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SHORT COMMUNICATION

IL-12-mediated STAT4 signaling and TCR signal strength cooperate in the induction of CD40L in human and mouse CD8$^+$ T cells

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CD40L is one of the key molecules bridging the activation of specific T cells and the maturation of professional and nonprofessional antigen-presenting cells including B cells. CD4$^+$ T cells have been regarded as the major T-cell subset that expresses CD40L upon cognate activation; however, we demonstrate here that a putative CD8$^+$ helper T-cell subset expressing CD40L is induced in human and murine CD8$^+$ T cells in vitro and in mice immunized with antigen-pulsed dendritic cells. IL-12 and STAT4-mediated signaling was the major instructive cytokine signal boosting the ability of CD8$^+$ T cells to express CD40L both in vitro and in vivo. Additionally, TCR signaling strength modulated CD40L expression in CD8$^+$ T cells after primary differentiation in vitro as well as in vivo. The induction of CD40L in CD8$^+$ T cells regulated by IL-12 and TCR signaling may enable CD8$^+$ T cells to respond autonomously of CD4$^+$ T cells. Thus, we propose that under proinflammatory conditions, a self-sustaining positive feedback loop could facilitate the efficient priming of T cells stimulated by high affinity peptide displaying APCs.

Keywords: CD40L · CD8$^+$ T cells · Costimulatory molecules · STAT4 signaling · TCR

See accompanying Commentary by Nolte and van Lier

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Introduction

The initial encounter of T cells with antigen-presenting cells (APCs) represents an essential primary event in adaptive immune responses. For a successful immune response, this initial priming must lead to fast and efficient differentiation of corresponding pathogen-specific T cells into appropriate effector T-cell types. T-cell differentiation is modulated by a variety of factors: The magnitude of the inflammatory response, the type of APCs presenting the pathogen-derived antigen, and the resulting cytokine milieu [1, 2]. In addition, T-cell differentiation is fundamentally regulated by the strength of the interaction of the T-cell receptor (TCR) with its specific peptide–MHC complex [3–6].

In the case of infection with intracellular pathogens such as viruses and bacteria, naive CD4$^+$ and CD8$^+$ T cells differentiate into proinflammatory Th1 and Tc1 cells, respectively. In this context, APCs presenting pathogen-derived antigens become

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activated by CD40L-expressing CD4$^+$ T cells, leading to IL-12 production that has been proposed to be essential for efficient priming of CD8$^+$ T cells [7, 8].

Previous data suggest that CD8$^+$ T cells can also activate dendritic cells (DCs) to produce IL-12 via CD40L [9]. However, it has remained an open question whether this is a common event and, most importantly, how CD40L expression of CD8$^+$ T cells is induced or modulated in the early phase of an immune response. Since appropriate CD8$^+$ T-cell activation and differentiation critically depends on the cognate TCR peptide–MHC complex interaction and early DC cytokines, we tested whether these two major instructive signals for T-cell differentiation induce and modulate CD40L expression in human and murine CD8$^+$ T cells.

Results and discussion

Induction of murine and human CD40L$^+$ CD8$^+$ T cells is regulated by IL-12

In order to identify signals that induce CD40L expression on CD8$^+$ T cells, we assessed a variety of inflammatory cytokines that are released by DCs during the early phase of an infection for their ability to induce CD40L during primary CD8$^+$ T-cell differentiation in vitro. Human naïve CD8$^+$ T cells were stimulated with αCD3/αCD28 antibodies in the presence of the indicated cytokines and cultured for 6–7 days. CD40L as well as IFN-γ expression was assessed after polyclonal restimulation with PMA/Iono. Among the tested cytokines, IL-2 and IL-12 most efficiently induced CD40L expression in human CD8$^+$ T cells (Fig. 1A) and both together synergistically enhanced CD40L induction (Fig. 1A–C). For naïve murine OT1 CD8$^+$ T cells stimulated with SIINFEKL and APCs, IL-12 was the most prominent inducer of CD40L expression of the early DC cytokines tested (Fig. 1D). In contrast to human cells, IL-2 did not influence CD40L expression on murine CD8$^+$ T cells (Fig. 1E and F).

