

IL-4 induces a suppressive IL-10-producing CD8⁺ T cell population via a Cdkn2a-dependent mechanism

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ABSTRACT

CD8⁺ T cells play an important role in immune regulation and effective immune responses against tumor cells, viral infection, and intracellular pathogens. In this report, using tiger or 10BiT mice, we defined a population of IL-10-producing CD8⁺ T cells that were induced by IL-4. These IL-10⁺CD8⁺ T cells possessed a strong inhibitory effect on the CD4⁺ T cell proliferation in an IL-10-dependent and cell contact-dependent fashion. In comparison with IL-10⁻CD8⁺ T cells, IL-10⁺CD8⁺ T cells expressed an array of Th2-like cytokines (IL-4, IL-5), perforin, and granzymes, as well as the cell cycle regulatory protein Cdkn2a. Interestingly, knockdown of *cdkn2a* using siRNA reduced IL-4-induced IL-10 production significantly. Furthermore, CD8⁺ T cells from *Cdkn2a*^{-/-} mice produced a significantly lower amount of IL-10, and the effect was limited to CD8⁺ T cells but not observed in CD4⁺ T cells and APCs. Finally, IL-10⁺CD8⁺ T cells played a protective role in the TNBS-induced murine colitis model, indicating a critical role of this population of CD8⁺ T cells in regulatory immune responses. Taken together, we have defined a population of IL-10-producing CD8⁺ Tregs induced by IL-4 and mediated by Cdkn2a. *J. Leukoc. Biol.* 94: 1103–1112; 2013.

Abbreviations: 10BiT=110 bacterial artificial chromosome in-transgene mice, Blimp1=B lymphocyte-induced maturation protein-1, Cdkn2a=cyclin-dependent kinase inhibitor 2a, *Cdkn2a*^{-/-} mice=B6.129-cyclin-dependent kinase inhibitor 2a^{tm1Fcdp} mice, Foxp3=forkhead box P3, GITR=glucocorticoid-induced TNFR, IBD=inflammatory bowel disease, iTreg=inducible regulatory T cell, N.C.=control small interfering RNA, PD-1=programmed death-1, rm=recombinant mouse, siRNA=small interfering RNA, Sp1/3=specificity protein 1/3, Tc=cytotoxic T lymphocyte, tiger=IL-10 IRES GFP-enhanced reporter mice, TNBS=2,4,6-trinitrobenzenesulfonic acid, Treg=regulatory T cell

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

Lymphocyte heterogeneity is required for optimal immune responses against pathogens and self-homeostasis. Several functionally distinct subsets of CD8⁺ T cells have been defined. Traditional Tc1 and Tc2 CD8⁺ T cells play overlapping and different roles in tumor immunity [1], viral infection [2], and some allergic diseases [3]. Tc17 cells can be generated in vitro and in vivo and exert critical functions in tumor rejection and viral clearance [4, 5]. CD8⁺ Tregs play an important role in maintaining immune self-homeostasis and resistance to autoimmune diseases. CD8⁺CD122⁺ Tregs prevent and cure naïve CD4⁺ T cell-induced IBD [6]. Moreover, transferring CD8⁺CD28⁻ T cells into CD8-deficient mice can suppress development of experimental autoimmune encephalomyelitis [7]. CD8⁺ IL-10-producing T cells were found in several murine disease models, including coronavirus-induced encephalitis [8], acute influenza virus infection [9], and *Salmonella* and *Leishmania* infection [10]. Also, they were found in human HIV-1 infection [11] and chronic hepatitis C virus infection [12].

IL-10 is a multifunctional cytokine produced by a variety of cell types, including Th2 cells, DCs, activated macrophages, B cells, and mast cells [13]. Recent reports have demonstrated that IL-10 is indispensable for Treg function [14] and suppresses proinflammatory T cell immunity [15]. Several groups, including us, have established that IL-10 is critical for maintaining the suppressive function of Tregs in arthritis [16] and colitis [17]. IL-10 suppresses TNF- α production by macrophages [18], Th1 cell cytokine production, and T cell proliferation [19]. IL-10 restrains Th17 cell-mediated pathology [20] and CD45RB^{lo} cell-mediated colitis [21]. Although Tregs [13] and type 1 Treg [22], macrophages [13], and various immune cells were found to be impor-

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tant sources of IL-10, the function of IL-10⁺CD8⁺ T cells in inflammation remains to be investigated.

A number of transcription factors involved in regulating IL-10 expression in CD4⁺ T cells and CD8⁺ T cells have been defined. GATA-3 [23] and Blimp1 [24] mainly regulate IL-10 expression through a specific signal pathway in CD4⁺ or CD8⁺ T cells. Several transcription factors have been found to regulate IL-10 expression through different mechanisms in macrophages or monocytes. c-Maf [25], stat1 [26], and stat3 [27] have been reported to be involved in IL-10 transcription regulation in the LPS signal transduction pathway, whereas Sp1 [28] or Sp3 [28] directly binds to some specific motifs of the IL-10 promoter to alter IL-10 mRNA levels. However, the comprehensive transcription networks that are responsible for IL-10 production and their regulation by the cytokine environments are still unknown.

The *cdkn2a* locus in mouse encodes two distinct tumor-suppressor proteins: p19^{ARF} and p16^{INK4a} [29]. p19^{ARF} mainly regulates p53 in response to aberrant growth or oncogenic stresses, such as c-Myc activation [29], whereas p16^{INK4a}, which has been mutated or deleted in several tumor tissues [30, 31], plays an important role in regulating the cell cycle. *Cdkn2a*^{-/-} mice [32] are susceptible to tumor generation and growth. However, whether p19^{ARF} and p16^{INK4a} have any roles in T cell differentiation is unclear.

The TNBS-induced murine colitis is an experimental model to be used to study the pathogenesis and therapy of human IBD. In this model, a combination of TNBS and colonic proteins induces excessive production of IFN- γ by Th1 cells, which then activates monocytes/macrophages to produce a series of cytokines and chemokines, such as TNF- α and IL-6 [33]. Down-regulation of IL-10 is also observed in this model, indicating an immunomodulatory role of cytokines in the pathogenesis of such an animal model [34].

In this report, we demonstrated that CD8⁺ T cells could be induced to produce IL-10 in the presence of IL-4 upon activation. These CD8⁺ Tregs suppressed CD4⁺ T cell proliferation in vitro through IL-10- and cell contact-dependent mechanisms. Furthermore, we determined that the cell cycle regulatory protein *Cdkn2a* controlled IL-4-induced IL-10 production in CD8⁺ T cells. Finally, these CD8⁺ T cells played a protective role in TNBS-induced murine colitis in vivo.

MATERIALS AND METHODS

Mice

10BiT mice, in which Thy1.1 expression is used to indicate IL-10 gene expression [35], were generated by Dr. Casey Weaver from University of Alabama and kindly provided by Dr. Susan Kaech from Yale School of Medicine. Tiger mice, in which GFP-positive cells are used to indicate cells expressing IL-10 [10] and CD4dnIL-10R α mice [21], were kindly provided by Dr. Richard Flavell from Yale School of Medicine. OT-1 mice (OVA²⁵⁷⁻²⁶⁴/H-2K^b-restricted) were crossed with 10BiT mice to obtain 10BiT.OT-1 dual-transgenic mice. *Cdkn2a*^{-/-} mice [32] were purchased from the NCI Mouse Repository (National Cancer Institute, Frederick, MD, USA) and were crossed with 10BiT mice to obtain *Cdkn2a*^{-/-}.10BiT mice. C57BL/6J (B6 WT) mice were purchased from the Academy of Military Medical Science (Beijing, China). All animals were maintained under specific pathogen-free conditions at Nankai University. All experiments were performed

in accordance with guidelines for animal care, created by Nankai University Experimental Animal Ethics Committee.

