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IL-2 modulates the TCR signaling threshold for CD8 but not CD4 T cell proliferation on a single cell level

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Abstract

Lymphocytes integrate antigen and cytokine receptor signals to make cell fate decisions. Using a specific reporter of TCR signaling that is insensitive to cytokine signaling, Nur77-eGFP, we identify a sharp, minimal threshold of cumulative TCR signaling required for proliferation in CD4 and CD8 T cells that is independent of both antigen concentration and affinity. Unexpectedly, IL-2 reduces this threshold in CD8 but not CD4 T cells, suggesting that integration of multiple mitogenic inputs may alter the minimal requirement for TCR signaling in CD8 T cells. Neither naïve CD4 nor naïve CD8 T cells are responsive to low doses of IL-2. We show that activated CD8 T cells become responsive to low doses of IL-2 more quickly than CD4 T cells, and propose that this relative delay in turn accounts for differential effects of IL-2 on the minimal TCR signaling threshold for proliferation in these populations. In contrast to Nur77-eGFP, c-Myc protein expression integrates mitogenic signals downstream of both IL-2 and the TCR, yet marks an invariant minimal threshold of cumulative mitogenic stimulation required for cell division. Our work provides a conceptual framework for understanding regulation of clonal expansion of CD8 T cells by sub-threshold TCR signaling in the context of mitogenic IL-2 signals, thereby rendering CD8 T cells exquisitely dependent upon environmental cues. Conversely, CD4 T cell proliferation requires an invariant minimal intensity of TCR signaling that is not modulated by IL-2, thereby restricting responses to low affinity or low abundance self-antigens even in the context of an inflammatory milieu.

Keywords

TCR; CD4 T cells; CD8 T cells; proliferation; IL-2; Nur77; NR4A1

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INTRODUCTION

Adaptive immune responses rely upon robust clonal expansion of rare antigen-specific lymphocyte populations. CD8 T cells can divide up to 15 times *in vivo,* as often as every 4–8 hours, to give rise to more than 10^4 progeny within 7 days (1). Lymphocytes integrate information about the concentration, affinity, and avidity of antigens, in addition to the presence of cytokines such as IL-2, to inform the decision to divide, and for how many times (2, 3). Tight regulation of this process is critical for both host defense and immune tolerance. T cell proliferation has been amenable to study *in vitro* through the use of TCR transgenic models and polyclonal stimulation with TCR cross-linking antibodies. Yet even uniformly stimulated populations of genetically identical lymphocytes exhibit enormous heterogeneity in proliferative responses, making it challenging to dissect how input signals are interpreted on a single cell level.

Nur77 is an orphan nuclear hormone receptor encoded by the primary response gene *Nr4a1*, and is robustly and rapidly induced by antigen receptor signaling (4–7). We have recently characterized a novel Nur77-eGFP BAC transgenic mouse line that recapitulates endogenous *Nr4a1* expression (6). Due to the long half-life of eGFP, reporter expression in T cells serves as a faithful and specific marker of intensity and duration of TCR signaling. Nevertheless, we observed that reporter T cells that have divided in response to TCR stimulation express a high and invariant amount of GFP regardless of antigen dose and modulation of TCR signal strength (7). These observations suggested that individual T cells must reach a minimal TCR signaling threshold for cell division. However, it is unknown whether extensive titration of antigen affinity alters the minimal TCR signaling threshold for proliferation. T cell proliferation is also modulated by cytokines such as IL-2 (2, 3), yet it is not clear how such environmental cues about the inflammatory milieu influence the TCR signaling threshold for T cell proliferation on a single cell level.

The Nur77-eGFP reporter facilitates dissection of the contribution of TCR signals to proliferation in different contexts, as it is sensitive to TCR but not cytokine-dependent JAK/STAT signals (4, 6, 7). Prior work has shown that Nur77 expression can be induced not only by TCR together with CD28 stimulation, but also by either ionomycin or PMA, and is both calcineurin- and PKC-dependent (6, 8). However, Nur77 reporter expression could not be induced by a constitutively active STAT5 construct, consistent with insensitivity of the reporter to IL-2 (4, 7).

Here we show that expression of Nur77-eGFP reporter sensitively reports relative differences in peptide affinity and dose in CD8 and CD4 cells. However, proliferating antigen-specific CD8 and CD4 T cells exhibit high and invariant GFP expression in response to broad titration of peptide potency and concentration, identifying a minimal threshold of cumulative TCR signaling required for proliferation. Though IL-2 is a known T cell growth factor, in this context it is surprising that provision of exogenous IL-2 markedly reduces the minimal TCR signaling threshold required by CD8, but not CD4, T cells to proliferate, particularly under conditions of suboptimal TCR stimulation. We show that CD4 T cells take more time to become responsive to low doses of IL-2 upon activation with antigen than CD8 T cells, and propose that this temporal delay accounts for differential effects of IL-2 upon

this TCR threshold. Finally, we show that expression of the primary response gene *c-myc* is coordinately regulated downstream of both antigenic and cytokine stimuli, and defines a minimal threshold for mitogenic stimulation that is invariant among individual proliferating lymphocytes. We propose that graded population proliferative responses are achieved in part by altering the fraction of responding cells that have received sufficient integrated mitogenic signaling to cross this threshold.

MATERIALS AND METHODS

Mice

Nur77-eGFP mice (both low and high expressing BAC Tg lines) have been previously described (6). All T cell assays were performed using low-GFP line except Figure 8A which used the high-GFP line. BoyJ and C3H/HeJ mice were obtained from Jackson labs. OTI, and OTII mice were previously described (9, 10). AND Tg mice on the C57BL/6 genetic background were generously provided by Paul Allen (Washington University), and have been previously described (11, 12). All strains were fully backcrossed to C57BL/6 genetic background. Mice were used at 5–9 weeks of age for all functional and biochemical experiments. All mice were housed in a specific pathogen free facility at UCSF according to the University Animal Care Committee and National Institutes of Health (NIH) guidelines.

Antibodies and Reagents

Antibodies for FACS: CD4, CD8, TCR vα2, CD25, CD69, CD122, CD127, CD132, lambda1, CD45.1, CD45.2 conjugated to FITC, biotin, PE, PerCP-Cy5.5, PE-Cy7, Pacific Blue, APC or Alexa 647 (eBiosciences or BD Biosciences); Antibodies for intra-cellular staining: c-MYC Ab (clone D84C12, Cell Signaling), STAT5 pY694 (BD, clone 47, BD biosciences), AKT pS473 (193H12 clone, Cell Signaling); Cytokines and other stimuli: anti-CD3ε (clone 145–2C11; Harlan), anti-CD28 (clone 37.5; UCSF flow core; dose 2μg/ml), anti–IL-2 (clone JES6-5H4; UCSF flow core; dose 10μg/ml), recombinant human IL-2 (rhIL-2) was from the NIH AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (Maurice Gately, Hoffmann-La Roche), recombinant IL-7 (variable doses for signaling), and IL-15 (100ng/ml) (R&D systems). Peptides: OTII-specific ovalbumin ($OVA_{323–339}$) peptide; OTI-specific ovalbumin (OVA257-264) peptide (aka SIINFEKL/“N4”), as well as altered peptide ligands “Q4R7”, and “G4” have been previously described (13), as have MCC and altered peptide ligands “K99E” and “T102L” (14) (Genscript). Inhibitor: Jak3i (15).

Dilutional dye loading

Lymphocytes were loaded with CellTrace Violet (Invitrogen) per the manufacturer’s instructions except 1ml/5 $\times 10^6$ cells rather than 1ml/1 $\times$ 10^6 cells.

Lymphocyte purification

CD8 or CD4 T cells were purified from single-cell suspensions of spleen and lymph nodes from male and female mice aged 6–12 weeks by negative selection with biotinylated antibodies to CD4 (GK1.5) or CD8 (53-6.7) respectively, CD19 (6D5), B220 (RA3-6B2), CD11b (M1/70), CD49b (DX5), Ly-76 (Ter119), CD24 (M1/69) (all BioLegend) and CD11c

(N418, Tonbo) and magnetic anti-biotin beads (MACSi Beads, Miltenyi Biotec) as previously described(15). Tregs were additionally depleted from CD4 T cells by adding 1:100 anti CD25 (PC61.5).

Fixable live/dead stain

in vitro cultured cells were stained to detect dead cells using fixable live/dead stain [near IR] (Life technologies) per manufacturer's instructions, compatible with both CellTrace Violet and MeOH permeabilization as needed.

Flow Cytometry and data analysis

Cells were stained with indicated antibodies and analyzed on a Fortessa (Becton Dickson) as previously described (16). Data analysis was performed using FlowJo (v9.7.6) software (Treestar Incorporated, Ashland, OR). 'Division index' and '% divided' parameters were calculated from CellTrace Violet-loaded lymphocytes using FlowJo (17). Statistical analysis and graphs were generated using Prism v6 (GraphPad Software, Inc). Figures were prepared using Illustrator CS6 v16.0.0.

Intracellular staining

Staining for intra-cellular c-MYC expression was performed as follows: after stimulation under various conditions as described below (with or without CellTrace Violet loading), 10^6 cells/well were resuspended in 96-well plates, stained with fixable live/dead dye as above, fixed in 2%PFA, washed in FACS buffer, and permeabilized with ice-cold 90% methanol at −20 °C overnight, or for at least 30 minutes on ice. Cells were then washed in FACS buffer, stained with anti-MYC antibody (1:200) x 45 minutes, washed in FACS buffer, and then stained with Alexa 647-conjugated secondary Goat anti-Rabbit antibody (Jackson Immunoresearch) along with directly conjugated antibodies to detect surface markers. Samples were washed and then refixed with 2%PFA prior to acquisition on BD Fortessa. Staining for intra-cellular pSTAT5 was performed as previously described (15), with the addition of pAKT staining (S473 193H12 Cell Signaling, diluted 1:50, and detected with anti-rabbit-PE 1:200, Jackson Immunoresearch).

