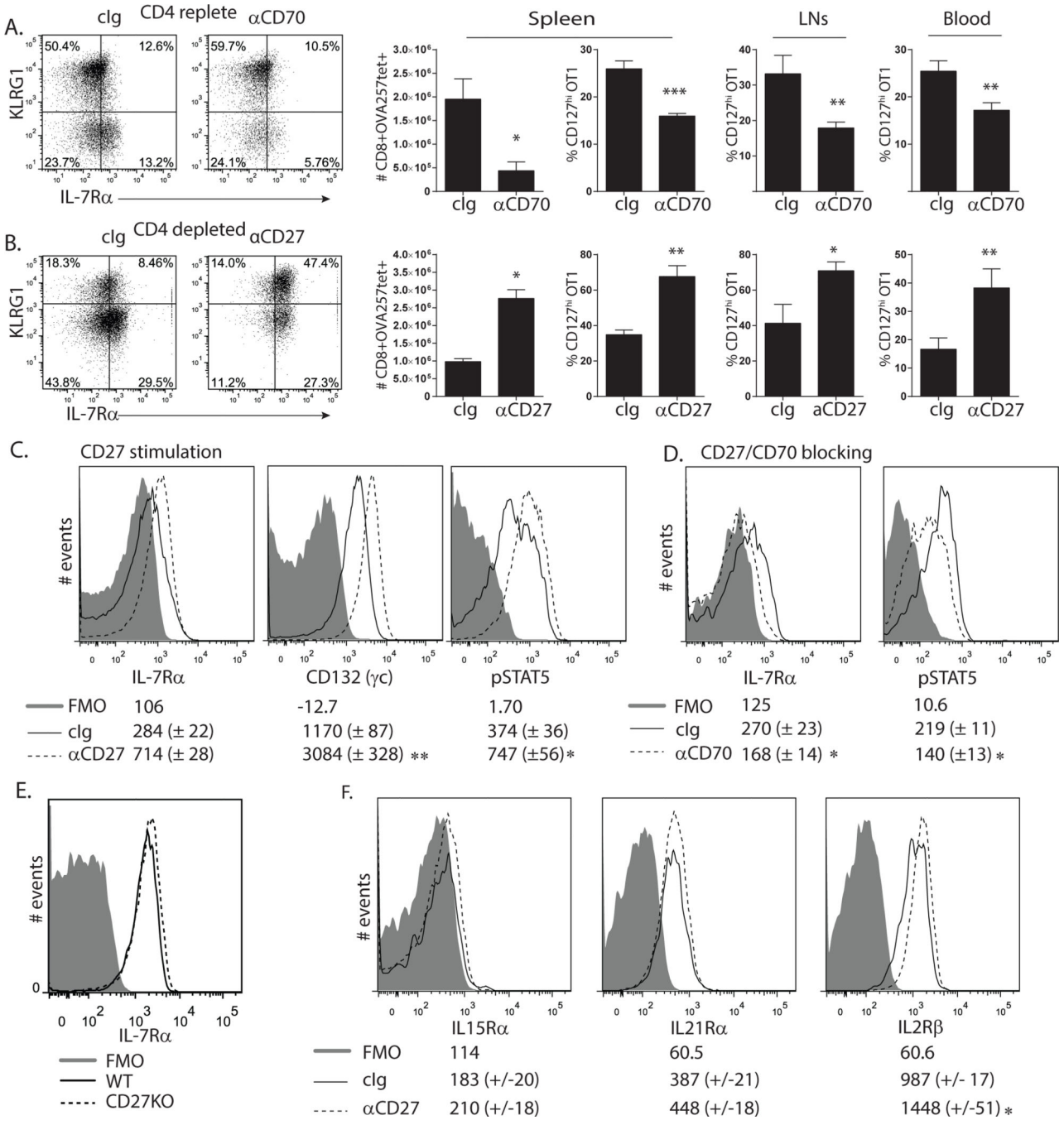


Figure 1. CD27 stimulation of CD8$^+$ T cells during primary response drives the presence of primary CD8+ T cells with enhanced expression of functional IL-7Rα.