Reagents

rmIL-2 and rmIL-4 and OVA²⁵⁷⁻²⁶⁴ peptide (SIINFEKL) were purchased from R&D Systems (Minneapolis, MN, USA). rmGM-CSF and rmM-CSF were purchased from PeproTech (London, UK). Purified IFN- γ mAb (clone XMG1.2) and purified CD210 mAb (clone 1B1.3a) were from eBioscience (San Diego, CA, USA). GolgiStop, anti-mouse CD3 mAb (clone 145-2C11) and anti-mouse CD28 mAb (clone PV1) were purchased from BD Biosciences (San Jose, CA, USA). FITC-conjugated anti-mouse CD11c (clone N418), PE-conjugated anti-mouse Thy1.1 (clone OX-7), PE-conjugated anti-mouse CD4 (clone RM45), PE-conjugated anti-mouse CD69 (clone H1.2F3), PE-conjugated anti-mouse CD122 (clone 5H4), PE-conjugated anti-mouse CD152 (CTLA-4; clone UC10-4B9), PE-conjugated anti-mouse CD357 (GITR; clone DTA-1), PE-conjugated anti-mouse/rat/human Foxp3 (clone 150D), PE-conjugated anti-mouse ICOS (clone 7E.17G9), PE-conjugated anti-mouse I-A/I-E (clone M5/114.15.2), PE-conjugated anti-mouse F4/80 (clone BM8), PE-conjugated rat IgG2b, κ isotype control antibody (clone RTK4530), PE-conjugated Rat Ig2a, κ isotype control antibody (clone RTK2758), PE-conjugated mouse IgG1, κ isotype control antibody (clone 150D), allophycocyanin-conjugated anti-mouse CD8 α (clone 53-6.7), and allophycocyanin-conjugated anti-mouse Thy1.1 (OX-7) were from BioLegend (San Diego, CA, USA). FITC-conjugated anti-mouse CD11b (clone ML/70) was from Sungene Biotech (Tianjin, China). PMA, ionomycin, CFSE, TNBS, Con A (C5275), and LPS were purchased from Sigma Chemical (St. Louis, MO, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Microarray analysis was performed by CapitalBio (Beijing, China).

CD8⁺ T cell differentiation

Naïve CD8⁺ T cells (CD8⁺CD62L^{hi}CD44^{lo}) were sorted from tiger or 10BiT mice and cultured with soluble anti-CD3 (2 μ g/ml) or Con A (2.5 μ g/ml) together with APCs in the presence of IL-4 (10 ng/ml) and IL-2 (2 ng/ml) for 48 h. After expansion for 2 days, cells were restimulated with PMA (50 ng/ml) and ionomycin (1 μ g/ml) for 6 h, and GFP⁺ or Thy1.1⁺ T cells were detected by flow cytometry. APCs were from bone marrow-derived DCs [10] or fixed splenocytes treated with mitomycin C, as described previously [36].

CD4⁺ T cell differentiation

In Th2 conditions, naïve CD4⁺ T cells (2.5 \times 10⁶/ml), sorted from reporter mice, were cultured with immobilized anti-CD3 (10 μ g/ml), soluble anti-CD28 (1 μ g/ml), IL-2 (2 ng/ml), IL-4 (10 ng/ml), and anti-IFN- γ (10 μ g/ml) for 48 h. After expansion for another 48 h, cells were restimulated for 6 h by PMA and ionomycin.

In Treg conditions, naïve CD4⁺ T cells (2.5 \times 10⁶/ml) were cultured with immobilized anti-CD3 (10 μ g/ml), soluble anti-CD28 (1 μ g/ml), IL-2 (2 ng/ml), and TGF- β (5 ng/ml) for 72 h [37].

Cell coculture assay

Naïve CD8⁺ T cells from 10BiT mice were activated with Con A together with APCs in the presence of IL-2 and IL-4 for 48 h. After expansion for 2 days, cells were restimulated with PMA and ionomycin as described above. Cells were sorted into CD8⁺Thy1.1⁺/Thy1.1⁻ T cells (2.5 \times 10⁵/well), which were then cocultured separately with CFSE-labeled, naïve CD4⁺ T cells (2.5 \times 10⁵/well) from B6 WT or CD4dnIL-10R α mice (the ratio was 1:1) in 96-well flat-bottom plates and mixed with APCs, IL-2, and soluble anti-CD3 (2 μ g/ml) in the culture media. After 72 h, cells were harvested; the CFSE profiles of the CD4⁺ T cells were analyzed by flow cytometry.

A 96-well Transwell plate with 0.4 μ m pore size was also used (Corning, Corning, NY, USA) for the coculture as described above. CFSE-labeled B6 WT naïve CD4⁺ T cells (2.5 \times 10⁵/well), mixed with APCs, IL-2, and soluble anti-CD3 (2 μ g/ml) in the culture media, were placed in the lower chamber alone or together with sorted CD8⁺Thy1.1⁺ T cells or with CD8⁺Thy1.1⁺ T cells (2.5 \times 10⁵/well) in the upper chamber. Cells were cul-

tured for 72 h, and CD4⁺ T cells in the lower chambers were collected and stained with PerCP-Cy5.5-conjugated anti-mouse CD4, and CFSE dilution was analyzed by flow cytometry.

ELISA

Mouse IL-10 ELISA kits were purchased from BioLegend, and ELISA was performed according to the manufacturer’s protocol.

Real-time PCR

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen) and reverse-transcribed by QuantScript RT Kit (Tiangen, Beijing, China). mRNA expression was quantified by real-time PCR. SYBR PreMix HotMaster Taq (Tiangen) was used in real-time PCR. The primer sequences used were listed in Supplemental Table 1.

Microarray analysis

For global gene expression analysis, sorted CD8⁺GFP⁺/GFP⁻ T cells were lysed by TRIzol Reagent (Invitrogen). Microarray analysis was performed as described in the CapitalBio manual. Microarray data are available in the Gene Expression Omnibus database (Accession Number GSE37739; www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37739).

siRNA transfection and detection

FAM-conjugated siRNAs were synthesized by GenePharma (Shanghai, China). The specific siRNA sequences used were listed in Supplemental Table 2, and Lipofectamine 2000 reagent (Invitrogen) was used for transfection. siRNA or N.C. was transfected into mouse splenocytes at a final concentration of 20 nM. Transfection was performed together with cells culturing in the presence of Con A, IL-2, and IL-4 for 48 h, and then cells