CD40L was induced in 2–30% of murine CD8$^+$ T cells in the absence of any added cytokines (Fig. 1C and F), but the magnitude of CD40L expression clearly also depended on the stimulation condition. OT1 CD8$^+$ T cells stimulated with αCD3/αCD28 antibodies (Fig. 1G) showed higher CD40L expression in the absence of exogenous IL-12 as compared with stimulation with SIINFEKL and APCs under the same conditions (Fig. 1F). Further, induction of CD40L clearly decreased in non-TCR transgenic naïve CD8$^+$ T cells stimulated with αCD3/αCD28 antibodies (Fig. 1H). These data indicate a role for TCR signaling in the induction of CD40L on CD8$^+$ T cells.

Under all conditions analyzed, addition of IL-12 significantly enhanced CD40L induction on CD8$^+$ T cells. The ability of IL-12 to induce expression of CD40L on CD8$^+$ T cells was dose dependent (Fig. 1I) and more efficient when the IL-12 signal was provided early after activation (Fig. 1J). CD8$^+$ T cells have the intrinsic ability to express CD40L to a certain degree, but we demonstrate that IL-12 is a major instructive signal to enhance CD40L expression in CD8$^+$ T cells after differentiation and reactivation.

STAT4 mediates induction of CD40L expression in CD8$^+$ T cells

The effect of IL-12 in cellular immunity is mainly exerted by the transcription factor STAT4. To assess the role of STAT4 in IL-12-mediated induction of CD40L expression in CD8$^+$ T cells, we primed STAT4^{ko} OT1 CD8$^+$ T cells and WT OT1 CD8$^+$ T cells in vitro with αCD3/αCD28 or SIINFEKL peptide and APCs. We demonstrated that the IL-12-mediated increase of CD40L expression in CD8$^+$ T cells was completely dependent on STAT4, irrespective of stimulation by αCD3/αCD28 (Fig. 2A) or by SIINFEKL peptide (Fig. 2B). In order to evaluate the role of IL-12 for CD40L induction in vivo, we transferred WT OT1 and STAT4^{ko} OT1 CD8$^+$ T cells into WT mice prior to immunization with SIINFEKL-loaded DCs. Even in the absence of STAT4, CD40L expression was still induced in SIINFEKL-specific CD8$^+$ T cells at day 7 after immunization (Fig. 2C). However, the frequencies were significantly reduced as compared with WT OT1 CD8$^+$ T cells (Fig. 2D). Three signals have been proven to be important for the generation of a proper CD8$^+$ T-cell response, namely TCR signaling, costimulation, and inflammatory cytokines like IL-12 [10, 11]. The effect of IL-12 signaling via STAT4 during priming of CD8$^+$ T cells has been shown to augment effector and memory cell generation and enhance survival [12, 13].

Our results support the notion that IL-12, the major instructive cytokine signal for efficient priming of proinflammatory T cells, also promotes the induction of CD40L expression on CD8$^+$ T cells. Since CD40L itself is a major signal for the induction of IL-12 by DCs, CD40L expressed by CD8$^+$ T cells might initiate a positive feedback loop to enhance differentiation of proinflammatory effector cells in the early phase of an immune response.