In vitro T cell stimulation

CD8 or CD4 T cells were purified from either OTI-Nur77-eGFP or AND-Nur77-eGFP mice as described above and loaded with CellTrace Violet. OTI cells were mixed in a 5:1 ratio with peptide-loaded splenocytes from Zap70-deficient mice to serve as APCs, plated at a concentration of 10^5 cells/200μl in round bottom 96 well plates in complete media (RPMI). AND cells were plated at a 1:1 ratio with peptide-loaded splenocytes from C3H/HeJ mice. Cells were stained and analyzed as described above after 1 or 3 days of culture. For all intra-cellular c-Myc staining assays, lymphocytes from OTI-Nur77-eGFP mice were loaded with CellTrace Violet, and directly incubated with peptides at varying concentrations. Cells were plated at a concentration of 10^6 cells/200ul in round bottom 96 well plates for incubations ≤48 hours, and 10^5 cells/200μl for 72 hour assays. For plate-bound T cell stimulation, flat-bottom plates were coated in 50μl PBS with varied doses of anti-CD3ε +/− anti-CD28

overnight at 4C, and washed in PBS prior to plating cells at a concentration of 2×10^5 cells/200μl.

In vivo T cell stimulation

CD8 T cells were purified from CD45.2 OTI-Nur77-eGFP reporter mice, and loaded with CellTrace Violet as described above. Cells (5×10^5) were transferred into CD45.1 BoyJ hosts via tail vein injection on d-1. APCs (total splenocytes) from Zap-70−/− mice were loaded with either N4, Q4R7, G4, or no peptide (10^{-8} M) via 2 hour incubation at 37C. 10^6 loaded APCs were transferred into recipients via tail vein injection on d0. Three days later splenocytes from recipients were surface stained to detect vα2, CD45.1, CD45.2, and CD8 expression, and analyzed by FACS to detect GFP expression, CellTrace Violet dilution, and surface marker expression.

Quantitative PCR (qPCR)

RNA was isolated from 1–2 × 10^6 $CD4^+$ T cell blasts per condition using an RNeasy kit (Qiagen), and cDNA was synthesized using qScript (Quanta Biosciences). mRNA was detected by Primetime (IDT) or Taqman (Life Technologies) predesigned qPCR assays. Assay ID: Taqman: il2ra (Mm01340213_m1); IDT: il2rg (Mm.PT.58.43694030), il2rb (Mm.PT.58.15857480), socs1 (Mm.PT.58.11527306.g), socs2 (Mm.PT.58.5195465), socs3 (Mm.PT.58.7804681), cish (Mm.PT.58.11557699). Data are from three replicates collected on a QuantStudio 12k (Life Technologies), plotted with 95% confidence intervals as calculated by QuantStudio (Life Technologies).

RESULTS

A sharp TCR signaling threshold for CD8 T cell proliferation is independent of peptide affinity *in vivo*

We previously reported that individual T cells integrate TCR signaling over time and require an invariant minimal amount and duration of signaling in order to divide, as detected by Nur77-eGFP reporter expression (7). Moreover, this threshold is not altered by stimulus dose or modulation of Zap70 kinase activity. In the present study, we determined whether peptide affinity, which can vary broadly, influences this threshold in T cells. We introduced the Nur77-eGFP reporter onto the OVA-specific OTI TCR transgenic (Tg) model, and took advantage of a well-characterized set of OVA-derived altered peptide ligands (APLs) with varying affinity for this TCR (10, 13). Purified OTI-Nur77-eGFP mature CD8 T cells were incubated with APCs loaded with varying concentrations of high, medium, and low affinity peptides (OVA a.k.a. N4/SIINFEKL, Q4R7, and G4, respectively), and Nur77-eGFP induction was assessed after 24-hour culture. Low concentrations of N4 peptide (10^{-12} M) were sufficient to induce GFP expression, while 10-fold and 1000-fold higher concentrations of Q4R7 and G4 peptides, respectively, were required to do so (Figure 1A, B), consistent with previous reports (13, 18). Although all OT1 T cells upregulate GFP at high doses of G4 peptide (10^{-6} M), GFP fluorescence intensity among responding cells remains low due to lower peptide affinity (Figure 1A, C).

We next sought to determine whether a minimal TCR signaling threshold *in vivo* was influenced by peptide affinity. To do so, we adoptively transferred OTI-Nur77-eGFP CD8 T cells that had been loaded with the dilutional dye CellTrace Violet into CD45.1+ congenic hosts. We subsequently adoptively transferred APCs loaded with a fixed concentration of either N4, Q4R7, or G4 peptides, selected based on *in vitro* titration, and recipient spleens were analyzed three days later. Low affinity G4 peptide did not induce either GFP upregulation or proliferation at the concentration used, while N4 robustly drove all transferred OTI cells to divide (Figure 1D). Intermediate affinity peptide Q4R7 induced GFP upregulation even among undivided OTI cells, and drove a small fraction of those cells to undergo 1–3 divisions. Importantly, despite the vast difference in potency between N4 and Q4R7, both peptides drive high GFP expression in proliferating OTI cells (Figure 1E, F). This is consistent with a high minimal threshold of accumulated TCR signaling that is required for T cell proliferation and is independent of peptide affinity. Interestingly, GFP MFI decreased with sequential divisions, but not by 50% as would be predicted by simple 2-fold dilution as seen with CellTrace Violet (Figure 1F). This result suggests that cells may receive ongoing TCR signals during early cell divisions *in vivo*.

IL-2 reduces the minimal threshold of TCR signaling required for OTI CD8 T cell proliferation

We showed previously that the threshold for CD4 T cell proliferation *in vitro* is not altered by neutralization or addition of exogenous IL-2 (7). CD8 T cells, in contrast to CD4 T cells, produce lower amounts of autocrine IL-2 and are exquisitely sensitive to IL-2 for proliferative responses (2, 19, 20). We therefore wanted to probe the effect of IL-2 on the minimal TCR signaling threshold for CD8 T cell proliferation. Purified OTI-Nur77-eGFP CD8 T cells loaded with CellTrace Violet were incubated *in vitro* with APCs loaded with varying concentrations of N4, Q4R7, or G4 peptides. Cells were cultured in the presence or absence of 50 U/ml IL-2, a concentration that maximally amplifies proliferative responses of OTI cell cultures (2). After 24 hours, we observed peptide affinity- and dose-dependent eGFP induction that was unaffected by exogenous IL-2 (Figure S1A). This is consistent with previous work suggesting that Nur77-eGFP reporter expression is insensitive to IL-2-dependent JAK-STAT signaling (4, 7). Upregulation of the high affinity IL-2Rα chain (CD25) was sensitive to both peptide concentration and, somewhat, to IL-2 treatment as expected (Figure S1B)(21).

We assessed CellTrace Violet dilution after 72 hours and quantified proliferation by “division index” (average number of cell divisions undergone by the entire population) and “% divided” (fraction of initial population estimated to have divided at least once) to capture information about both the responder population and the amplitude of the response under these conditions (Figures 2A, S1C)(17). Addition of IL-2 to cultures reduced the peptide EC_{50} for these indices, and this effect was most evident at low peptide doses and with low affinity peptide (Figures 2A, S1C).

Although GFP expression in OT1 T cells varied broadly across several orders of magnitude in proportion to stimulus intensity after 24 hours of stimulation before any cell divisions have occurred (Figure 1A, B, S1A), in the absence of exogenous IL-2, GFP expression

among divided cells was consistently high regardless of peptide dose and affinity (Figure 2B, C, S1D, E). Under no stimulatory conditions, in the absence of IL-2, did we detect any T cell that had undergone 1 cell division with GFP fluorescence substantially lower than 10,000 (arbitrary fluorescence units), suggesting that a minimal threshold of integrated TCR signaling essential for proliferation corresponded to this value (Figure 2B, C, S1D, E). Importantly, high doses of N4 or Q4R7 drove GFP expression above the perceived threshold among divided cells, suggesting that this minimal signaling threshold could be superseded, and might in turn account for subtle differences in GFP observed *in vivo* (Figures 1E, 2B, C, S1D, E). By contrast, CD25 expression in divided cells correlated with peptide dose and affinity, but not with proliferation *per se*, suggesting that CD25 expression did not impose a minimal boundary condition (Figure S1D).

We next wanted to explore the impact of exogenous IL-2 on the minimal TCR signaling threshold required for CD8 T cell proliferation. Low dose IL-2 drives minimal proliferation in the absence of peptide stimulation (<1% dividing cells), but synergistically enhances proliferation of T cells stimulated with suboptimal peptide dose and/or affinity (Figure 2A, B, S1C, D). Importantly, in IL-2 treated cultures with low affinity and/or low doses of peptide, we observed division of T cells with markedly lower GFP expression relative to matched cultures without IL-2 supplementation (closer to 2,000 MFI) (Figure 2B, C, S1D, E). As before, T cells from cultures treated with high doses and/or high affinity peptide expressed high GFP well above this minimal threshold (Figure 2B, C, S1D, E). Since IL-2 signaling does not directly modulate GFP expression (Figure S1A), we reasoned that this was due to a reduced “minimum” of integrated TCR signaling required to drive cell division in the presence of IL-2.

IL-15 also reduces the minimal threshold of TCR signaling required for OTI CD8 T cell proliferation, while treatment with Jak3 inhibition increases it

CD8 T cells can produce IL-2 in response to TCR stimulation sufficient to drive quorum-sensing behavior (22). To isolate the contribution of autocrine IL-2 production on the minimal TCR signaling threshold required for CD8 T cell proliferation, we took advantage of a novel and specific JAK3 inhibitor (15). We incubated CellTrace Violet-loaded Nur77-eGFP OTI T cells together with APCs pulsed with varying concentrations of N4 and treated the cultures with either inhibitor or DMSO. Indeed, inhibitor-treated samples exhibit reduced CD25 expression consistent with inhibition of IL-2-dependent signaling, and concomitant increase in GFP expression among divided lymphocytes (Figure 3A, B). This shows that even autocrine production of IL-2 by CD8 T cells is sufficient to reduce the minimal TCR signaling threshold required for proliferation.