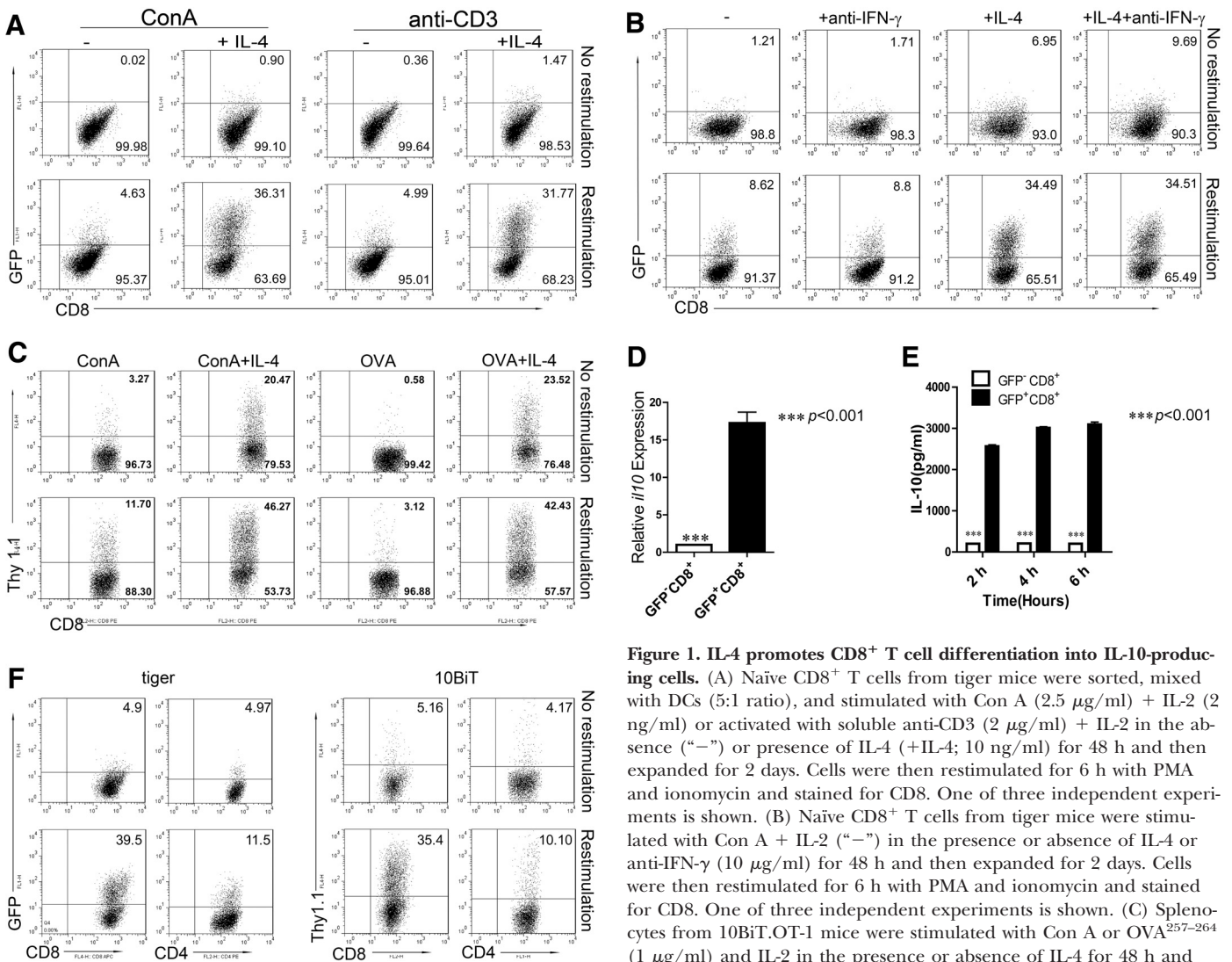


Figure 1. IL-4 promotes CD8⁺ T cell differentiation into IL-10-producing cells. (A) Naïve CD8⁺ T cells from tiger mice were sorted, mixed with DCs (5:1 ratio), and stimulated with Con A (2.5 μg/ml) + IL-2 (2 ng/ml) or activated with soluble anti-CD3 (2 μg/ml) + IL-2 in the absence (“-”) or presence of IL-4 (+IL-4; 10 ng/ml) for 48 h and then expanded for 2 days. Cells were then restimulated for 6 h with PMA and ionomycin and stained for CD8. One of three independent experiments is shown. (B) Naïve CD8⁺ T cells from tiger mice were stimulated with Con A + IL-2 (“-”) in the presence or absence of IL-4 or anti-IFN-γ (10 μg/ml) for 48 h and then expanded for 2 days. Cells were then restimulated for 6 h with PMA and ionomycin and stained for CD8. One of three independent experiments is shown. (C) Splenocytes from 10BiT.OT-1 mice were stimulated with Con A or OVA^{257–264} (1 μg/ml) and IL-2 in the presence or absence of IL-4 for 48 h and then expanded for 2 days. Cells were then restimulated as described above and stained for CD8 and Thy1.1. One of three reproducible experiments is shown. (D) GFP⁺CD8⁺ T cells and GFP⁻CD8⁺ T cells from A were sorted for IL-10 mRNA quantification by real-time PCR. Data are shown as the mean + SEM of three replicate measurements from three independent experiments. ***P < 0.001. (E) GFP⁺CD8⁺ T cells and GFP⁻CD8⁺ T cells were sorted and cultured without IL-2. Supernatants were collected at different time-points for IL-10 ELISA. Data are shown as mean + SEM of triplicate of three experiments performed. ***P < 0.001. (F) Naïve CD4⁺ and CD8⁺ T cells were sorted from tiger and 10BiT reporter mice, cultured as described above, and analyzed by flow cytometry. One representative experiment of three is shown. FL1/2-H, Fluorescence 1/2-height; APC, allophycocyanin.

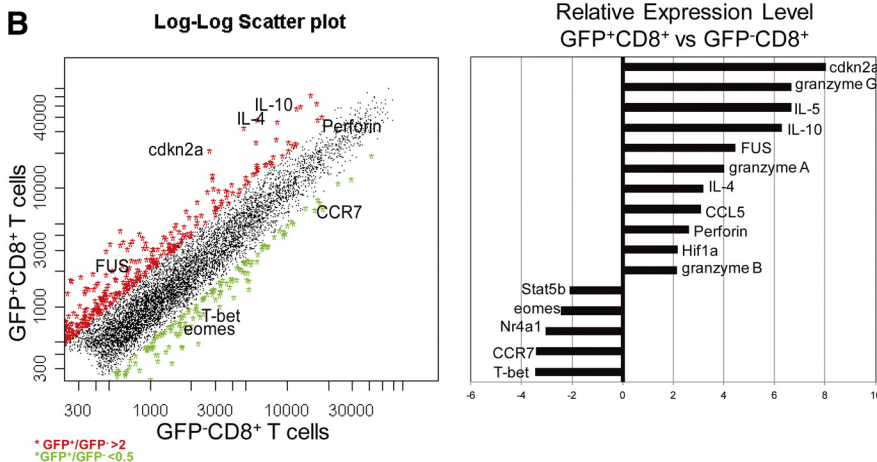
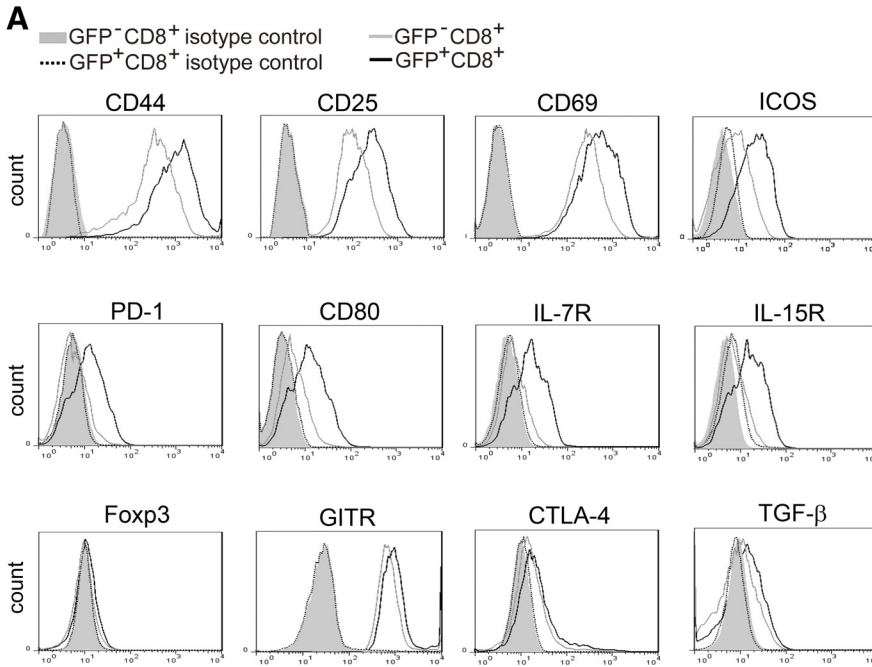
transfected with FAM-conjugated siRNA (FAM⁺ cells) were sorted. Gene-silencing efficiency was detected by real-time PCR.