TCR signaling strength modulates CD40L induction in CD8$^+$ T cells

The increased ability of transgenic OT1 CD8$^+$ T cells to express CD40L compared with that of polyclonal CD8$^+$ T cells, even in the absence of IL-12 as the instructive signal (compare with Fig. 1G and H), prompted us to evaluate the role of TCR signaling strength on the induction of CD40L expression in CD8$^+$ T cells. For variation of TCR signaling strength, we employed a model in which TCR transgenic OT-1 cells are stimulated by different altered peptide ligands (APLs) that induce reduced TCR signaling compared with the high affinity epitope SIINFEKL [14]. Naïve OT1 CD8$^+$ T cells were cultured with different concentrations of the high affinity epitope SIINFEKL (N4) or the lower affinity peptides SIYNFEKL (Y3) or SIITFEKL (T4). The Y3 and T4 variations of SIINFEKL have been shown to be fourfold and 70-fold less potent stimulators of OT1 CD8$^+$ T cells, respectively [14].

The ability of CD8$^+$ T cells to induce CD40L expression was strongly dependent on the TCR signaling strength provided

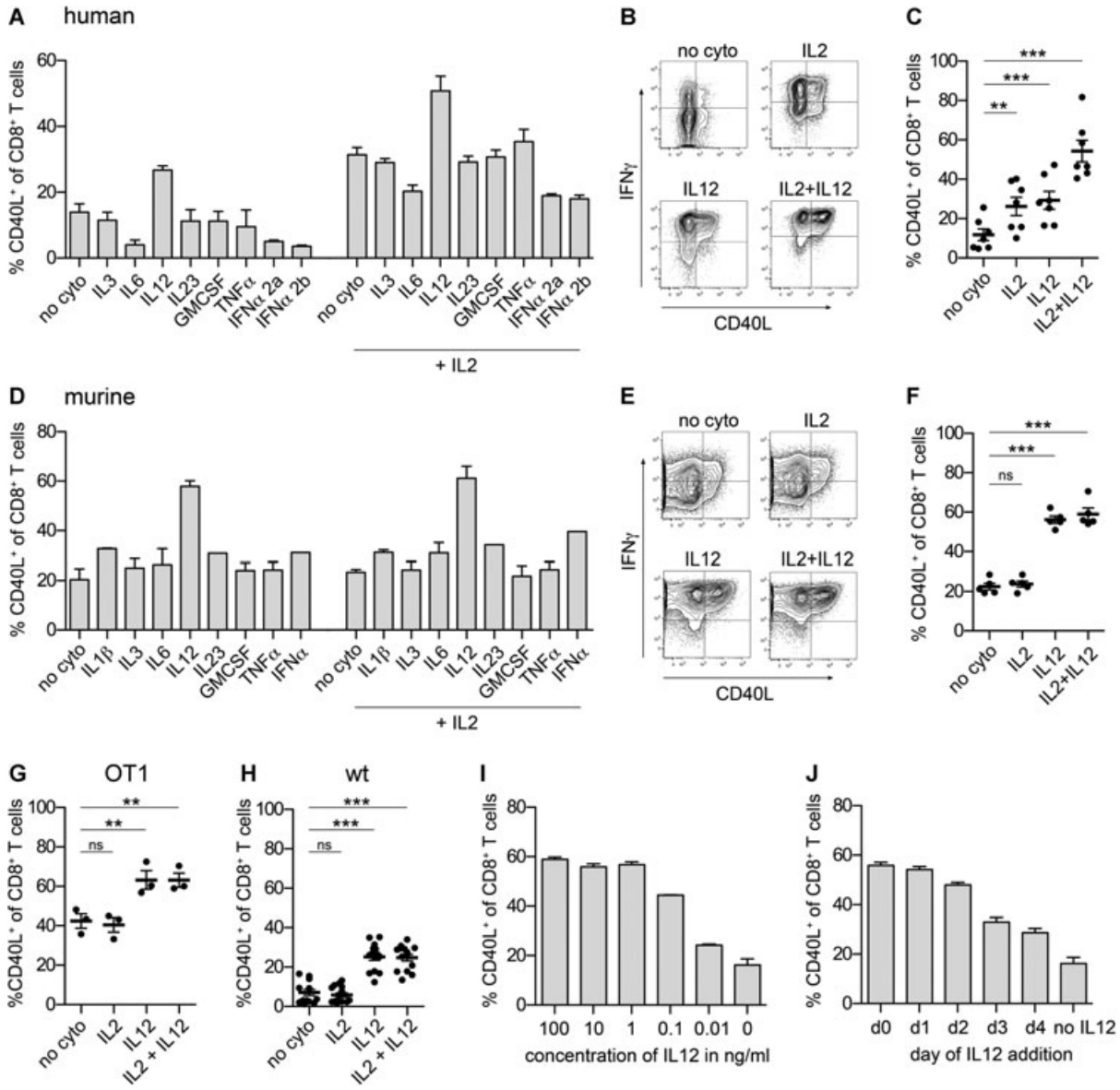

Figure 1. Induction of murine and human CD40L$^+$ CD8$^+$ T cells is regulated by IL-12. Naïve CD8$^+$ T cells were cultured in the presence of cytokines for 6–7 days. Cells were stimulated as indicated and intracellular CD40L expression was analyzed by flow cytometry. (A) Human CD8$^+$ T cells were stimulated with αCD3/αCD28 for 2 days in the presence of early DC cytokines, cultured for 4–5 more days and were than restimulated with PMA/Iono. Data are shown as mean + SEM of two donors and are representative of three experiments performed. (B) Representative dot plots and (C) summary of seven donors are shown as mean ± SEM. (D) Murine OT1 CD8$^+$ T cells were stimulated with SIINFEKL and APCs for 6–7 days and restimulated with SIINFEKL. Data are shown as mean + SEM and are pooled from three