We next wanted to determine whether another critical common gamma chain cytokine, IL-15, could also alter the minimal TCR signaling threshold for CD8 T cell proliferation. Like IL-2, IL-15 also signals through the common gamma chain, JAK1/3, and STAT5 to drive T cell proliferation (3). Importantly, the IL-15Rα chain is expressed on naïve T cells along with the common gamma chain (3). We incubated CellTrace Violet-loaded Nur77-eGFP OTI T cells together with APCs pulsed with varying concentrations of N4 in the presence or absence of low doses of IL-2 or IL-15. As expected, IL-2 and IL-15 could

enhance T cell proliferation at suboptimal doses of N4 (Figure 3C). Under these conditions, we observed reduced GFP expression among dividing T cells, suggesting that IL-15 and IL-2 could both reduce the minimal amount of integrated TCR signaling required for proliferation.

IL-2 does not modulate the minimal TCR signaling threshold for CD4 T cell proliferation

In light of observations with the OTI model system (Figure 2), we hypothesized that analogous modulation of the minimal TCR signaling threshold for proliferation by IL-2 might be revealed in an MHC class-II restricted system using low affinity peptides. To test this hypothesis, we took advantage of the AND TCR Tg specific for moth cytochrome c peptide (MCC) and a series of altered peptide ligands with varying potency (11, 12, 14). AND Tg CD4 T cells harboring the Nur77-eGFP reporter were incubated with APCs that had been loaded with varying concentrations of high, medium, or low potency peptides (MCC, K99E, or T102L respectively). After 24 hours of culture with addition of either exogenous IL-2 or anti-IL-2 neutralizing antibodies (to block autocrine effects of endogenously produced IL-2), we observed a dose response of GFP induction that corresponded to peptide potency, such that MCC was approximately 10-fold more potent than K99E, and 1000-fold more potent than T102L (Figure 4A). Consistent with prior observations, IL-2 induced CD25 expression (most evident with weaker potency peptides), but did not potentiate GFP (Figure 4A, B).

After 72 hours of culture, we observed clear titration of proliferative responses across dose and affinity of peptide (Figure 4C). Moreover, GFP expression in undivided T cells varied with dose and peptide affinity as seen after 24 hours of culture. However, among divided cells, GFP expression was relatively uniform and high irrespective of peptide affinity or concentration, confirming in an independent model system the existence of a high and invariant minimal TCR signaling threshold for proliferation (Figure 4C–E). Most strikingly, addition of IL-2 to these cultures markedly upregulated CD25 as expected, but did not alter the high GFP threshold for proliferation, even with low peptide concentration or weak peptide affinity (Figure 4C–F).

We next wanted to determine whether insensitivity of the TCR signaling threshold to IL-2 in CD4 T cells could be observed in an independent system. We therefore took advantage of OTII OVA-specific TCR Tg mice harboring the Nur77-eGFP reporter. We incubated CellTrace Violet-loaded lymphocytes from OTII-Nur77-eGFP mice with vaxrying concentrations of OVA peptide in the presence of either 50 U/ml exogenous IL-2 or anti-IL-2 neutralizing antibodies. We found that anti-IL-2 treatment profoundly reduced surface expression of CD25 and inhibited proliferation on a population level, particularly at near-threshold doses of OVA peptide (Figure S2A–C). However, among proliferating OTII T cells, GFP expression was high and invariant on a single cell level for any given cell division, regardless of IL-2 signaling (Figure S2A–C), consistent with observations in the AND Tg model system (Figure 4C–E). Taken together, these data suggest that IL-2 treatment modulates the TCR signaling threshold for proliferation of CD8 (OTI) but not CD4 (AND, OTII) T cells.

CD28 co-stimulation does not modulate the minimal TCR signaling threshold for T cell proliferation independently of IL-2

We have shown that the minimal TCR signaling threshold required for T cell proliferation is independent of antigen dose and affinity. In order to dissect the role of CD28 co-stimulation in regulating this threshold, we studied polyclonal Nur77-eGFP reporter T cells stimulated with plate-bound anti-CD3ε with or without concomitant anti-CD28 stimulation. We further treated cultures with either 50 U/ml IL-2 or anti-IL-2 neutralizing antibodies to isolate the contribution of CD28 signaling independent of autocrine IL-2 production. We again observed that in the presence of IL-2, the TCR signaling threshold for proliferation was reduced in CD8, but not CD4 T cells, suggesting that this difference is generalizable across a range of TCR specificities (Figure S2D–F). However, we found that CD28 costimulation, while enhancing proliferation, had no impact on this threshold independent of IL-2 (Figure S2D). Furthermore, IL-2 can modulate this threshold in the presence or absence of CD28 co-stimulation. Finally, we confirmed the biological activity of reagents used to modulate IL-2 by assessing CD25 expression in cultured T cells (Figure S2G, H).

Naïve CD8 and CD4 T cells are unresponsive to low doses of IL-2

To understand why proliferation of CD8 and CD4 T cells were differentially responsive to IL-2, we first assessed IL-2 signaling competence in naïve OTI and OTII T cells. Consistent with the lack of high affinity IL-2 receptor complex expression (comprised of IL-2Rα, β, and γ) (Figure 5A)(21, 23), neither naïve OTI nor naïve OTII T cells phosphorylated STAT5 in response to the low dose of IL-2 used in our proliferation assays (Figures 5B, C). In contrast to IL-2 stimulation, low doses of the common gamma chain cytokine IL-7 drove STAT5 phosphorylation in both naïve CD4 and CD8 T cells at a comparable EC_{50}, albeit to different maximal levels (Figure 5D, E). This argues that the common gamma chain is expressed and not limiting for signaling, consistent with prior reports (23). Higher doses of IL-2 (>500 U/mL) stimulate the intermediate affinity IL-2 receptor complex of IL-2Rβ (CD122) and IL-2Rγ (CD132)(21), which we observed when stimulating naïve CD8 OTI T-cells with IL-2 doses over 300 U/mL. However, naïve CD4 OTII T cells did not respond even to extremely high doses of IL-2, which may reflect minimal expression of CD122, in contrast to naïve OTI cells (Figure 5A), as previously reported (23). Taken together, these data suggest that neither naïve CD4s nor naïve CD8s can respond to the dose of IL-2 used in our proliferation assay, and presumably require upregulation of CD25 (as well as CD122 in CD4 T cells) expression to do so.

Activated CD8 and CD4 T cells exhibit differential capacity to signal in response to low dose IL-2

We next asked whether induction of CD25 or signaling through the high affinity IL-2 receptor complex differed in activated CD4 and CD8 T cells. We stimulated polyclonal CD4 and CD8 T cells with plate-bound anti-CD3ε for 24 hours, and assessed IL-2 receptor chain expression. CD25 and CD132 were robustly induced in both cell types at both the transcript and protein levels (Figure 6A, S3A–C). By contrast, although *il2rb* transcript was highly induced by TCR stimulation in both cell types, surface expression of CD122 (IL-2Rβ) was not upregulated in CD8 T cells. Although CD122 surface expression was slightly increased

by TCR ligation in CD4 T cells, it remained reproducibly lower than in CD8 T cells (Figure 6A, S3A–C). Although it remains possible that CD122 expression remains limiting on activated CD4 T cells, both activated CD4 and CD8 T cells express the components of the high affinity IL-2 receptor.

We re-stimulated activated CD4 and CD8 T cells with a broad range of IL-2 doses, and observed high basal and robust inducible STAT5 phosphorylation among $CD25^{hi}$ CD8 T cells even at low IL-2 doses. $CD25^{lo}$ CD8 T cells also responded, but at doses of 200 U/ml or higher (Figure 6B–D). By contrast, IL-2 treatment treatment induced only weak STAT5 phosphorylation in a subset of $CD25^{hi}$CD4 T-cells. We assessed phosphorylation of Akt at Ser473 in response to titration of IL-2, and observed a very similar pattern of response (Figure S3D–F). We propose that the distinct IL-2 response in CD4 and CD8 T cells at early time points (≥ 24 hours of culture) may account for the differential sensitivity of the TCR signaling threshold to cytokine supplementation in these cell types.

Since activated CD4 and CD8 T cells both express the high affinity IL-2 receptor chains, we next explored whether differential expression of a negative regulator of JAK-STAT signaling could account for distinct sensitivity to IL-2. We assessed expression of the SOCS transcripts, *socs1, 2, 3*, and *cish* in naïve and TCR-stimulated CD4 and CD8 T cells (Figure S3G, H). *Socs1, 2, and 3* mRNA levels are downregulated by TCR stimulation, while *cish* mRNA is robustly induced. However, we found no marked differences in transcript expression in activated CD4 and CD8 T cells. It remains possible that CD4 and CD8 T cells exhibit post-transcriptional differences in abundance or activity of these gene products.

c-Myc protein is cooperatively regulated by TCR and IL-2 signals

Because Nur77-eGFP reporter expression is sensitive to TCR, but not JAK-STAT signaling, we can visualize antigen-dependent signals in the context of various cytokine milieus on a single cell level. This approach has revealed that co-stimulatory signals through IL-2 in CD8 T cells but not CD4 T cells markedly reduce the minimal threshold of antigen receptor signaling required for cell division on a single cell level. We next wanted to identify a regulator of cell division that was cooperatively controlled by multiple mitogenic inputs and might mark a truly invariant mitogenic signaling threshold for cell division. *Nr4a* genes are among a small set of rapidly-induced primary response genes (PRGs) that are regulated by multiple mitogenic inputs(24). Among these PRGs are the well-studied cell cycle regulators c-Myc, c-Fos, and JunB. Cantrell and colleagues have recently shown that c-Myc is induced in a digital manner by TCR signals in OTI T cells via transcriptional regulation, but argue that IL-2 markedly increases c-Myc protein expression in a graded manner by enhancing protein translation (25). We focused attention on c-Myc as a likely integrator of TCR and IL-2-dependent signaling because it drives metabolic reprogramming and cell cycle progression that is essential for rapid clonal expansion (26–28).

Intracellular staining enables simultaneous single cell analysis of c-Myc protein expression and Nur77-eGFP expression in a heterogeneous population of proliferating lymphocytes stimulated with plate-bound anti-CD3ε. We observed initial c-Myc induction in a small subset of CD4 and CD8 T cells as early as 2 hours after stimulation, with the proportion of c-Myc+ cells increasing over the next several hours (Figure 7A, B). The distributions of GFP

and c-Myc expression in stimulated T cells were each bimodal and tightly correlated (Figure 7A, B).