(A, B) 1,000 OT-1 cells were adoptively transferred into replete (A) - or CD4-depleted (B) WT recipients1d before vac-OVA (1e7 pfu i.p.) priming. Mice (n=3–5/cohort) were then treated with either (A) CD70 blocking antibodies (FR70) or (B) CD27 stimulating antibodies (AT124.1)), in comparison to control IgG (cIg). Spleens, or lymph nodes and blood, were harvested at d7, and OT-1 cells were analyzed by flow cytometry for surface expression of IL-7Rα. (C, D) Adeno-OVA primed mice (n=3–5/cohort) were treated with either (C) CD27 stimulating antibodies (αCD27) or (D) CD70 blocking antibodies (αCD70)

in comparison with control IgG (cIg). Spleens were harvested at d7, and OVA-specific CD8$^+$ T cells were analyzed by flow cytometry for surface expression of IL-7Rα and γc, and levels of pSTAT5 upon *in vitro* stimulation with rIL-7, as described in the Methods. Overlapping histograms showed representative data from triplicates per group and numbers show mean+/− SD. Experiments were independently repeated > 3 times. (E) Steady state expression of IL-7Rα on naïve CD8+ T cells in WT and CD27-knockout mice (G). Limited impact of CD27 stimulation on the expression of other γc cytokine receptors. Adeno-OVA primed mice (n=3/cohort) were treated with either CD27 stimulating antibodies (αCD27) or control IgG (cIg), and d7 spleens were harvested and effector CD8$^+$ T cells were analyzed by flow cytometry for surface expression of cytokine receptors. Experiments were independently repeated 2 times. *p<0.05; **p<0.01; ***p<0.001 Student t-test for bar charts.