experiments performed (E) Representative dot plots and (F) summary of five experiments are shown as mean ± SEM. (G) Murine OT1 CD8$^+$ T cells or (H) wt CD8$^+$ T cells were stimulated with αCD3/αCD28 for 2 days and after culturing for 4–5 more days restimulated with PMA/Iono. Data are shown as mean ± SEM of three experiments and 14 experiments, respectively. (I) Murine OT1 CD8$^+$ T cells were stimulated with SIINFEKL, APCs, and varying concentrations of IL-12 and were restimulated after 6 days with SIINFEKL. (J) Murine OT1 CD8$^+$ T cells were stimulated with SIINFEKL and APCs, IL-12 was added at different time points and cells were restimulated after 6 days with SIINFEKL. (I, J) Data are shown as mean + SEM of two replicates and are representative of three experiments performed. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, ANOVA and Bonferroni's multiple comparison test.

during the primary activation over a broad range of peptide concentrations (Fig. 3A and B). Interestingly, in the same cultures, the induction of IFN-γ$^+$ T cells was not affected (Fig. 3A and Supporting Information Fig. 1). Also, by using decreasing amounts of αCD3 antibody for primary stimulation, an MHC class I independent variation of TCR signal strength, we demonstrated a direct correlation between TCR signal strength and CD40L induction in CD8$^+$ T cells (Fig. 3C). TCR signaling strength modulated the induction of CD40L but not IFN-γ in OT1 CD8$^+$ T cells also in vivo when antigenic challenges were provided by immunizations with DCs pulsed with SIINFEKL or APLs (Fig. 3D and Supporting Information Fig. 2). The decreased ability of STAT4^{ko} OT1 CD8$^+$ T cells to induce CD40L was not only observed when CD8$^+$ T cells were primed with DCs loaded with SIINFEKL but also when using APLs (Fig. 3E). Hence, IL-12 signaling is instructive for CD40L induction in CD8$^+$ T cells also upon weaker TCR signaling.