We next sought to determine whether regulation of c-Myc expression reflected integration of both TCR and IL-2 signaling, and under what conditions this could be observed. Induction of c-Myc in a sub-population of OTI T cells was detectable after 4 hours of stimulation, and the MFI varied correspondingly with peptide dose and affinity, (Figure 7C–E). As expected, low dose IL-2 treatment had no effect on c-Myc induction after 4 hours of stimulation as CD25 upregulation is essential and occurs significantly later (Figure 7E). At later time points (24 and 48 hours), c-Myc protein expression was not sustained in cultures treated with peptide alone, except with high doses of high affinity of N4 peptide (Figure 7E). Upon addition of IL-2 to these cultures, high c-Myc protein expression was maintained at later time points (Figure 7E). Of note, IL-2 induced CD25 upregulation at 24 and 48 hours as expected (Figure 7E), and c-Myc protein expression strongly correlated with CD25 expression (Figure S4A). This pattern bears a striking resemblance to stimulatory conditions under which IL-2 enhances proliferative responses. These observations suggest that c-Myc protein expression in CD8 T cells is sustained by IL-2 and accounts for the reduced TCR signaling threshold for proliferation (Figure 2B, C).

c-Myc protein expression marks an invariant minimal threshold of mitogenic stimulation required for lymphocyte proliferation

We hypothesized that c-Myc was representative of a gene expression program that integrates antigen and costimulatory inputs to impose a minimal mitogenic signaling threshold for proliferation on a single cell level. We sought to determine whether c-Myc protein expression marked an “invariant” minimal mitogenic signaling threshold among proliferating OTI T cells cultured with a broad range of peptide dose and affinity, in the presence or absence of IL-2. As we reported earlier, we again observed markedly enhanced proliferation in the presence of IL-2 at low dose and low affinity peptide conditions, with a concurrently reduced expression of GFP in dividing cells (Figure 8A, B). Like GFP, c-Myc expression could be induced to supra-threshold levels by high affinity, high dose peptide. Most importantly, in G4-treated samples with weak antigen stimulation, c-Myc expression did not drop below an invariant minimal threshold among dividing CD8 T cells irrespective of IL-2 supplementation (Figure 8A, C).

To confirm that our observations were not specific to the OT1 TCR transgenic model system, we assessed Nur77-eGFP and c-Myc expression in proliferating polyclonal CD8 T cells cultured in the presence or absence of exogenous low dose IL-2. IL-2 supplementation allowed T cells with low integrated TCR signaling as read out by GFP expression to enter the first division, but all cells in the first division expressed high and invariant levels of c-Myc protein irrespective of IL-2 treatment (Figures S4B–E).

These data suggest that expression of c-Myc, and perhaps coordinately regulated induction of other primary response genes downstream of mitogenic inputs, might function to enforce a minimal threshold of integrated mitogenic signaling and reduce the minimal requirement for antigen receptor signaling in CD8 T cells on a single cell level in the presence of co-stimulation.

DISCUSSION

It is not clear how early biochemical events triggered in lymphocytes by antigen and other stimuli are translated over time into complex cellular responses such as cell division that require hours or days, rather than minutes. We explore how lymphocyte proliferation is regulated in response to cumulative TCR signaling integrated over prolonged periods of time. We show here, and in prior work, that Nur77-eGFP reporter expression – by virtue of its rapid induction and long half-life – serves as a specific and faithful read-out of TCR signaling intensity summed over time in primary lymphocytes (6, 7). This reflects a broad gene expression program that is sensitive to dose and affinity of pMHC stimulation on a single cell level (29). Yet, even uniformly stimulated, genetically identical lymphocytes with a single TCR express a broad distribution of Nur77-eGFP, consistent with stochastic cell-to-cell variation in pMHC contact and signal transduction. Despite this, we have observed a consistent sharp lower “boundary” or minimal “threshold” of cumulative TCR signaling for proliferation, independent of antigen abundance, affinity, or avidity. Although TCR signal strength-dependent thresholds for proliferation (as well as effector functions) have long been postulated (30), the Nur77-eGFP reporter uniquely allows such thresholds to be visualized. We propose that graded population responses to titration of antigenic stimuli are achieved in part by altering the fraction of responding cells that have received sufficient TCR signaling to cross this minimal threshold (**see model** Figure 9). Only the cells in a population that have accumulated sufficient TCR signaling to cross this putative threshold are licensed to divide; conversely, lymphocytes that accumulate sub-threshold amounts of TCR signaling are entirely excluded from participating in proliferative responses.

We further demonstrate using the Nur77-eGFP reporter that the single cell TCR signaling threshold for proliferation is markedly reduced by mitogenic cytokines and signals in CD8 T cells, but not CD4 T cells. We propose that cytokine input disproportionately enhances lymphocyte proliferation in the context of suboptimal antigen abundance and/or potency by permitting individual cells with low accumulated antigen receptor signaling to cross a “reduced” minimal signaling threshold (**see model** Figure 9). Mitogenic cytokines may license low affinity CD8 T cells to mount productive immune responses even in response to low amounts of cumulative AgR signaling that would fall “below threshold” in isolation. Conversely, by sequestering IL-2, Tregs may do the opposite, thereby restricting primary CTL responses by low affinity clones or in response to low abundance antigens (31). Indeed, the Rudensky lab has recently shown that capture of IL-2 by mature Tregs is dispensible for the control of CD4 T cells, but is vital for limiting the activation of CD8 T cells(32). Our observations may help explain why CD8 but not CD4 T cells begin to mount responses to presumably low affinity self-antigens in this context. A model for quorum sensing by T cells that is mediated by secreted IL-2 and modulated by IL-2 sequestration has been proposed to operate at precisely such suboptimal stimulation conditions with low affinity or low abundance antigen (33). Recent work by Altan-Bonnet and colleagues similarly demonstrates *in vitro* bystander activation (or “co-optation”) of low affinity CD8 T cell clones mediated by IL-2 secretion from strongly activated high affinity CD8 T cells (19, 22). Supply of co-stimulatory signals by either CD4 or CD8 T cells could thus alter not only the magnitude but the *quality* of resulting immune responses by recruiting low affinity clones to

participate (30, 34). By contrast, we find that CD4 T cells maintain a high TCR signaling threshold for Ag-dependent proliferation that is not reduced by cytokine input. In this manner, low affinity, self-antigen-responsive CD4 T cells are restrained from clonal expansion via bystander elaboration of cytokines in the context of a physiologic immune response. This could provide a critical added layer of protection against autoimmunity.

Hodgkin and colleagues have proposed a “calculus” of T cell responses in which average number of cell divisions in a population before quiescence or death (“mean division destiny”) sum linearly when TCR and co-stimulatory signals are combined (2). However, this model of linear summation focuses on analysis of strong TCR stimuli (N4 and Q4), which differ in potency only by a factor of 40 (13), and consequently does not capture an important feature of co-stimulatory signals - synergistic effects with weak stimuli. We show that a wider range of peptide affinity and dose unmasks non-linear synergy between IL-2 and suboptimal TCR stimulation. We propose that a vital function of IL-2 is to transform suboptimal stimuli into productive immune responses. We further argue that this effect permits weakly-stimulated lymphocytes to participate in an immune response. In a physiologic context with T cell clones of varying affinity, IL-2 should permit low affinity clones to participate significantly in an immune response from which they would otherwise be excluded.

Mitogenic stimuli in lymphocytes trigger expression of an overlapping set of immediate-early or primary response genes (PRGs), such as *c-myc, c-fos,* and *Jun-B* that are essential to direct cell cycle progression (24). Not surprisingly, protein expression driven by the primary response genes is under exquisite post-transcriptional and post-translational regulation. Recent work has identified a robust layer of post-transcriptional regulation of c-Myc by cytokine signaling (25). We show that expression of c-Myc protein integrates AgR and cytokine inputs in T cells. We suggest that c-Myc protein accumulation may serve as an integrator of mitogenic input, and in turn marks a seemingly invariant minimal threshold essential for initiation of cell division. The Hodgkin group has recently proposed that c-Myc expression declines over time in proliferating lymphocyte populations, and when it drops below a minimal threshold, cell division ceases (35). Similarly, the PRG *c-fos* has been proposed to serve as a “counter” of intermittent TCR signaling events *in vivo*, and phospho-c-Jun is sensitive to strength and duration of TCR signaling, although it is not known whether their expression marks a similar threshold for proliferation (36, 37). *c-myc* and related PRGs, such as *c-fos* and c-*Jun-B*, may collectively enforce such a threshold by directing a broad program of metabolic reprogramming and proliferation (28). Indeed, recent work shows that the amount of c-Myc protein expressed in a CD8 T cell population correlates with proliferative potential (26, 27). It has been proposed that the PI3K pathway links mitogenic inputs with c-Myc and PRG expression, but this remains an open area of investigation (19, 38).

How is a high and invariant minimal threshold of TCR signaling maintained by CD4 but not CD8 T cells in the face of co-stimulatory IL-2? We show that neither CD4 nor CD8 naïve T cells phosphorylate STAT5 in response to low doses of IL-2. However, despite comparable strong CD25 upregulation on activated CD4 and CD8 T cells after 24 hours of stimulation, only CD8 but not CD4 T cells robustly phosphorylate STAT5 at this time point. Further, this

difference is not attributable to differences in CD132 expression at this time point. Since CD4 T cells can respond sensitively to IL-2 by 72 hours as evidenced by increased expression of CD25 at that time point, we propose that CD4 T cells acquire IL-2 responsiveness more slowly than CD8 T cells, perhaps because of limiting expression of CD122, or differential expression of a regulator of JAK-dependent signaling. We do not identify differential expression of *socs* gene transcripts in CD4 as compared to CD8 T cells, but it remains possible that post-transcriptional differences in abundance or activity of these gene products may contribute to our observations. Such a wiring scheme would prolong the duration of TCR-dependent regulation of CD4 T cell proliferation, and thereby impose a high TCR signaling threshold on CD4 T cells that is not modulated by mitogenic cytokine supplementation (Figure 9).