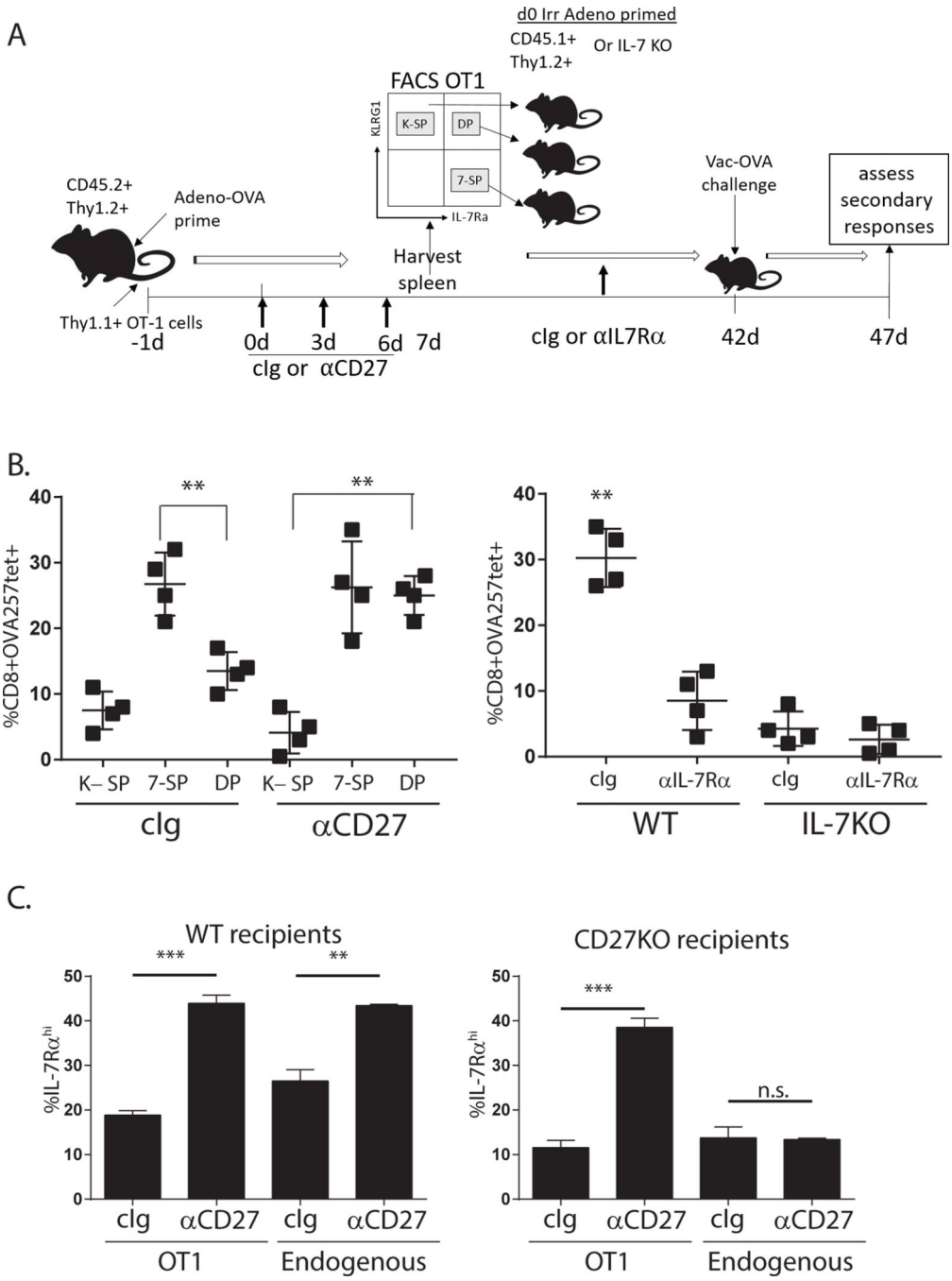


Figure 2. Enhanced expression of IL-7Rα after CD27 stimulation increases memory cell potential in effector cells in a IL-7 dependent manner.

(A) Schema of stimulation and transfer of OT-1 cells into WT, IL-7KO or CD27 KO recipient mice. (B) Expansion of OT-1 secondary effectors from KLRG1-single positive (K-SP), IL-7Rα-single positive (7-SP) or KLRG1-IL-7Rα double positive (DP) CD8+ T cells. Primary OT-1 effectors were sorted at d7 after adeno-OVA immunization concurrent with either cIg or αCD27, transferred to Thy1mismatched WT (left panel), or WT mice or IL-7KO mice treated with cIg or a blocking antibody to IL-7Rα (right panel), and challenged 35d later with vac-OVA (n=3–5 per group). The magnitude of the secondary

responses was determined 5d later by staining with, CD8, CD44, OVA257-multimers and the Thy1.1 congenic marker. (C) Direct stimulation of CD27 on CD8+ T cells drives IL-7Rα expression. 500 OT-1 were transferred into either WT or $CD27^{-/-}$ recipients (n=3/group), immunized with adeno-OVA and treated with either cIg or anti-CD27. Both OT-1 and endogenous OVA-specific $CD8^+$ T cells were analyzed at d7 for surface expression of IL7Rα. Dot plots was pre-gated on OT1 cells (live $Thy1.1^+$ $CD8^+$) and showed representative mouse for each group. Bar charts showed combined results in each group from a representative experiment. Experiments were independently repeated 2~5 times with 3 mice per group. Student t-test for bar charts in (A) and (B), and one-way ANOVA for (C). *p<0. 05, **p<0. 01, and ***p<0. 001.