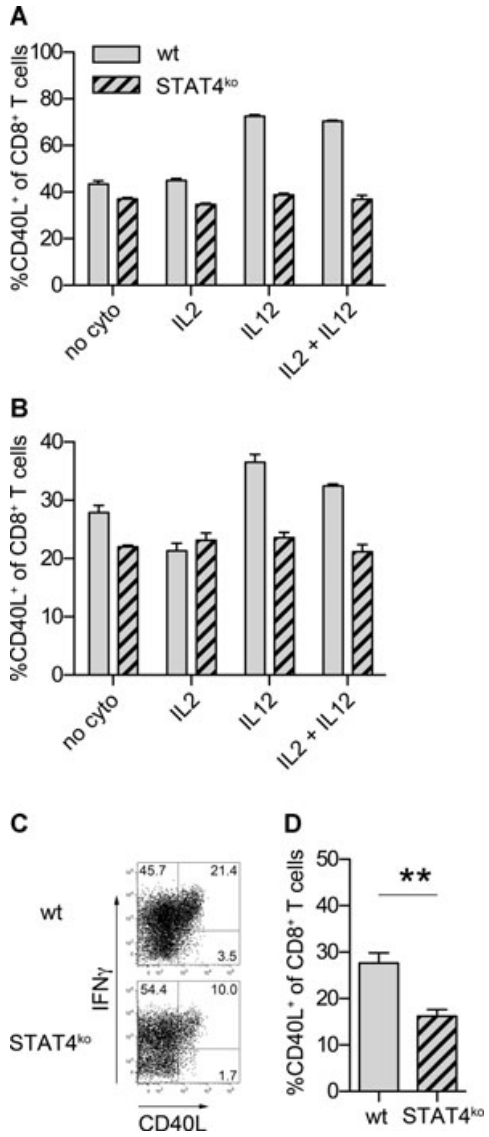

Figure 2. STAT4 mediates induction of CD40L expression in CD8$^+$ T cells (A and B) Naïve murine CD8$^+$ T cells of OT1 or STAT4^{ko} OT1 mice were (A) stimulated with αCD3/αCD28, cultured for 6 days with IL-2 and/ or IL-12 and restimulated with PMA/Iono or (B) stimulated with SIINFEKL and APCs cultured for 6 days with IL-2 and/ or IL-12 and restimulated with SIINFEKL. Subsequently CD40L expression was determined by flow cytometry. Data are shown as mean + SEM of two replicates and are representative of two experiments performed. (C and D) Wt mice were transferred with a mix of equal amounts of naïve CD8$^+$ T cells of OT1 or STAT4^{ko} OT1 mice and 1 day later immunized with SIINFEKL-pulsed DCs. Day 7 post immunization (p.i.) splenocytes were restimulated with SIINFEKL and CD40L induction of OT1 and STAT4^{ko} OT1 CD8$^+$ T cells was analyzed. (C) Representative dot plots and (D) a summary of five mice pooled from two experiments are shown as mean + SEM. $^{**}p<0.01$, paired *t*-test.

IL-12Rβ2 expression and recognition of IL-12 by naïve CD8$^+$ T cells was shown to be enhanced with increased TCR signaling [15], making high affinity CD8$^+$ T cells more susceptible to IL-12 signals. ChIP-on-chip experiments by Good et al. [16] have already demonstrated STAT4 binding to the CD40L promotor. Furthermore, cooperative binding of STAT4 and c-Jun, an important transcription factor downstream of TCR signaling, has been shown to enhance IFN-γ production [17]. Similarly, according to our data, integration of TCR and IL-12 signaling may also result in induction of CD40L on CD8$^+$ T cells.

Although CD40L expression by CD8$^+$ T cells has been reported in selected experimental settings [9, 18–20], CD4$^+$ T-helper cells are regarded as the primary source of CD40L [21, 22], the main mediator of helper functions. Helper functions of CD8$^+$ T cells have been proposed earlier [20] and it was demonstrated that CD8$^+$ T cells expressing CD40L in vitro induce DC maturation and IL-12 secretion [9] but no reports have clarified how such CD8$^+$ T-cell helper function could be induced early following T-cell activation. Our data now reveal that strong TCR signaling and IL-12 increase CD8$^+$ T-cell expression of CD40L after an initial primary activation phase. Accordingly, it is possible that memory/effector CD8$^+$ T cells during a second encounter with the pathogen also increase CD40L expression and thereby may assist to provide sufficient CD40L-mediated activation of APCs in memory recall responses.