Taken together, our model provides a conceptual framework for understanding synergistic regulation of clonal expansion by “sub-threshold” antigen receptor signaling in the context of mitogenic co-stimulatory signals, thereby rendering CD8 T cells exquisitely dependent upon environmental cues such as IL-2. By contrast, we show that CD4 T cells maintain an invariant minimal TCR signaling threshold for proliferation that is independent of antigen affinity, abundance, and IL-2 supply, thereby restricting responses to low affinity self-antigens irrespective of IL-2 supply.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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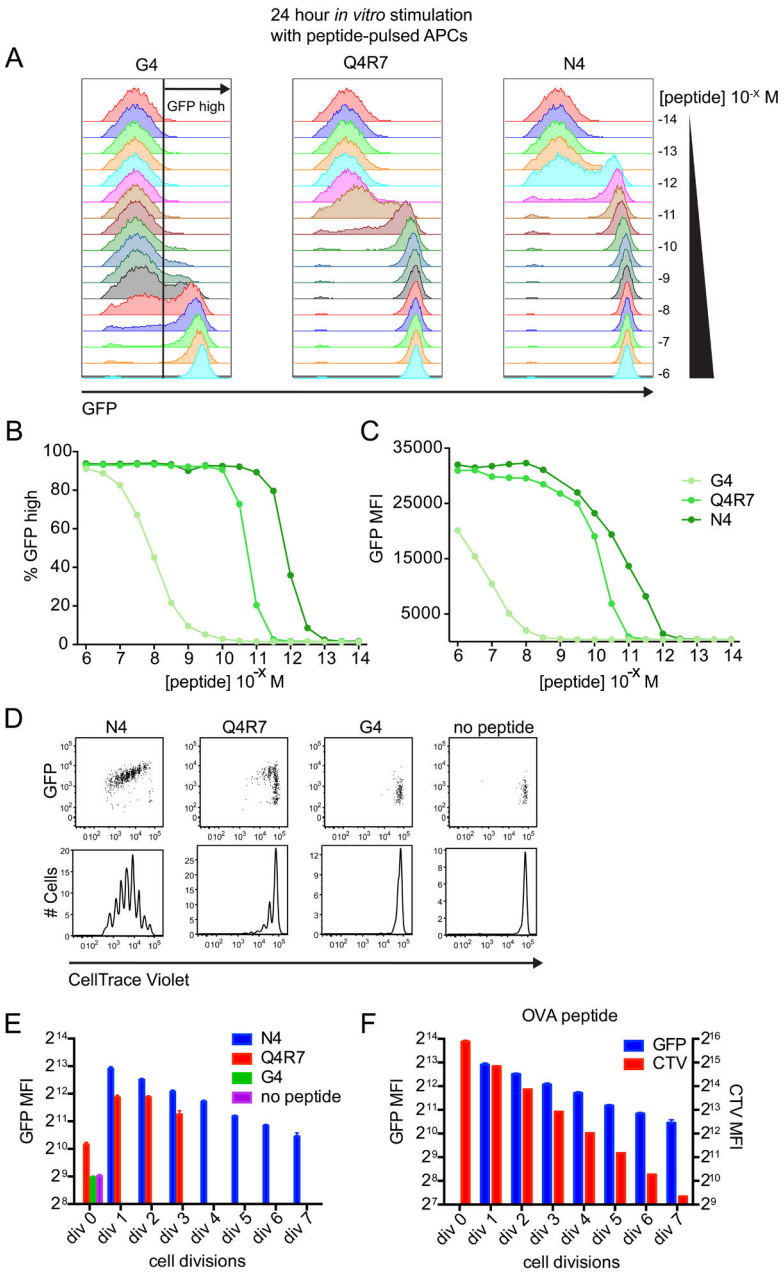

Fig. 1. A sharp TCR signaling threshold for CD8 T cell proliferation is independent of antigen affinity in vivo

A–C. Purified Nur77-eGFP OTI CD8 T cells were incubated in vitro with peptide-pulsed splenic APCs for 24 hours, and GFP expression was assessed by FACS. (A) Histograms depict GFP distribution among stimulated CD8+ T cells. (B) Graphs depict % live CD8 T cells that have upregulated GFP above baseline, as gated above, (C) and GFP MFI in live CD8 T cells. Data is representative of 3 independent experiments.

D–E. Purified CellTrace Violet-loaded Nur77-eGFP OTI CD8 T cells were adoptively transferred into host CD45.1+ BoyJ mice, followed 24 hours later by adoptive transfer of peptide-loaded APCs (pulsed with 10^{-8} M N4, Q4R7, or G4). Splenocytes were harvested

after 72 hours, surface stained to detect TCR Va2, CD45.1, CD45.2, and CD8 expression, and analyzed by FACS to detect GFP expression, CellTrace Violet dilution, and surface marker expression. (D) Plots and histograms depict GFP and CellTrace Violet expression in transferred CD8 T cells. (E, F) Graphs depict quantification of GFP MFI and/or CellTrace Violet (CTV) in CD8 T cells gated on the basis of cell division +/− SEM. Data in D–F reflects 3 biological replicates per peptide condition.

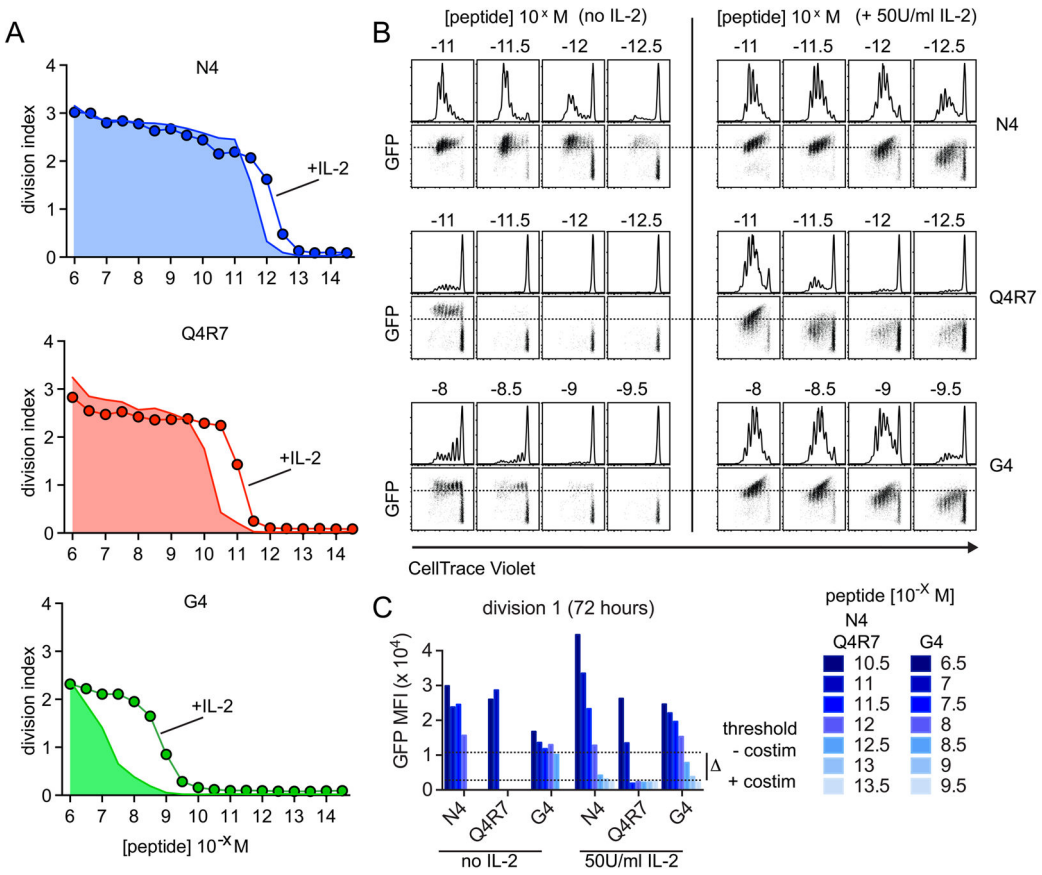

Fig. 2. IL-2 but not antigen affinity or dose modulates the TCR signaling threshold for CD8 T cell proliferation in vitro

A–C. Purified Nur77-eGFP OTI CD8 T cells were loaded with CellTrace Violet and incubated in vitro with peptide-pulsed splenic APCs for 24 hours in the presence or absence of 50U/ml rhIL-2. After 72 hours, cells were stained to detect CD8 and CD25 expression. (A) Graphs depict division index of CD8 T cells cultured with different concentrations of peptides ± IL-2. (B) Plots and histograms depict GFP and CellTrace Violet expression in cultured CD8 T cells. (C) Graph depicts GFP MFI in CD8 T cells that had undergone one cell division at time of harvest under different peptide and cytokine conditions. All data are representative of at least N=3 independent experiments.

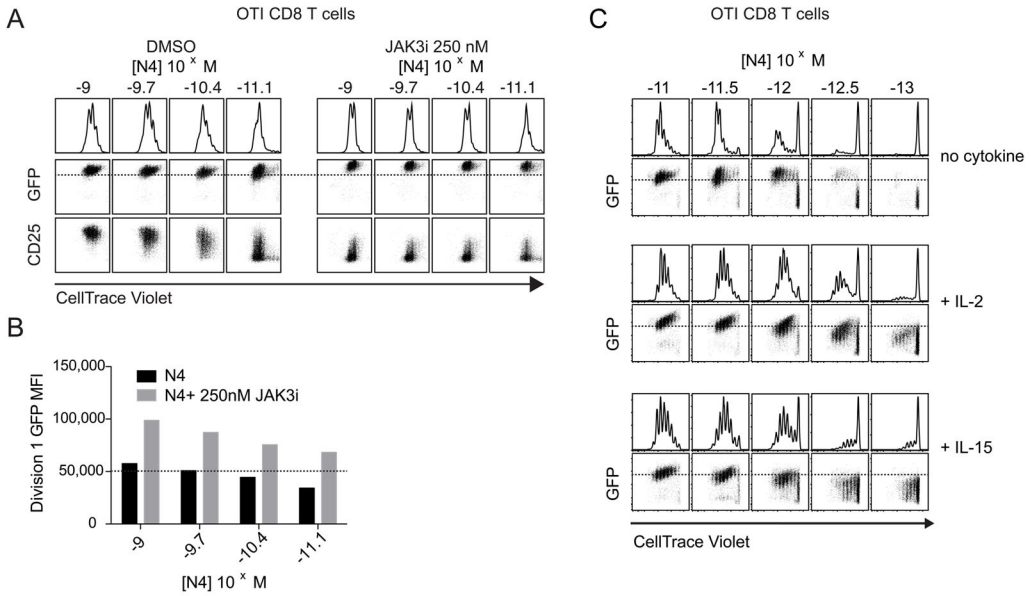

Fig. 3. JAK3 blockade raises the TCR signaling threshold for proliferation, while IL-2 and IL-15 reduce it in CD8 T cells

A, B. Purified OTI-Nur77-GFP T-cells were incubated with peptide pulsed splenocytes from TCRα−/− mice for 3 days in the presence of DMSO or JAK3i (250 nM) and subsequently stained for CD8 and CD25. (A) Plots and histograms depict CellTrace Violet dilution, Nur77GFP and CD25 levels in titrations of N4 peptide. (B) Median Nur77-GFP of cells completing one division. Data are representative of two independent experiments.