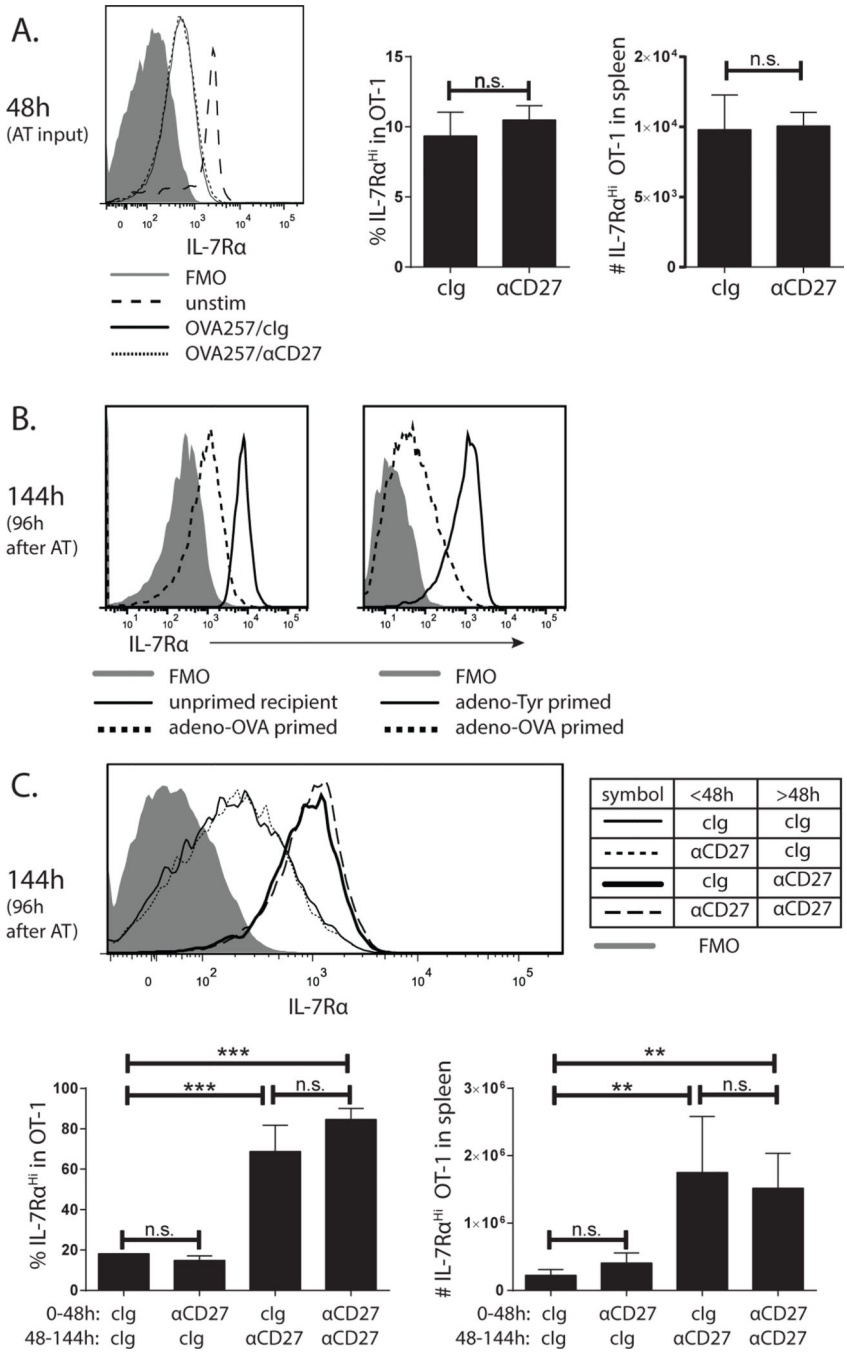


Figure 3. Early CD27 stimulation has no impact on IL-7Rα down-regulation, yet late CD27 stimulation facilitates IL-7Rα re-expression.

(A) OT-1 cells were co-cultured with irradiated APC pulsed with OVA_{257} peptide, in the presence of either CD27 stimulating antibodies (αCD27) or control IgG (cIg). Frequency and absolute number of IL-7Rα-expressing OT-1 cells were assessed at 48h. (B) 48h *in vitro*-stimulated, IL-7Rα-down-regulated OT-1 cells described in (A) were adoptively transferred into mice pre-primed with adeno-OVA, or adeno-Tyr (adenovirus recombinant with tyrosinase) or plain PBS as indicated. Spleens were harvested 96h after the adoptive transfer and the expression of IL-7Rα on OT-1 cells were identified by flow cytometry. (C)

Similar setting as in (B), except recipient mice were infected with adeno-OVA, and after the adoptive transfer they were then treated with either CD27 stimulating antibodies (αCD27) or control IgG (cIg). Spleens were harvested 96h later after the adoptive transfer and the expression of IL-7Rα on OT-1 cells were identified by flow cytometry. Each experiment was independently repeated 2 or 3 times with 3 mice per group. Student t-test for bar charts in (A), and one-way ANOVA for bar charts in (C). **p<0. 01, and ***p<0. 001.