TNBS-induced colitis

Colitis was induced by administration of TNBS in B6 WT mice on Day 0, as described previously [38]. Mice were i.v.-injected with GFP⁺CD8⁺ (1×10⁶ cells/mouse) or GFP⁻CD8⁺ (1×10⁶ cells/mouse) T cells or PBS vehicle, 24 h before and 24 h after TNBS administration. Mice treated with 50% ethanol were used as a negative control. Mice were weighed daily and killed on Day 5. Colon tissues were harvested, fixed for H&E staining, and RNA-extracted for IFN-γ mRNA level analysis. Histopathological scoring rules of colon tissue sections were as described [39].

Flow cytometry

A BD FACSCalibur (BD Biosciences) instrument was used for flow cytometry analysis; the software was CellQuest Pro (BD Biosciences) or FlowJo (Tree Star, Ashland, OR, USA). BD FACSAria (BD Biosciences) was used for cell sorting; the software was FACSDiva (BD Biosciences).



Statistics

Statistical significance was evaluated by a two-tailed unpaired Student's *t*-test using InStat version 3.06 software for Windows (GraphPad, San Diego, CA, USA).

RESULTS

IL-4 promotes CD8⁺ T cell differentiation into IL-10-producing cells

To define the role of IL-4 in CD8⁺ T cell differentiation, naïve (CD44^{lo}CD62L^{hi}) CD8⁺ T cells from tiger mice, in which GFP-positive cells are used to indicate cells expressing IL-10, were cultured with Con A or soluble anti-CD3 in the presence of IL-4. We demonstrated that IL-4 induced a significantly higher number of GFP⁺CD8⁺ T cells under Con A and anti-CD3 conditions (Fig. 1A). In our preliminary studies, this effect was

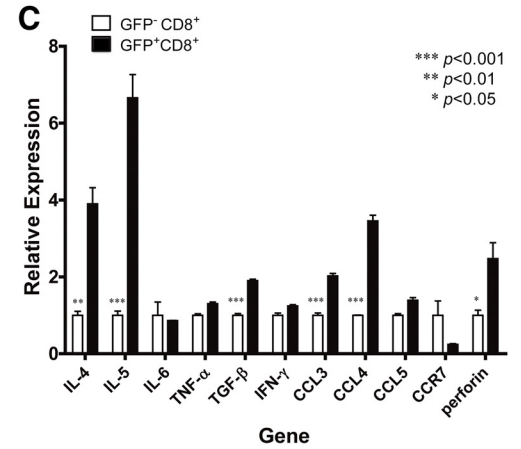


Figure 2. Characteristics of IL-4-induced, IL-10-producing CD8⁺ T cells. (A) Naïve CD8⁺ T cells from tiger mice were cultured as in Fig. 1, and GFP⁻CD8⁺ T cells (gray, solid-line histograms) or GFP⁺CD8⁺ T cells (black, solid-line histograms) were gated, and surface molecules, as indicated, were analyzed. Isotype-matched control antibody staining for GFP⁻CD8⁺ T cells (gray-filled histograms) or GFP⁺CD8⁺ T cells (black, dotted-line histograms) were also shown. One typical staining of three is shown. (B) Gene expression analysis comparing GFP⁺CD8⁺ with GFP⁻CD8⁺ T cells from the above cultures. (C) GFP⁺CD8⁺ and GFP⁻CD8⁺ T cells were sorted from the above cultures, and cDNA was prepared for analyzing the expression levels of indicated genes. Data represent mean + SEM of three combined experiments: **P* < 0.05; ***P* < 0.01; ****P* < 0.001. eomes, Eomesodermin; FUS, fused in sarcoma; T-bet, T-box expressed in T cells; Hif1a, hypoxia-inducible factor 1α; Nr4a1, nuclear receptor subfamily 4 group A member 1.

IL-4 dose-dependent (data not shown). The induction of IL-10 by IL-4 was independent of IFN- γ (Fig. 1B). Interestingly, using CD8⁺ T cells from 10BiT.OT-1 transgenic mice, in which Thy1.1 expression is used to indicate IL-10 gene expression,

we determined that IL-4 also induced antigen-responding IL-10 production by CD8⁺ T cells (Fig. 1C). To confirm the correlation between GFP and IL-10 expression level, GFP⁺ and GFP⁻ CD8⁺ T cells were sorted from the culture, and the ex-