C. Purified Nur77-eGFP OTI CD8 T cells were loaded with CellTrace Violet and cultured with peptide-pulsed splenic APCs mice for 72 hours in the presence or absence of 50U/ml rhIL-2, or 100 ng/ml IL-15. Plots and histograms depict GFP, CD25, and CellTrace Violet expression in cultured CD8 T cells. Data are representative of two independent experiments.

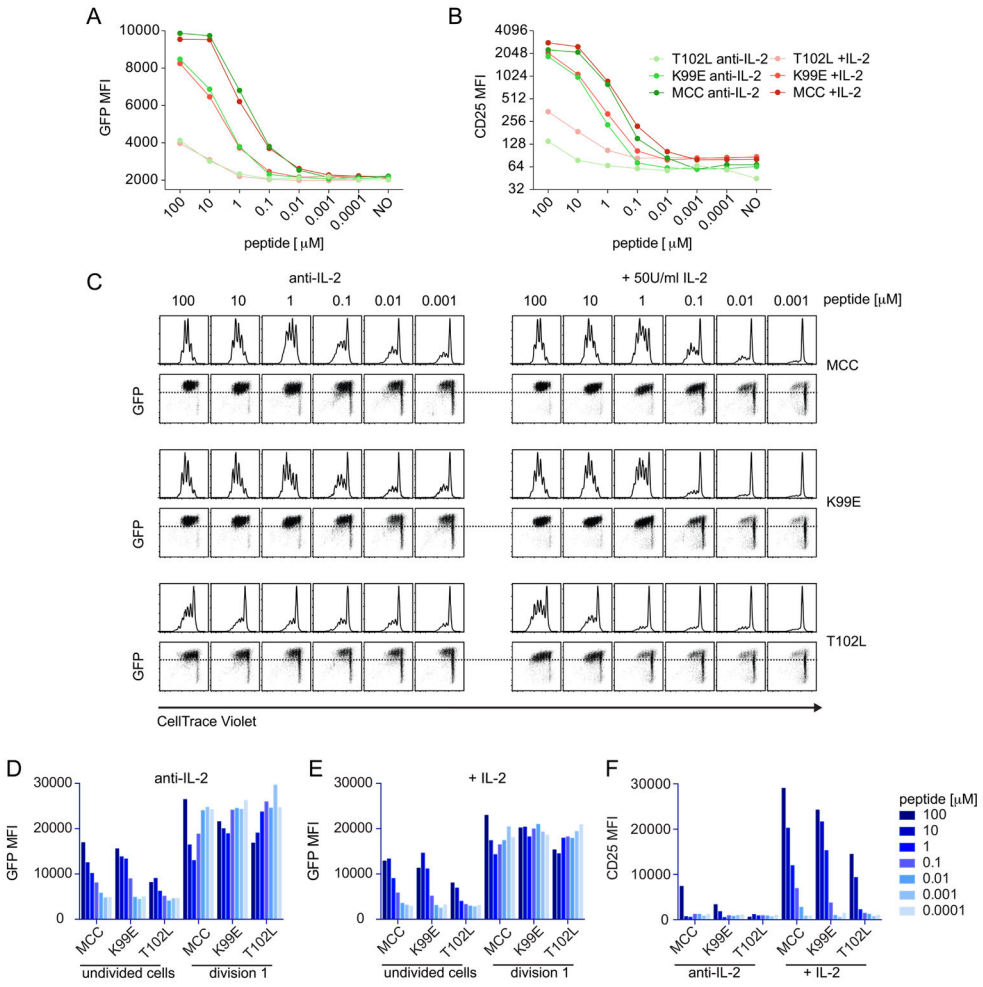

Fig. 4. Neither IL-2 nor antigen affinity modulates the TCR signaling threshold for AND Tg CD4 T cell proliferation in vitro

A, B. Purified Nur77-eGFP AND CD4 T cells were incubated in vitro with peptide-pulsed splenocytes from C3H mice for 24 hours in the presence of either anti-mouse IL-2 or 50U/ml rhIL-2, and subsequently stained to detect CD4 and CD25 expression.

A. Graphs depict GFP or (B) CD25 MFI in live CD8 T cells.

C–F. Purified Nur77-eGFP AND TCR Tg CD4 T cells were loaded with CellTrace Violet and cultured as described above for 72 hours and subsequently stained to detect CD4 and CD25 expression. (C) Plots and histograms depict GFP and CellTrace Violet expression in cultured CD4 T cells. (D, E) Graphs depicts GFP MFI in CD4 T cells that had undergone either no cell divisions or one cell division at time of harvest under different peptide and cytokine conditions. (F) Graph depicts CD25 MFI in bulk CD4 T cells cultured under different peptide conditions and cytokine conditions. All data are representative of at least N=3 independent experiments.

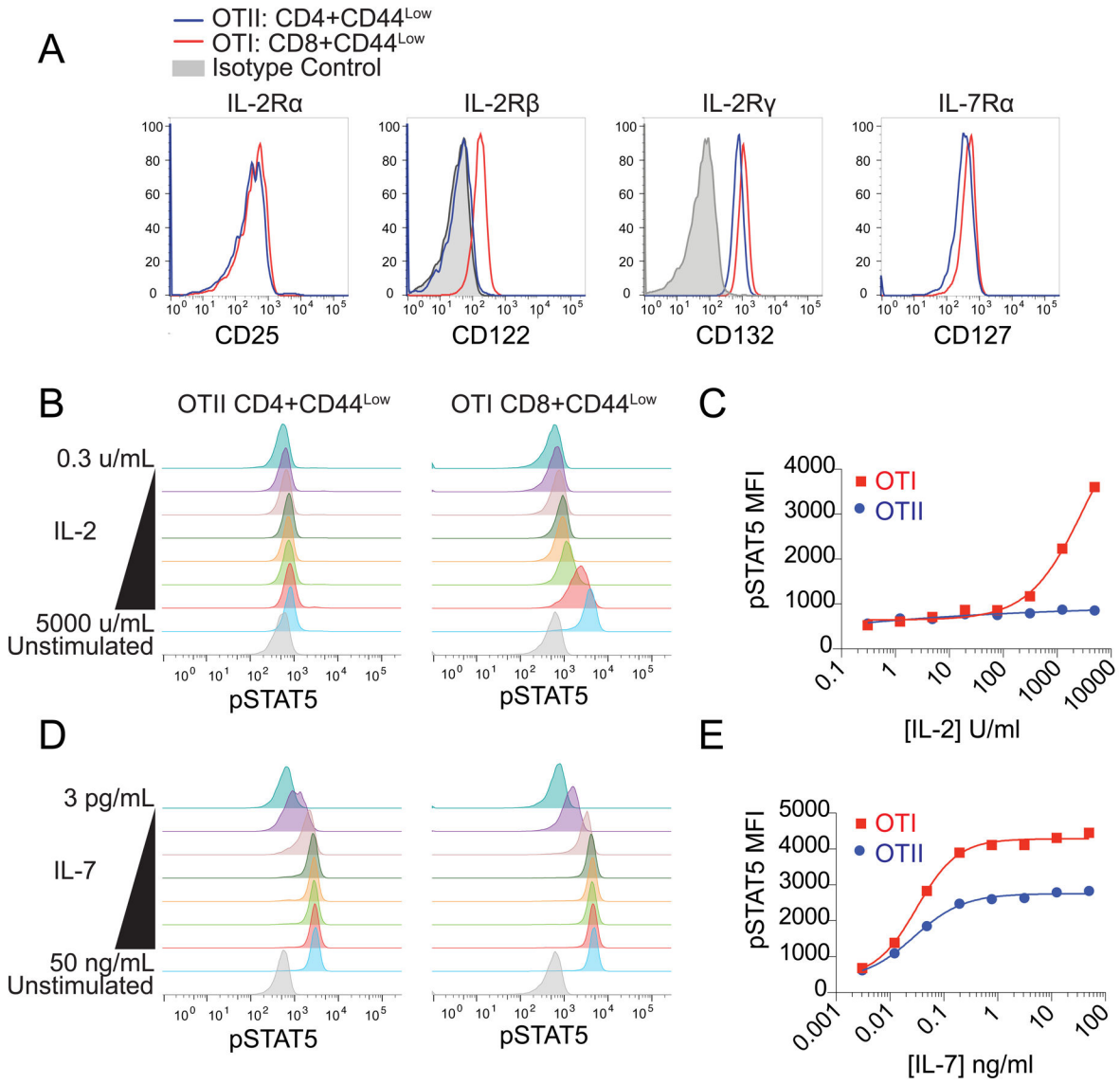

Fig. 5. Naive CD4 and CD8 T cells cannot respond to low dose IL-2

A–E. Purified CD8 and CD4 T cells from OTI and OTII mice respectively were mixed together. (A) Mixed cells were stained to detect CD62LhiCD44low naïve CD8+ (red) and CD4+ T cells (blue) as well as surface expression of CD25, CD122, CD132 and CD127. Gray-shaded histograms represent isotype control. (B–E) Mixed cells were stimulated with a range of IL-2 or IL-7 concentrations for 15 minutes, fixed, and stained to detect intracellular pSTAT5 as well as CD44, Foxp3, CD4 and CD8 expression. (B, D) Histograms represent pSTAT5 expression in naïve CD4 or CD8 T cells gated to exclude Foxp3+ Tregs. (C, E) Graphs depict quantification of pSTAT5 from histograms in (B, D). All data are representative of at least N=2 independent experiments.