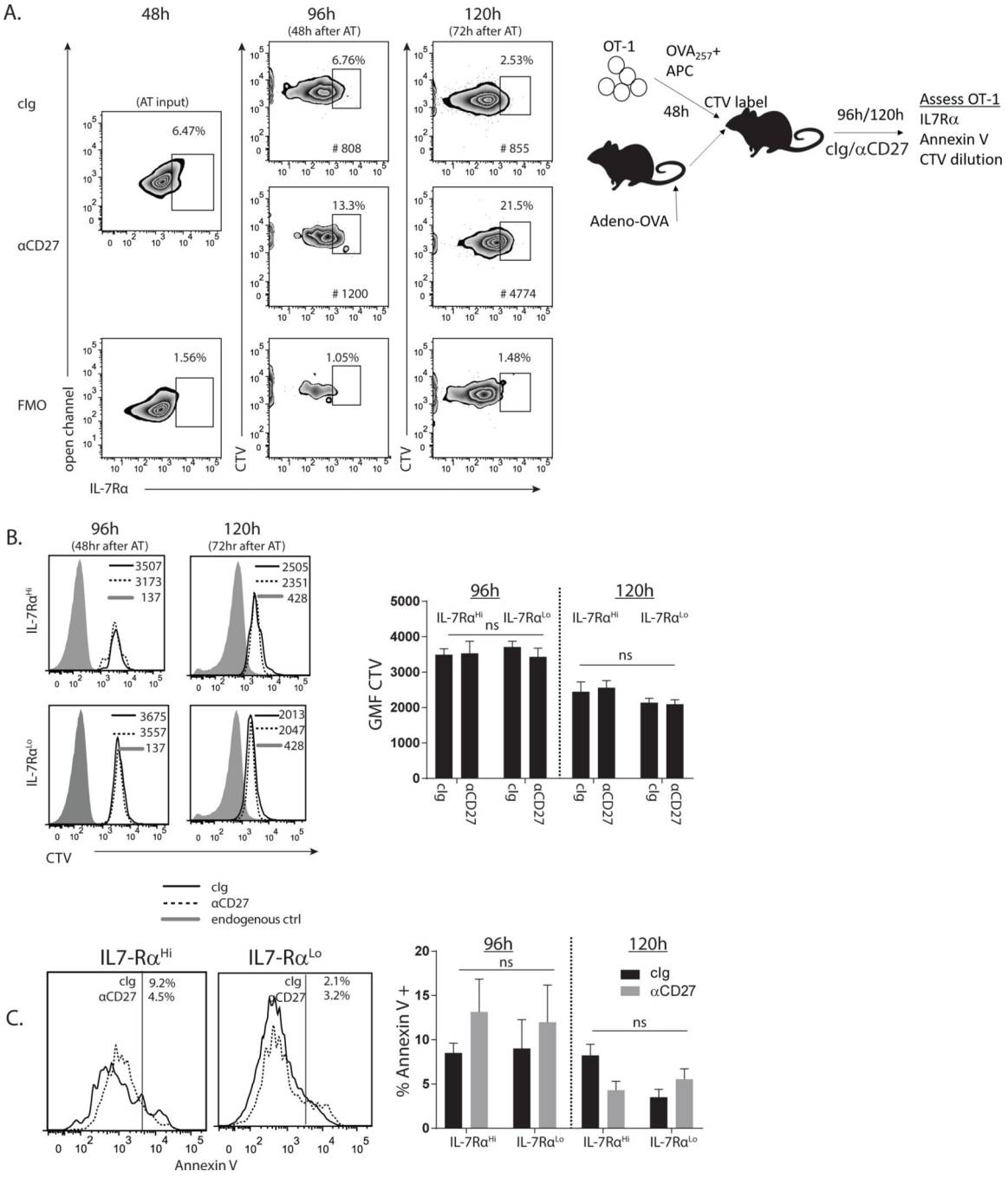


Figure 4. The increased frequency and absolute number of IL-7Rα-expressing T cells driven by CD27 stimulation are not associated with accelerated cell division or enhanced T cell survival.