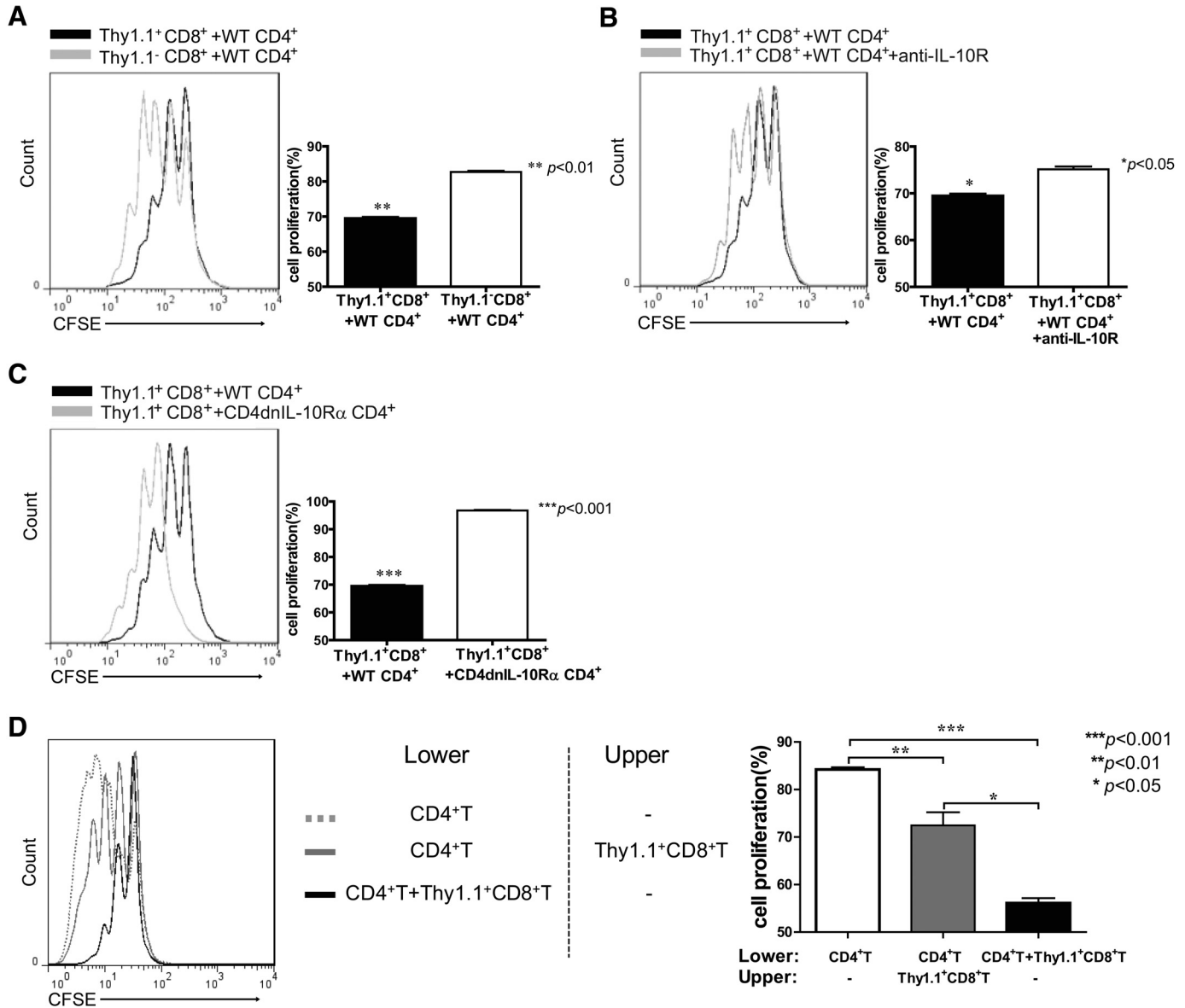


Figure 3. IL-10-producing CD8⁺ T cells suppress CD4⁺ T cell proliferation in IL-10- and cell contact-dependent mechanisms. (A) Naïve CD4⁺ T cells from B6 WT mice were sorted, labeled with CFSE, mixed with APCs (5:1 ratio), and stimulated with soluble anti-CD3 (2 μ g/ml) + IL-2 (2 ng/ml) in the presence of sorted Thy1.1⁺CD8⁺ or Thy1.1⁻CD8⁺ T cells, produced as described in Materials and Methods. After 72 h, cells were harvested and stained for CD4. CFSE profiles of the CD4⁺ T cells were analyzed. The proliferation of naïve CD4⁺ T cells in the presence of Thy1.1⁺CD8⁺ T cells (black-line histogram) were overlaid with those cultured with Thy1.1⁻CD8⁺ T cells (gray-line histogram). One representative experiment of three is shown. Cell proliferation was analyzed according to CFSE dilution. Data are representative of three independent experiments (mean \pm SEM; **P<0.01). (B) In the parallel culture as described above (A), sorted Thy1.1⁺CD8⁺ T cells and responders were cultured in the presence (gray-line histogram) or absence (black-line histogram) of anti-IL-10R mAb. The black-line histograms in A and B show the identical data that represent proliferation of naïve CD4⁺ T cells from B6 WT mice cocultured with Thy1.1⁺CD8⁺ T cells. One representative experiment of three is shown. Data represent three independent experiments (mean \pm SEM; *P<0.05). (C) Responder cells from B6 WT (black-line histogram) or CD4dnIL-10R α (gray-line histogram) mice were cocultured with sorted Thy1.1⁺CD8⁺ T cells. One representative experiment of three is shown. Data represent three independent experiments (mean \pm SEM; ***P<0.001). (D) Naïve CD4⁺ T cells (2.5 \times 10⁵/well) mixed with APCs (5:1 ratio), IL-2, and soluble anti-CD3 (2 μ g/ml) were cultured in the lower chambers of transwell plates, and sorted Thy1.1⁺CD8⁺ T cells (2.5 \times 10⁵/well) were cultured in the lower (black, solid-line histogram) or upper chambers (gray, solid-line histogram). The gray dotted-line histogram is the control of naïve CD4⁺ T cells without effector cells. One typical experiment of three is shown. Data represent three independent experiments (mean \pm SEM; *P<0.05; **P<0.01; ***P<0.001).

pression level of IL-10 was analyzed by real-time PCR. Indeed, GFP⁺CD8⁺ T cells expressed a significantly higher level of IL-10 (Fig. 1D), which was confirmed further by detection of IL-10 protein levels using ELISA (Fig. 1E). Interestingly, under the same culture conditions, CD4⁺ T cells expressed a significantly lower level of IL-10 (Fig. 1F), and these two reporter mice (tiger and 10BiT) showed similar results. Therefore, both of these reporter mice were used in our following studies. Our results present new evidence that CD8⁺ T cells could be induced to produce IL-10 by IL-4 upon activation.

Characterization of IL-4-induced, IL-10-producing CD8⁺ T cells

To characterize these IL-4-induced, IL-10-producing CD8⁺ T cells, a series of surface markers was analyzed by taking advantage of GFP⁺/GFP⁻ CD8⁺ T cells (Fig. 2A). GFP⁺CD8⁺ T cells showed an activated phenotype, with higher expression of CD44, CD25, and CD69. These GFP⁺CD8⁺ T cells also expressed higher levels of ICOS and PD-1. Moreover, these cells expressed higher levels of IL-7R and IL-15R, indicating a stronger ability for survival of these CD8⁺ T cells. Whereas these cells did not express Foxp3, no differences in the expression of GITR, CTLA4, and TGF-β were observed between GFP⁺CD8⁺ T and GFP⁻CD8⁺ T cells.

To compare the broad gene profiles between these two types of CD8⁺ T cells under the identical conditions, GFP⁺ and GFP⁻CD8⁺ T cells were sorted, and cDNA microarray was performed. A series of functional genes and transcription factors was found to be differentially expressed between these two populations of CD8⁺ T cells (Fig. 2B). To confirm the expression profiles, cDNA samples prepared from these two cell populations were used for real-time PCR analysis. IL-10-producing CD8⁺ T cells were confirmed to express higher levels of IL-4, IL-5, CCL3, CCL4, and perforin (Fig. 2C).

IL-10-producing CD8⁺ T cells suppress CD4⁺ T cell proliferation through IL-10- and cell contact-dependent mechanisms

Based on the fact that IL-10 is a suppressive cytokine, we sought to determine the suppressive function of IL-10-producing CD8⁺ T cells. Naïve CD8⁺ T cells from 10BiT mice were activated with Con A together with APCs in the presence of IL-2 and IL-4 to induce Thy1.1⁺ expression. Cultured CD8⁺ T cells were sorted into Thy1.1⁺ and Thy1.1⁻ cells, followed by the coculture experiment. The proliferation of naïve CD4⁺ T cells in the presence of Thy1.1⁺CD8⁺T cells were overlaid with those cultured with Thy1.1⁻CD8⁺ T cells. Indeed, Thy1.1⁺ CD8⁺ T cells dramatically suppressed proliferation of CD4⁺ T cells (Fig. 3A). Interestingly, addition of an anti-IL-10R antibody blocked the suppression (Fig. 3B). The suppression effect was also impaired when using CD4⁺ T cells isolated from CD4dnIL-10Rα mice [21], in which CD4⁺ T cells express a high level of IL-10R but without the cytoplasmic domain for signaling through these receptors as the responders (Fig. 3C). Our results thus demonstrate a critical role of IL-10 in mediating the suppressive effect of these IL-10-producing CD8⁺ T cells.