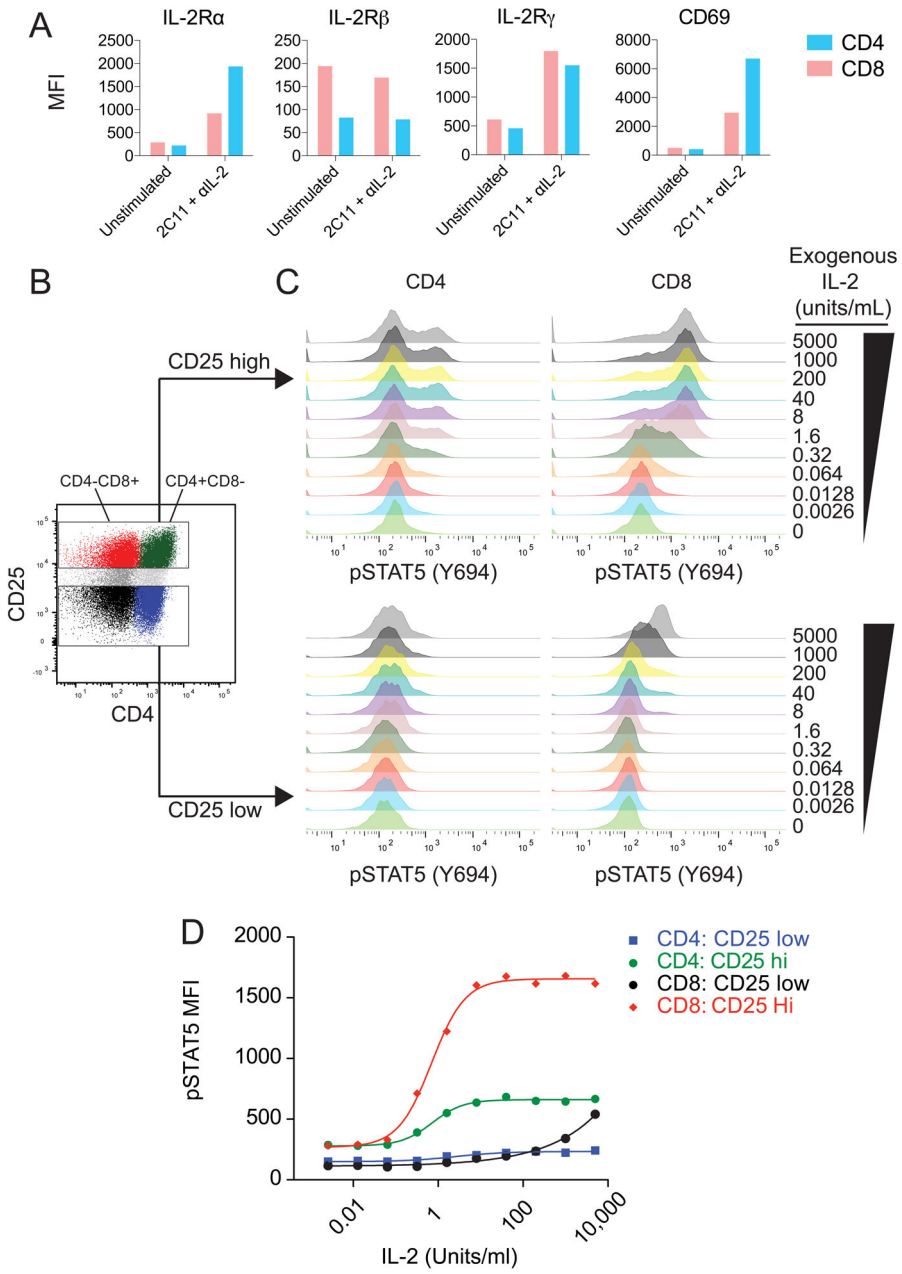

Fig. 6. Activated CD8 and CD4 T cells exhibit differential capacity to signal in response to low dose IL-2

A–D. Purified CD8 and CD4 T cells depleted of Tregs were cultured for 24 hours with or without plate-bound anti-CD3ε 0.5μg/ml and neutralizing anti-IL-2 blocking antibody. (A) Cells were then stained to detect surface expression of CD25, CD122, and CD132. Graphs depict MFI for the IL-2 chains in cells cultured in media alone or after 24 hours of stimulation. B–D. Anti-CD3ε-stimulated cells were mixed, stimulated for 15 minutes with varying doses of rhIL-2, and fixed. Samples were then stained to detect CD25, CD4, CD8 and intra-cellular pSTAT5 expression. (B) Plot depicts gating on CD25 hi and lo populations within the CD4+CD8− and CD4−CD8+ populations. (C) Histograms represent pSTAT5

expression induced by varying doses of IL-2 in gated populations. (D) Graphs depict quantification of pSTAT5 from histograms in (C). All data are representative of at least N=5 independent experiments.

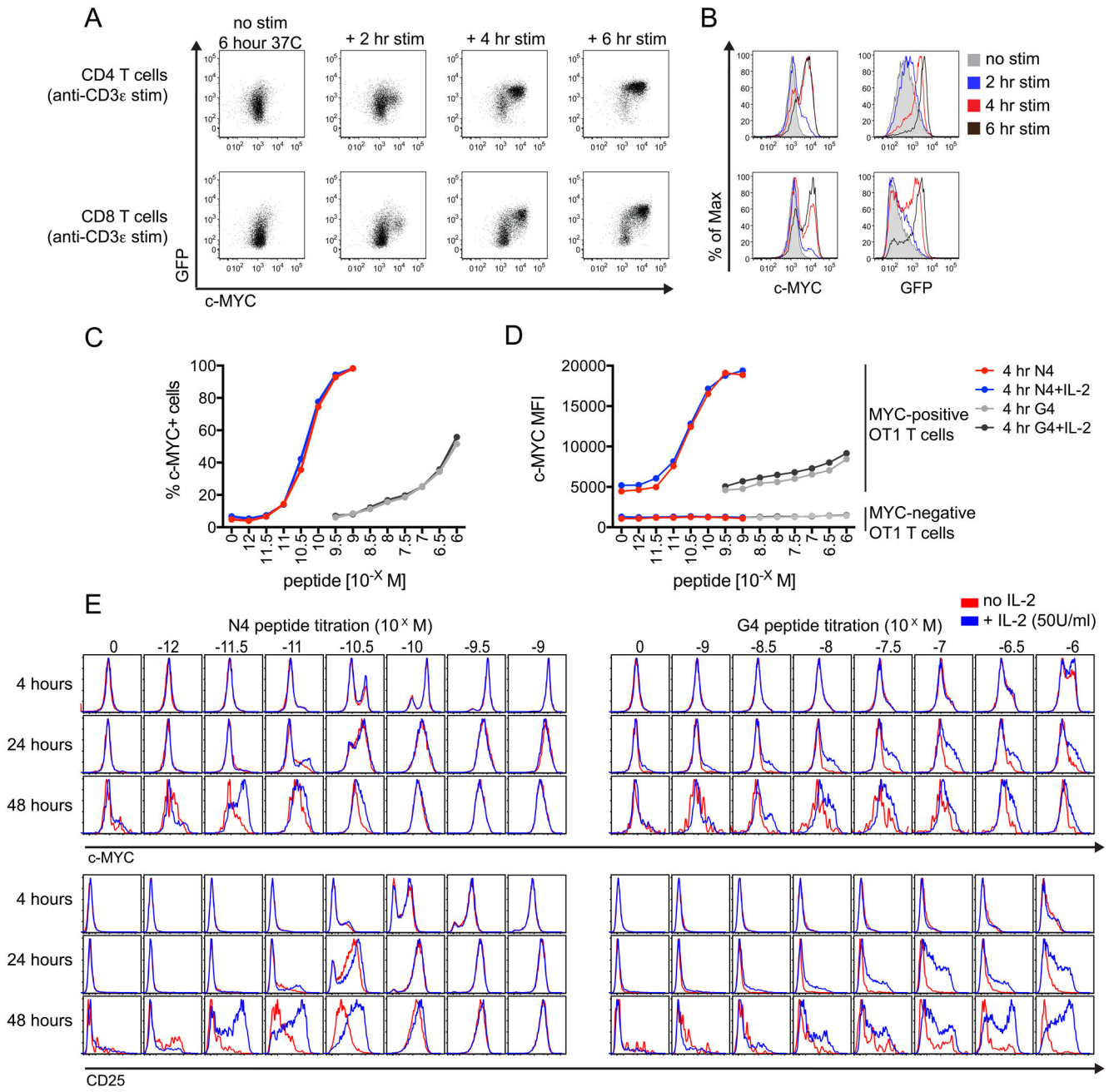

Fig. 7. c-Myc protein is upregulated rapidly in response to antigen receptor signaling, and sustained by IL-2

A, B. Nur77-eGFP splenocytes were stimulated with platebound 5μg/ml anti-CD3e for 0, 2, 4, or 6 hours in vitro, and subsequently fixed, permeabilized, and stained to detect intra-cellular c-Myc protein as well as CD4 and CD8. Plots (A) and histograms (B) depict GFP and c-myc expression in CD4, CD8, and B220+ B cells stimulated as described above. (C–E) OTI lymphocytes were cultured with varying concentrations of N4 or G4 peptides with or without rhIL-2 50U/ml for 4, 24, or 48 hours, and subsequently fixed, permeabilized, and stained to detect intra-cellular c-Myc protein as well as CD8 and CD25 expression. (C)

Graph depicts % CD8 T cells that had upregulated c-Myc protein under various culture conditions after 4 hours. (D) Graph depicts c-Myc MFI in CD8 T cells that had upregulated c-Myc or had not. (E) Histograms depict either c-Myc (top panels) or CD25 surface expression (bottom panels) in CD8 T cells cultured as described above. All data are representative of at least 3 independent experiments.