After 48h *in vitro* stimulation to down-regulate their IL-7Rα, CTV-labelled OT-1 cells and transferred into adeno-OVA pre-primed recipients (n=3/cohort). Mice were then treated with either CD27 stimulating antibodies (αCD27) or control IgG (cIg). 48 and 72h after the adoptive transfer, (A) IL-7Rα expression on OT-1 cells, and (B) proliferation and (C) cell apoptosis in OT-1 subsets were analyzed by flow cytometry. Flow cytometry plots showed representative data from duplicates, with subset percentage, cell number, or fluorescence

geometric mean indicated. Similar experiments were performed twice. ns= not significant by one way ANOVA.

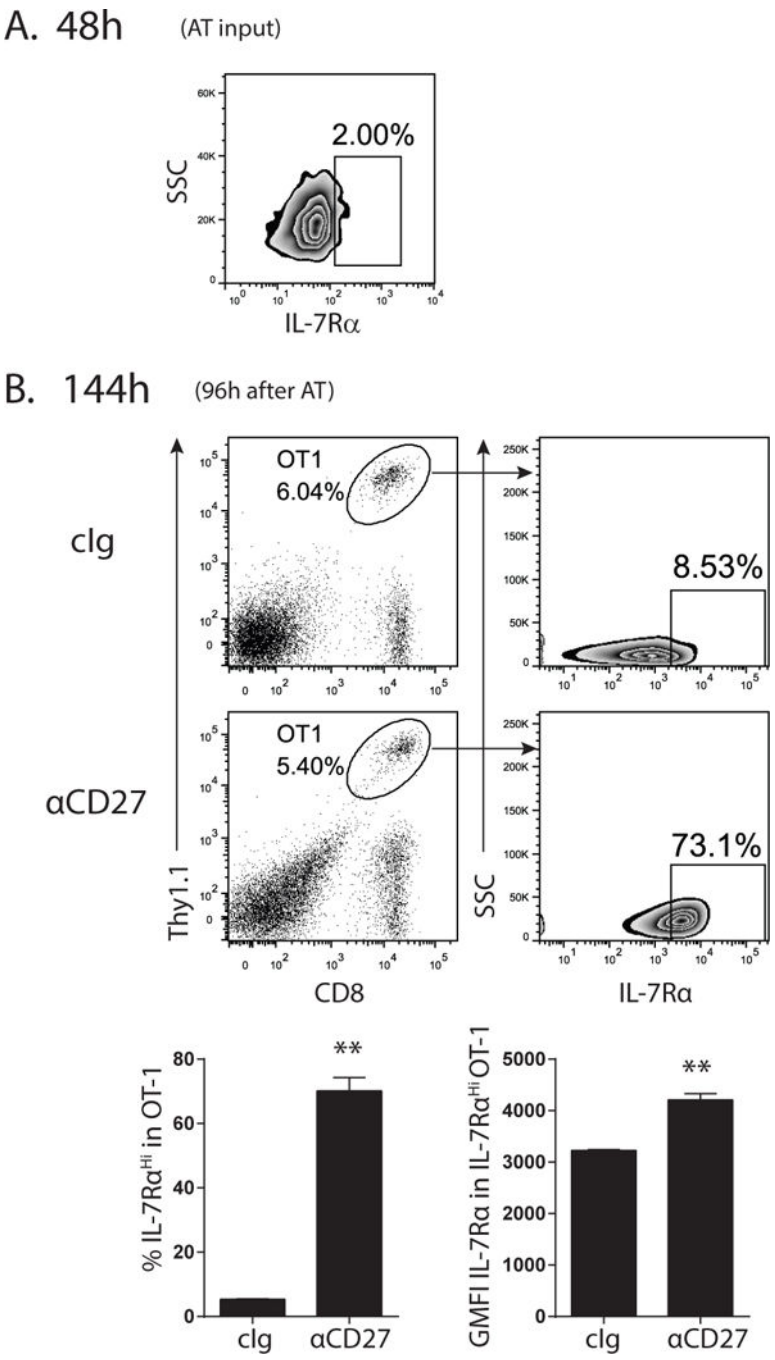


Figure 5. CD27 stimulation directly modulates IL-7Rα re-expression.