Concluding remarks

We here demonstrate that CD40L expression in proinflammatory CD8$^+$ T cells is modulated in vitro and in vivo via STAT4 and TCR signal strength. Accordingly, one can hypothesize that under proinflammatory conditions not only CD4$^+$ but also high affinity CD8$^+$ T cells are able to express CD40L ensuring professional DC licensing. This may result in a self-sustaining positive feedback loop in which CD40L-expressing CD8$^+$ T cells increase IL-12 production by DCs, which in turn would facilitate the differentiation of proinflammatory T cells. Viruses or bacteria can replicate fast and if they successfully penetrate the first line of defense formed by innate immunity mechanisms, one may hypothesize that slow activation and maturation of T cells at an early point during an immune response cannot be risked or such infections would not be contained. Thus the induction of putative CD8$^+$ helper T cells able to activate the immune system after recognition of antigens presented in MHC class I molecules would be beneficial to host defense.

Materials and methods

Human cell preparation and media

All human blood samples were obtained from healthy volunteers after informed consent was provided. PBMCs were separated from heparinized whole blood using a Ficoll-Hypaque (PAA, Pasching, Austria) gradient and were cultured in RPMI 1640 medium (Gibco) supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.3 mg/mL glutamine, and 10% heat-inactivated human AB serum (PAA). All experiments followed protocols approved by Institutional Ethics Committee.