To further determine whether the suppressive effect of IL-10-producing CD8⁺ T cells was cell contact-dependent, transwell plates

were used to separate the effector cells and responders. The separation indeed reduced the suppression effect, indicating a contact-dependent mechanism (Fig. 3D). Overall, IL-10-producing CD8⁺ T cells suppressed CD4⁺ T cell proliferation through IL-10- and cell contact-dependent mechanisms.

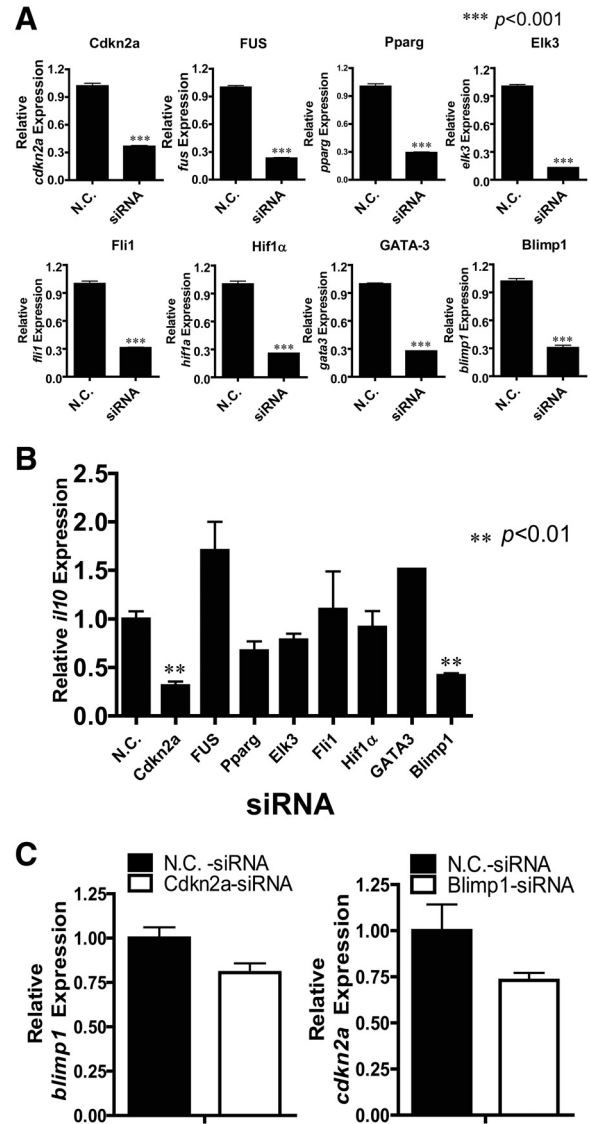


Figure 4. Cdkn2a is critical for IL-4-induced IL-10 production. (A) Splenocytes from B6 WT mice were transfected with FAM-conjugated siRNAs and cultured under the Con A + IL-2 + IL-4 condition for 48 h; cells transfected with siRNA (FAM⁺ cells) were sorted and lysed for real-time PCR to detect the expression level of the indicated gene. The cDNA was also used to analyze the mRNA levels of IL-10 by real-time PCR (B). Data are shown as mean + SEM of three independent experiments performed; ***P* < 0.01; ****P* < 0.001. (C) Splenocytes from B6 WT mice were transfected with Cdkn2a or blimp1 siRNA and cultured with Con A + IL-2 + IL-4 for 48 h; FAM⁺ cells were sorted for real-time PCR analysis of blimp1 and Cdkn2a, respectively. Data are representative of at least three independent experiments (mean+SEM). No statistical difference was observed. Pparg, Peroxisome proliferator-activated receptor γ; Elk3, a member of ETS oncogene family; Fli1, Friend leukemia integration 1 transcription factor.

Cdkn2a is a critical factor in mediating the IL-4-induced IL-10 expression

To define the key transcription factors that might mediate the induction of IL-10 by IL-4, several siRNA sequences were screened to knock down those transcription factors that were up-regulated in GFP⁺CD8⁺ T cells based on the cDNA microarray results (Fig. 2B). The efficiency of these siRNAs reached >70%, as confirmed by real-time PCR (Fig. 4A). Strikingly, only knock-down of *cdkn2a* and *blimp1* significantly reduced IL-4-induced IL-10 expression (Fig. 4B). To examine whether *Cdkn2a* and *blimp1* had any interaction, the expression of *blimp1* or *Cdkn2a* in the opposite siRNA-treated cells was analyzed. Interestingly, no direct effect on the expression level of IL-10 induced by one factor was caused by the opposite factor (Fig. 4C).

Cdkn2a significantly regulates the IL-4-induced IL-10 production in CD8⁺ T cells

To further study the role of *Cdkn2a* in IL-4-induced IL-10 expression, naïve CD8⁺ T cells were sorted from B6 WT and *Cdkn2a*^{-/-} mice, and we demonstrated by real-time PCR that CD8⁺ T cells expressed significantly less IL-10 in the absence of *Cdkn2a* (Fig. 5A). This conclusion was confirmed further by detection of IL-10 protein levels using ELISA (Fig. 5B). Interestingly, IL-10 expression by iTregs and Th2-differentiated CD4⁺ T cells was not affected (Fig. 5C and D). Moreover, IL-10 expression from splenic macrophages and CD11c⁺ DCs triggered by LPS was also not affected (Fig. 5E), and the same result was also obtained from the bone marrow-derived DCs and macrophages (data not shown). Taken together, our results thus define a unique role for *Cdkn2a* in CD8⁺ T cell differentiation toward IL-10 production induced by IL-4.

IL-10-producing CD8⁺ T cells play a protective role in a TNBS-induced murine colitis model

To define the regulatory role of IL-10-producing CD8⁺ T cells in vivo, a TNBS-induced colitis model was adapted with different populations of CD8⁺ T cells transferred at different time-points of disease induction, as described in Materials and Methods. Mice injected with GFP⁺CD8⁺ T cells recovered their body weight more rapidly than mice treated with GFP⁻CD8⁺ T cells or PBS at the later stage of colitis (Days 4 and 5; Fig. 6A). The reduced inflammation in the GFP⁺CD8⁺ T cell-treated group was also confirmed by colon histopathology (Fig. 6B). With the use of a standard scoring system, the GFP⁺CD8⁺ T cell-treated group reduced the severity of the disease significantly (Fig. 6C). Finally, colon lengths of GFP⁺CD8⁺ T cell-treated mice were also obviously longer than those in the GFP⁻CD8⁺ T cells or PBS-injected groups and had no significant differences from those in the control group (Fig. 6D). These results indicate that IL-10-producing CD8⁺ T cells play a protective role in the TNBS-induced murine colitis model.