(A) 48h *in vitro*-stimulated OT-1 cells were stringently sorted for minimal IL-7Rα expression, and then adoptively transferred into mice pre-primed with adeno-OVA. Mice were then treated with either CD27 stimulating antibodies (α27) or control IgG (cIg). (B) Spleens were harvested 96h later after the adoptive transfer and the percentage of IL-7Rα-re-expressing OT-1 cells, and per-cell levels of IL-7Rα within the IL-7Rα-re-expressing OT-1 cells were estimated by flow cytometry. Bar charts showed the combined result of

three mice per group from a representative experiment. Experiments were independently repeated 3 times. Student t test, **p<0. 01.

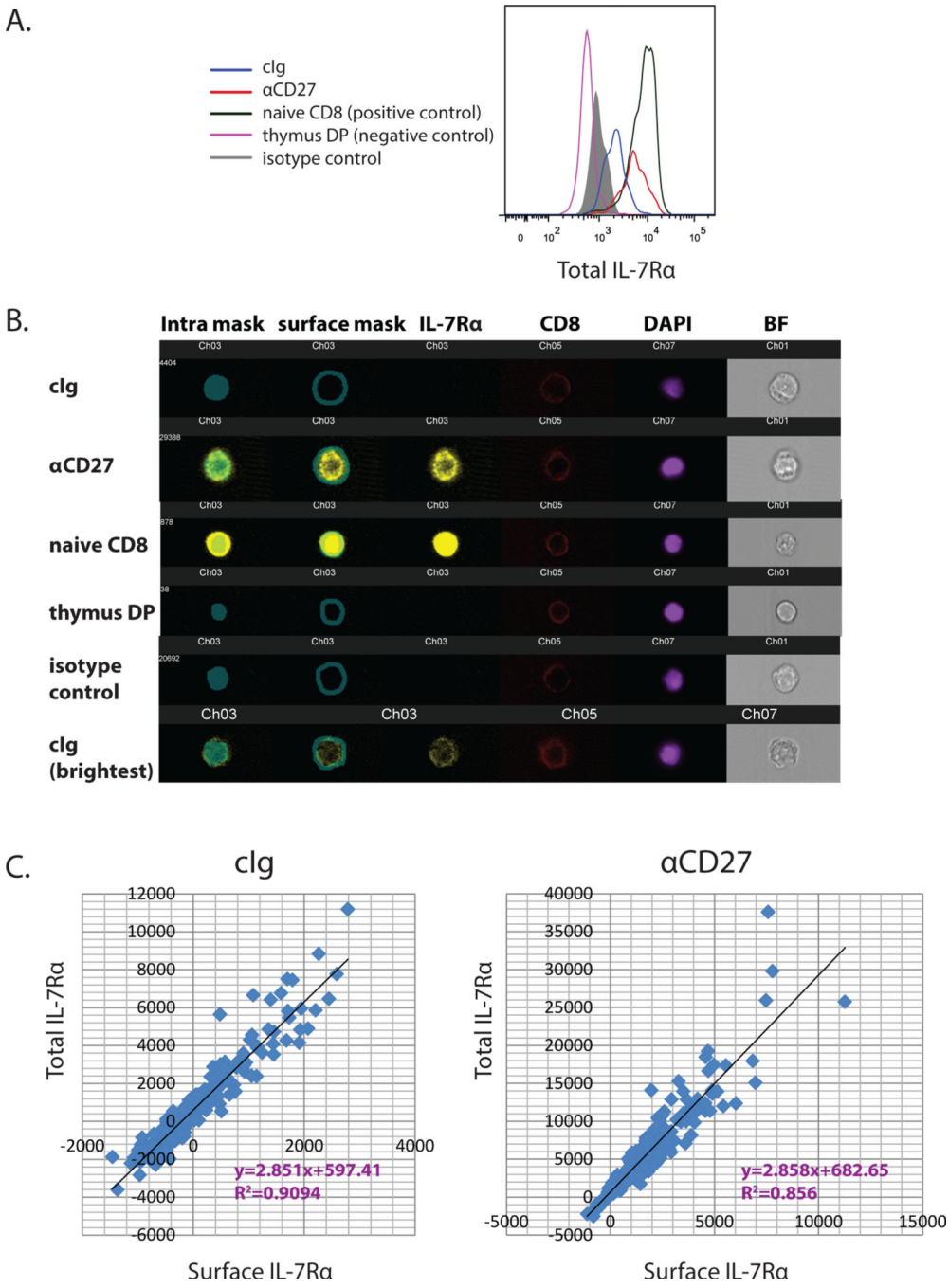


Figure 6. CD27 stimulation concomitantly promotes IL-7Rα expression in both surface and intracellular compartments without skewing the proportion of each compartment.

To assess the total (surface + intracellular) expression of IL-7Rα, splenocytes were harvested from either CD27 stimulating antibodies (αCD27) or control IgG (cIg) treated