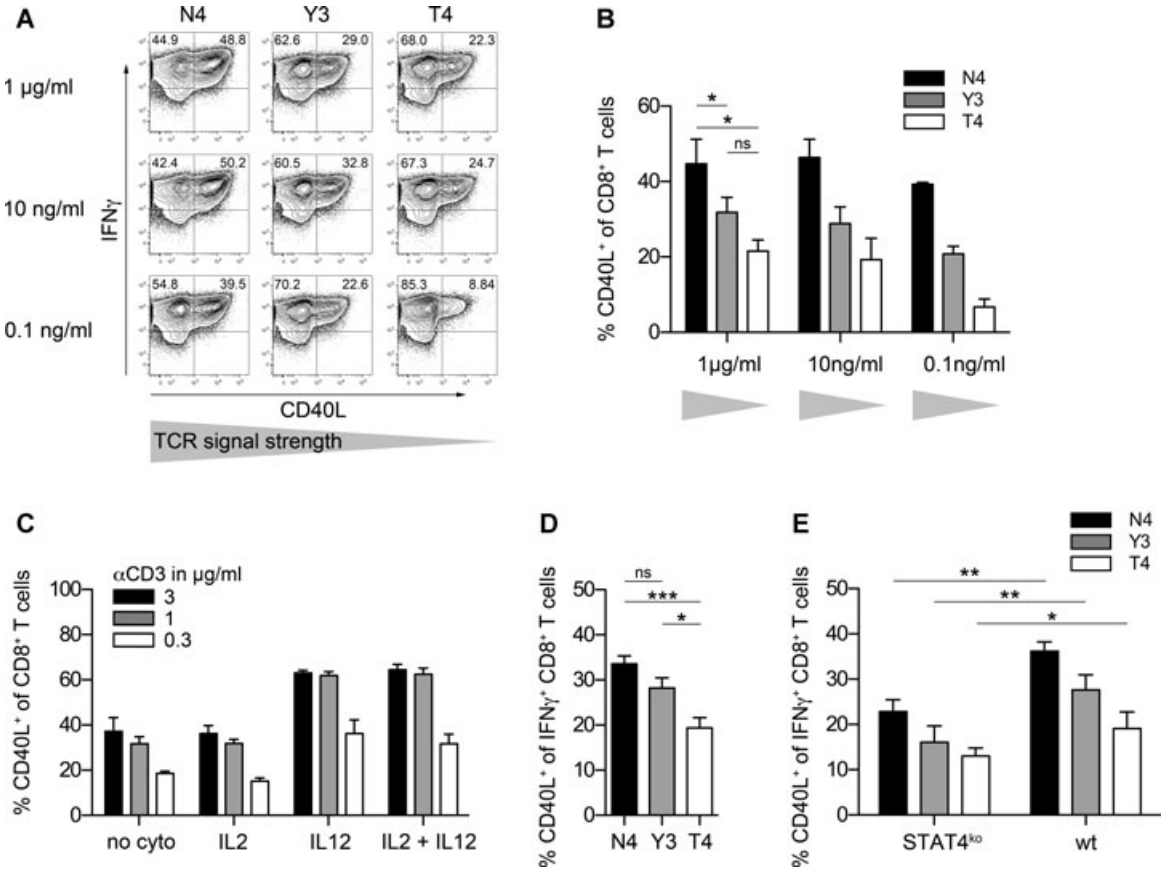

Figure 3. TCR signaling strength modulates CD40L induction in $CD8^+$ T cells. (A and B) Naïve murine $CD8^+$ T cells of OT1 mice were stimulated with SIINFEKL (N4) or APLs (SIYNFEKL (Y3); SIITFEKL (T4)) and APCs, cultured for 6 days with IL-2 and IL-12, restimulated with PMA/Iono and CD40L expression was determined by flow cytometry. (A) Representative dot plots are shown. (B) Titration of peptide concentration used for initial stimulation is shown as mean + SEM of two to five experiments. $^*p<0.05$, paired *t*-test. (C) Naïve murine $CD8^+$ T cells of OT1 mice were stimulated with αCD28 and different doses of αCD3, cultured for 6 days with IL-2 and/or IL-12, restimulated with PMA/Iono and CD40L expression was determined by flow cytometry. Data are shown as mean + SEM of four experiments. (D) Wt mice were transferred with naïve OT1 $CD8^+$ T cells and one day later immunized with DCs pulsed with SIINFEKL or APL. Day 7 p.i. splenocytes were restimulated with SIINFEKL and CD40L induction of OT1 $CD8^+$ T cells was analyzed; data are shown as mean + SEM of nine mice per group pooled from three experiments. $^*p<0.05$, $^{***}p<0.001$, unpaired *t*-test. (E) Wt mice were transferred with a mix of equal amounts of naïve $CD8^+$ T cells of WT OT1 and $STAT4^{ko}$ OT1 mice and 1 day later immunized with DCs pulsed with SIINFEKL or APL. Day 7 p.i. splenocytes were restimulated with SIINFEKL and CD40L induction in WT OT1 and $STAT4^{ko}$ OT1 $CD8^+$ T cells was analyzed; data are shown as mean + SEM of five to six mice per group pooled from two experiments. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, paired *t*-test.

Mice cell preparation and media

C57BL/6, CD45.1, CD90.1, and OT1 mice on the C57BL/6 background were obtained from the Jackson Laboratory. $STAT4^{-/-}$ OT1 mice were kindly provided by Protul A. Shrikant. All mice were bred, housed, and used at the Charité in accordance with the German law for animal protection with permission from the local veterinary offices.

The mice were infected or immunized at 6–12 weeks of age and sacrificed at indicated time points. Single-cell suspensions were obtained from spleens and cells were cultured in RPMI 1640 medium (Gibco) supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.3 mg/mL glutamine, 50 µM β-ME (Sigma-Aldrich, Seelze, Germany) and 10% heat-inactivated FCS (PAA).