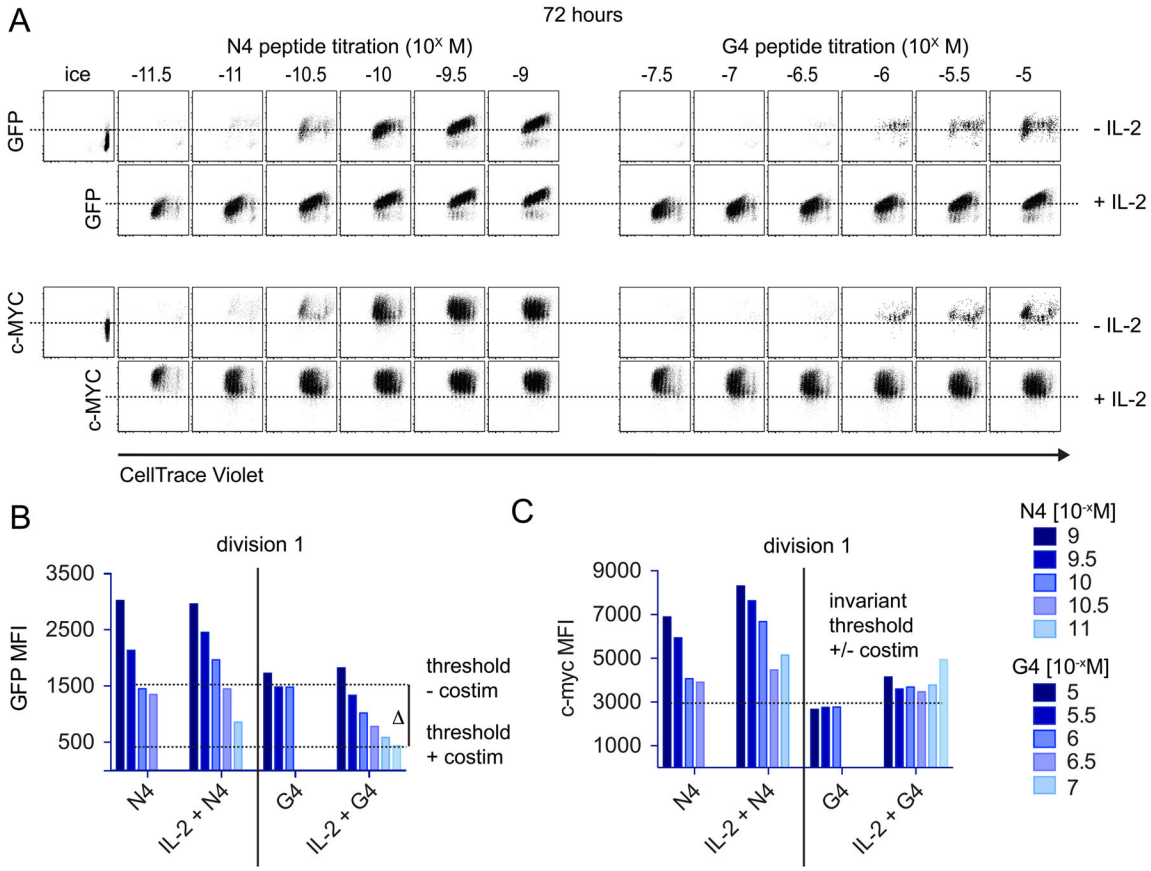

Figure 8. C-Myc expression marks an invariant threshold for T cell proliferation

Nur77-eGFP OTI lymphocytes were loaded with CellTrace Violet and cultured with varying concentrations of N4 or G4 peptides with or without rhIL-2 50U/ml for 72 hours, and subsequently fixed, permeabilized, and stained to detect intra-cellular c-Myc protein as well as CD8 and CD25 expression. (A, B) Plots depict CD25, c-myc, GFP, and/or CellTrace Violet from CD8 T cells cultured as described above. (C, D) Graphs depict GFP MFI (C) or c-Myc MFI (D) in CD8+ T cells that had undergone 1 cell division at time of harvest. All data in this figure are representative of at least 2 independent experiments.

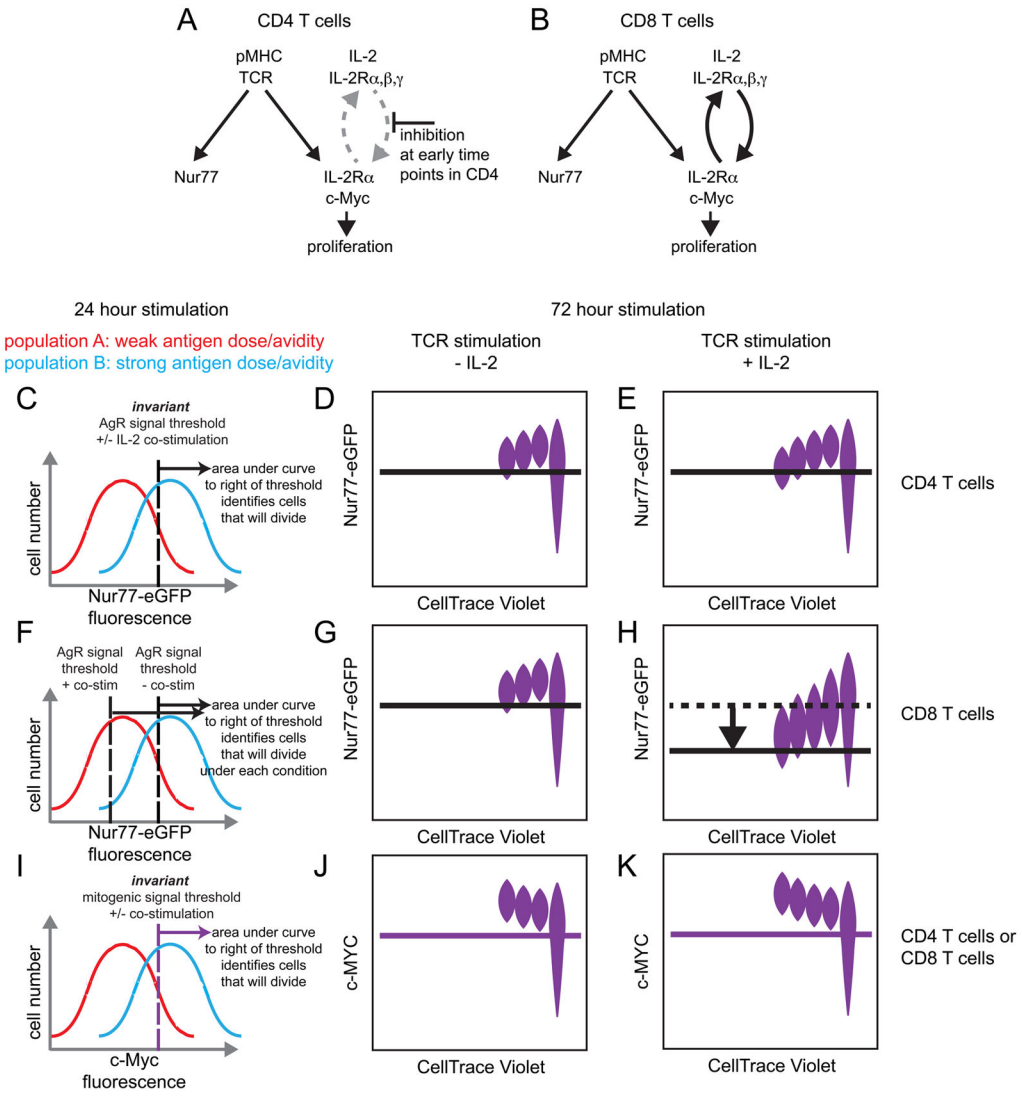

Figure 9. Figure S4. Model of signaling thresholds for antigen-dependent lymphocyte proliferation

A. CD4 T cells upregulate IL-2Rα (CD25) in response to TCR signaling, but at early time points are refractory to low dose IL-2 stimulation despite CD25 expression. Thus c-myc induction and proliferation are dependent upon a robust minimal amount of TCR signaling that is not modulated by the presence of exogenous IL-2. (B) CD8 T cells also upregulate IL-2Rα in response to TCR signaling, and are consequently able to respond to low doses of IL-2, which further boosts IL-2Rα expression. This imposes a positive feedback loop that permits CD8 T cells to mount synergistic proliferative responses to extremely weak TCR stimuli in the presence of IL-2, but remain dependent upon a minimal amount of TCR signal to trigger the positive feedback. (C–E) Because Nur77-eGFP expression is a specific reporter of cumulative AgR signaling but is insensitive to cytokine signaling, (C) CD4 T cell populations treated with either low (red histogram) or high (blue histogram) TCR stimulation express different distribution of Nur77-eGFP after 24 hours irrespective of IL-2 treatment. Our model predicts that only T cells expressing GFP above a minimal threshold

(i.e. only those cells that have integrated cumulative TCR signaling sufficient to drive a minimal amount of reporter expression) are able to proliferate, regardless of peptide or IL-2 input. (D, E) All divided CD4 T cells express GFP above this threshold irrespective of IL-2 supplementation. (A) We argue that this is due to insensitivity of CD4 T cells to low dose IL-2 signaling at early time points despite high CD25 induction. (F–H) CD8 T cells also express low or high amounts of Nur77-eGFP depending upon strength of AgR stimulation, and require high cumulative AgR signaling in order to proliferate. However, because CD8 T cells are responsive at early time points to co-stimuli, the minimal amount of AgR signaling required for proliferation is reduced by co-stimulation. (G, H) This model predicts that under these conditions even lymphocytes expressing low levels of Nur77-eGFP can divide, and is supported by our findings that costimulation indeed results in dividing lymphocytes expressing lower Nur77-eGFP expression. (I–K) Finally, we propose that c-Myc protein expression is regulated by both AgR and cytokine mitogenic inputs, and marks a threshold for minimal cumulative mitogenic stimulation required for proliferation. (J, K) This model predicts that c-Myc protein expression among dividing lymphocytes conforms to a minimal threshold that is independent of modulation of individual input signals, consistent with our observations.