mice, permeabilized and stained for IL-7Rα. (A) Levels of IL-7Rα on OT-1 cells identified by regular flow cytometry. (B) The imaging of IL-7Rα expression by imaging flow cytometry using the identical samples from (A). (C) Further analysis of (B) revealed the proportion of surface IL-7Rα in the total expression. Similar experiments were independently repeated twice, with duplicates per group.

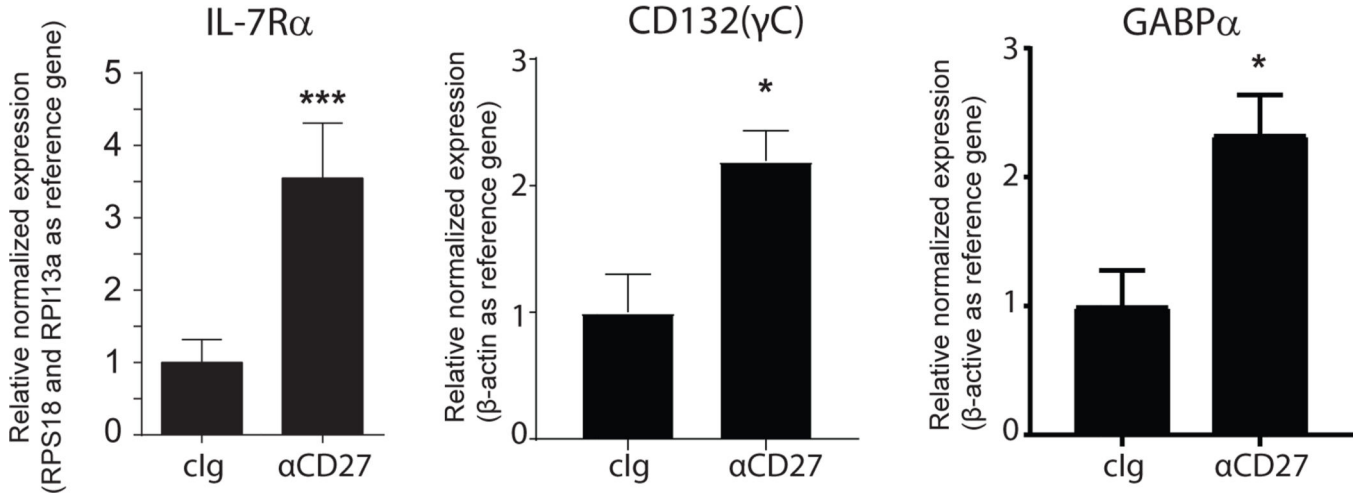


Figure 7. CD27 stimulation enhances both IL-7Rα and γc expression on effector CD8$^+$ T cells and is coordinated with an increase in GABPα expression.

CD27 signals regulate IL-7Rα re-expression by promoting its mRNA and the transcription factor GABPα. d5~6 effector CD8$^+$ T cells from CD27 stimulating antibodies (αCD27) or control IgG (cIg) treated mice were sorted and their *il7ra, CD132* and *gabpa* mRNA levels were compared by quantitative PCR. Data showed combined results from 3 independent experiments, with duplicates or triplicates in each experiment. Student t test, *p<0.05; ***p<0. 001