In vitro T-cell priming (human)

PBMCs were positively enriched for $CD8^+$ T cells using microbeads (Miltenyi) and naïve $CD8^+$ T cells isolated as $CD3^+/CD4^-/CD8^+/CD45RA^+/CCR7^+$ by FACS Aria II (BD). Naïve $CD8^+$ T cells were cultured on plate-bound αCD3 (NA/LE, 1 µg/mL, BD, UCHT1) and αCD28 (NA/LE, 1 µg/mL, BD, CD28.2) in the presence of one or combinations of the following cytokines (all 10 ng/mL): rhIL-2, rhIL-3, rhIL-6, rhIL-12, rhIL-23, rhGMCSF, rhTNF-α, rhIFN-α 2a, or rhIFN-α 2b (all Miltenyi). $CD8^+$ T cells were removed from αCD3/αCD28 stimulus after 2 days, cultured for another 4–5 days. $CD8^+$ T cells were than restimulated with PMA (10 ng/mL) and Ionomycin (1 µg/mL) (P/I) (both Sigma) in the presence of Brefeldin A (10 µg/mL) for 6 h.

In vitro T-cell priming (murine)

Cells harvested from lymphoid organs were positively enriched for $CD8^+$ T cells using microbeads (Miltenyi) and naïve $CD8^+$ T cells were isolated as $CD3^+/CD4^-/CD8^+/CD44^{low}/CD62L^+$ by FACS Aria II (BD). Naïve $CD8^+$ T cells were cultured on plate-bound αCD3 (NA/LE, 3 μg/mL, unless indicated otherwise, BD, 17A2) and αCD28 (NA/LE, 1 μg/mL, BD, 37.51) or with irradiated $CD8^-$ splenocytes and peptides (SIINFEKL (N4), SIYNFEKL (Y3) or SIITFEKL (T4), JPT, 1 μg/mL unless indicated otherwise) with one or combinations of the following cytokines (all 10 ng/mL): rhIL-1β, rmIL-2, rmIL-3, rmIL-12, rhIL-23, rmGMCSF, rhTNF-α, rmIFN-α (all Miltenyi), or rmIL-6 (Peprotech). $CD8^+$ T cells were removed from αCD3/αCD28 stimulus after 2 days, rmIL-2 was added 3 days after stimulation, and all $CD8^+$ T cells were restimulated after a total culture time of 6–7 days with PMA (10 ng/mL), Ionomycin (1 μg/mL) (both Sigma), or SIINFEKL (1 μg/mL, JPT) in the presence of Brefeldin A (10 μg/mL) for 6 h.

Infection and immunization of mice

5e4 naïve OT1 $CD8^+$ T cells or a 1:1 mixture of 5e4 naïve OT1 $CD8^+$ T cells and 5e4 naïve $STAT4^{ko}$ OT1 $CD8^+$ T cells with different congenic background ($CD90.1^+/CD90.2^+$ or $CD90.1^+/CD90.2^-$) were transferred to WT mice. Mice were immunized i.v. with 1e6 $CD11c^+$ DCs pulsed for 2 h with the peptide SIINFEKL, SIYNFEKL, or SIITFEKL (JPT) at 5 μg/mL in the presence of 1 μg/mL LPS (*E. coli*, Sigma). DCs were isolated from WT mice with CD11c Microbeads (Miltenyi). At indicated time points, splenocytes of immunized mice were isolated, single-cell suspensions generated, and cells restimulated with SIINFEKL (5 μg/mL) in the presence of Brefeldin A (10 μg/mL) for 6 h.

Flow cytometry

For intracellular analysis, cells were fixed with FACS-Lysing solution (BD Pharmingen) and permeabilized afterward with FACS-Perm2 (BD Pharmingen) according to the manufacturer's instructions. After washing, cells were stained for 20 min at 4°C in the dark, washed, and analyzed on CantoII or LSRII (both BD). All FACS data were analyzed with FlowJo (Tree–Star, OR, USA).

Statistics

For statistical analysis, GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, CA, USA) was used.

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Conflict of interest: The authors declare no competing financial interests.

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Abbreviation: **APL**: altered peptide ligand

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