To investigate further the mechanisms underlying the protective role of IL-10-producing CD8⁺ T cells in TNBS-induced colitis model, we analyzed IFN- γ mRNA expression levels in colon tissues among different groups. The results showed that IFN- γ mRNA levels in the GFP⁺CD8⁺ T cell-treated group were significantly lower than that of GFP⁻CD8⁺ T cells or PBS groups, which were similar to the control group and naïve mice (Fig. 6E). Taken together, IL-10-producing CD8⁺ T cells possibly protect mice from TNBS-induced colitis through inhibition of IFN- γ production.

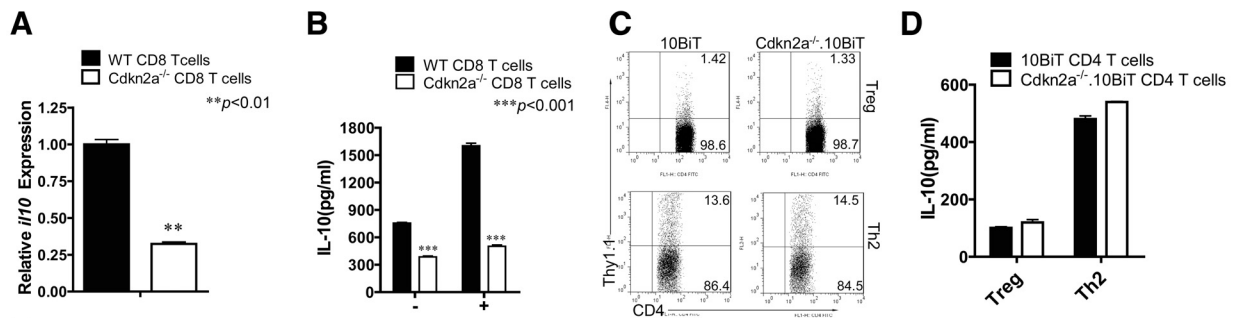


Figure 5. *Cdkn2a* is required for IL-10 expression only in CD8⁺ but not CD4⁺ T cells, macrophages, and DCs. (A) Naïve CD8⁺ T cells from B6 WT (black bar) and *Cdkn2a*^{-/-} (white bar) mice were sorted and stimulated with immobilized anti-CD3 (10 μ g/ml) and soluble anti-CD28 (1 μ g/ml) in the presence of IL-2 and IL-4 for 48 h, expanded 2 days. Cells were then restimulated by PMA and ionomycin for 6 h and then analyzed IL-10 expression by real-time PCR. Data are representative of three independent experiments (mean+SEM; ***P*<0.01). (B) Supernatants from the parallel, naïve CD8⁺ T cell, cultured in A at 96 h (“-”, no restimulation) and 6 h after restimulation (“+”), were collected for IL-10 detection by ELISA. Results from three independent experiments were combined and presented (mean+SEM; ****P*<0.001). (C) Naïve CD4⁺ T cells were sorted from 10BiT mice and *Cdkn2a*^{-/-}. 10BiT mice, cultured under iTreg conditions or Th2 conditions, as described in Materials and Methods. Cells were then analyzed by flow cytometry. Data were representative of three independent experiments. (D) Supernatants from iTreg and Th2 cultures of C were collected and used for IL-10 detection by ELISA. Data (mean+SEM) from three combined experiments were shown. No statistical difference was observed. (E) Splenocytes from 10BiT mice or *Cdkn2a*^{-/-}.10BiT mice cultured with LPS (1 μ g/ml) for 24 h. Cells were then analyzed by flow cytometry. Data are representative of three independent experiments.

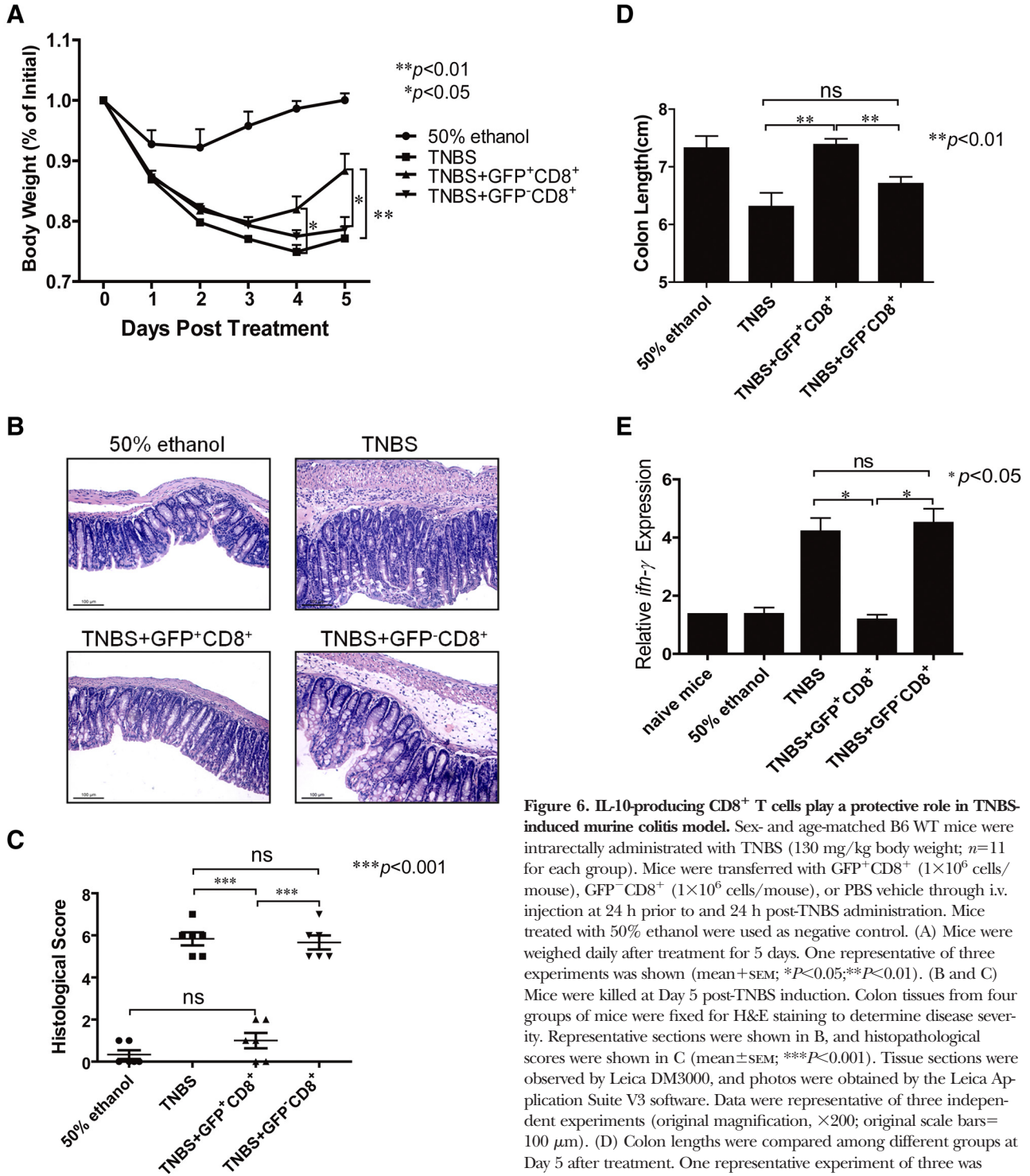


Figure 6. IL-10-producing CD8⁺ T cells play a protective role in TNBS-induced murine colitis model. Sex- and age-matched B6 WT mice were intrarectally administered with TNBS (130 mg/kg body weight; $n=11$ for each group). Mice were transferred with GFP⁺CD8⁺ (1×10^6 cells/mouse), GFP⁻CD8⁺ (1×10^6 cells/mouse), or PBS vehicle through i.v. injection at 24 h prior to and 24 h post-TNBS administration. Mice treated with 50% ethanol were used as negative control. (A) Mice were weighed daily after treatment for 5 days. One representative of three experiments was shown (mean \pm SEM; * $P < 0.05$; ** $P < 0.01$). (B and C) Mice were killed at Day 5 post-TNBS induction. Colon tissues from four groups of mice were fixed for H&E staining to determine disease severity. Representative sections were shown in B, and histopathological scores were shown in C (mean \pm SEM; *** $P < 0.001$). Tissue sections were observed by Leica DM3000, and photos were obtained by the Leica Application Suite V3 software. Data were representative of three independent experiments (original magnification, $\times 200$; original scale bars = 100 μ m). (D) Colon lengths were compared among different groups at Day 5 after treatment. One representative experiment of three was shown (mean \pm SEM; ** $P < 0.01$). (E) RNA was extracted from colon tissues, and the cDNA was then prepared for analyzing the expression levels of *ifn-γ* among different groups. One representative experiment of three was shown (mean \pm SEM; * $P < 0.05$).

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DISCUSSION

CD8⁺ T cells play an important role in protective immune responses against intracellular viral, bacterial infection, and tumor cells [1, 2, 40–42]. Similar to CD4⁺ T cells, CD8⁺ T cells have divergent, functional subsets, according to their cytokine profiles, including Tc1,

Tc2, Tc17, and Tregs [43, 44]. However, less attention has been paid to the study of differentiation and transcriptional-controlling mechanisms of CD8⁺ Tregs. In this study, we observed that CD8⁺ T cells were induced to produce IL-10 by IL-4 and controlled by the cell cycle regulatory protein Cdkn2a.

IL-4 is a critical cytokine in the induction of CD4⁺ T cell Th2 differentiation, and these CD4⁺ T cells produce IL-4, IL-5, IL-13, and IL-10 cytokines [45, 46]. It has also been reported that together with IL-12, IL-4 also induces IL-10 production by CD8⁺ T cells [47]. Interestingly, we demonstrated that together with IL-2, IL-4 was sufficient to induce naïve CD8⁺ T cells to differentiate into IL-10-producing cells (Fig. 1). Either IL-2 alone or IL-4 alone failed to induce significant levels of IL-10 (data not shown). In our preliminary experiments, cells cultured in the absence of IL-2 failed to survive and grow. We therefore speculated that IL-2 served as the growth and surviving factor in our system, and IL-4 played a critical role in inducing IL-10 production by CD8⁺ T cells. Also, this process was dependent on TCR signaling-mediated activation, as even cytokine alone in the absence of T cell activation could lead to cell death (data not shown). Furthermore, this process was not affected by the presence of endogenous IFN- γ , as neutralizing IFN- γ did not change the expression level of IL-10 (Fig. 1). Our results thus provide an additional pathway for inducing IL-10 production by CD8⁺ T cells.

One of the key findings in our study was the identification of the specific role of Cdkn2a in CD8⁺ T cell differentiation, especially in IL-4-induced IL-10 secretion. Although quite a few signaling factors were up-regulated upon IL-4 treatment, only knockdown of *cdkn2a* and *blimp1* suppressed IL-10 expression significantly (Fig. 4), and it was evident that these two pathways had no direct interaction (Fig. 4). The critical role of Cdkn2a was confirmed further using Cdkn2a^{-/-} mice, and the effect was restricted to CD8⁺ T cells but was not identified in CD4⁺ T cells or APCs (Fig. 5). Interestingly, knockdown of *gata3* failed to affect IL-4-induced IL-10 expression (Fig. 4), implying that Cdkn2a is not downstream of GATA3. It is unclear whether Cdkn2a is downstream of phosphorylated Stat6 or directly downstream of TCR signaling. To the best of our knowledge, this is the first report to define the role of this cell cycle regulatory protein in CD8⁺ T cell differentiation, and further studies are required to explore the function of Cdkn2a in other cytokine-induced differentiation of CD8⁺ T cells and their detailed signaling pathways.

Defining the function of IL-10-producing T cells is a challenge. Recently, several reporter mice have been made to use GFP or Thyl.1 to indicate IL-10-expressing cells, such as tiger mice [10] and 10BiT mice [35]. By taking advantage of IL-10 GFP or Thyl.1 reporter mice, we demonstrated that IL-10-producing CD8⁺ T cells possessed a strong, suppressive function in vitro via IL-10- and cell contact-dependent mechanisms (Fig. 3) and in vivo through suppressing IFN- γ production (Fig. 6). Interestingly, these IL-4-induced, IL-10-producing CD8⁺ T cells expressed suppressing molecules (PD-1, CTLA-4), as well as a high level of Tc2 cytokines (Fig. 2). Several subsets of CD8⁺ Tregs have been identified, such as CD8⁺CD122⁺ Tregs [6], CD8⁺CD28⁻ T cells [7], and Qa-1-restricted CD8⁺ T cells [48]. It is interesting to define whether this population of IL-10-producing CD8⁺ T cells is a new subset of CD8⁺ Tregs. Further validation would be needed to study the gene expression profiles and functions of these IL-4-induced, IL-10-producing CD8⁺ T cells.

It has been shown that IL-10 suppresses Th1-mediated inflammation in several animal models [49–52]. TNBS-induced colitis is a typical Th1-mediated inflammation [53]. We demonstrated a protective role of these IL-10-producing CD8⁺ T cells in this colitis model (Fig. 6). Interestingly, colon tissue in mice injected with IL-10⁺CD8⁺ T cells showed a significantly decreased level of IFN- γ (Fig. 6), implying a possible mechanism of these IL-10⁺CD8⁺ T cells in TNBS-induced colitis through suppressing the Th1 response. Further studies are in progress to test the detailed molecular mechanisms of this population of CD8⁺ Tregs in the TNBS-induced colitis model and its function in other inflammatory models.

In summary, we have defined a population of CD8⁺ Tregs that was induced by IL-4 and controlled by the cell cycle regulatory protein Cdkn2a, which is the first time it was reported to be involved in T cell differentiation. These IL-10-producing CD8⁺ T cells played a protective role in TNBS-induced colitis. Further studies to understand the pathophysiological function of this subset of CD8⁺ T cells will enable us to facilitate the effective CD8⁺ T cells in protective immune responses against pathogens and tumors.

AUTHORSHIP

Y.Z. and H.Z. designed the project, performed experiments, collected and analyzed the data, and wrote the manuscript. Y.S. performed mouse model and real-time PCR. J.H. helped to design the project and write the manuscript. X.Q. performed cell culture and arranged the figures. X.Z. performed flow cytometry analysis. Z.W., P.W., and Z. Yao supervised and coordinated this work. S.M.K., C.T.W., and R.A.F. kindly provided transgenic mice. Z. Yin and L.Z. designed the overall research and helped to write the manuscript. All authors have read, commented on, and approved the final manuscript.

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DISCLOSURES

The authors have no financial or commercial conflicts of interest.

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KEY WORDS:
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