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Letter to the editor

**Generation of Helios reporter mice and an evaluation of the suppressive capacity of
Helios⁺ regulatory T cells *in vitro***

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Background

Allergic contact dermatitis (ACD) is one of the most common skin diseases, affecting 15 to 20% of the general population worldwide (1). It is classified as a delayed-type hypersensitivity response. Murine contact hypersensitivity (CHS) is a frequently used animal models of ACD (2). Recent evidences show that $Foxp3^+$ regulatory T cells (Tregs) play essential roles in the regulation of various immune responses including CHS (3-5).

Helios, an Ikaros family transcription factor, is expressed at a high level in Tregs and has been suggested to play an important role in the suppressive function (6). It was proposed to be a good marker for thymus-derived Tregs (7). In addition, Helios augments transactivation of *Foxp3* by binding directly to the *Foxp3* promoter (6). Therefore, Helios is a potential modulator of Treg functions.

These notions led to increased interests in potential roles of Helios in inflammatory skin diseases, such as CHS. However, the function of $Helios^+$ Tregs has yet to be determined, since both *Foxp3* and Helios are intracellular markers and cannot be used for isolation of Tregs subsets.

Questions addressed

To solve these problems, we established a Helios reporter mouse strain that enabled us to analyze live $Helios^+$ Tregs. Using this strain, we assessed the suppressive activity of $Helios^+$ Tregs in skin draining lymph nodes (DLNs) responding to CHS.

Experimental design

Venus cDNA, encoding a variant of green fluorescent protein (GFP), was inserted downstream of the first ATG of Helios through a homologous recombination in the KY1.1 ES cell-line, which was established from F₁ blastocysts derived by mating C57BL/6J and 129S6/SvEvTac (Fig. 1a) (8).

Results

The expression of Foxp3 and Helios of CD4⁺ T cells were differentiated in various tissues of Helios^{Venus}/Foxp3^{hCD2/hCD52} reporter mice. In the thymus, most of the Foxp3⁺ cells were Helios⁺. In contrast, the expression level of Helios varied in the peripheral lymphoid organs, such as the spleen and the mesenteric lymph nodes (mLNs) (Fig. 1b). Nearly all Foxp3⁺ Tregs in the thymus expressed Helios, whereas around 60 % of Foxp3⁺ Tregs expressed Helios in the spleen and the mLN (Fig. 1c). These observations were consistent with those by Thornton et al. (7). We also determined the absolute number of Helios⁺ Tregs (Fig. 1d). Intriguingly, Helios⁺ Tregs showed a relative increase in CD25 expression as well as CTLA-4 compared to Helios⁻ Tregs (Fig. 1e and f). These data imply that Helios⁺ Tregs may possess higher suppressor activity than Helios⁻ Tregs.

We focused on the DLNs of ear skin and assessed the number of T cells that express Helios and/or Foxp3. We challenged the ear skin of C57BL/6 mice with 2,4-dinitro-1-fluorobenzene (DNFB) and collected the DLNs between day 0 and 7 after the challenge. The number of both Helios⁺ and Helios⁻ subsets was increased in the DLNs after the challenge (Fig. S1). There was a tendency that the number of Helios⁺ Tregs was dominated over that of the Helios⁻ subset during the CHS.

Next, we investigated whether Helios expression correlated with the suppressive function of Tregs residing in the DLNs during CHS. We sorted Tregs into Helios⁺ and Helios⁻ fractions using FACS Aria II (Fig. 2a). We assessed the suppressive activity of the sorted fractions by culturing them with regional LN cells of DNFB-sensitized mice, which contained antigen-specific non-Tregs as well. T cells in the regional LNs proliferated in response to 2,4-dinitrobenzene sulfonic acid (DNBS), a water-soluble compound with the same antigenicity as DNFB. Addition of Tregs inhibited the proliferation of DNBS-responding T cells in a dose dependent manner (Fig. 2b, left). Helios⁺ Tregs suppressed the T cell-proliferation at fewer numbers than Helios⁻ Tregs did, indicating that antigen- or hapten-specific Tregs were enriched in the Helios⁺ population (Fig. 2b, left). Furthermore, superiority of suppressive function of Helios⁺ Tregs over Helios⁻ counterpart was observed for T cell proliferation mediated by plate-bound anti-CD3 ϵ (Fig. 2b, right). This result indicates that Helios⁺ Treg-population is poised to inhibit immune response and is enriched with antigen-specific clones.

Since IFN- γ and IL-17 are known to be involved in the effector phase of CHS and to lead to recruitment of T cells in the skin (S2-4), we measured these cytokines in supernatants. We found that Helios⁺ Tregs inhibited the production of inflammatory cytokines by LN cells more efficiently than Helios⁻ Tregs did (Fig. 2c). The significance of suppression was comparable between Helios⁺ and Helios⁻ Tregs when T cells were stimulated with DNBS, whereas Helios⁺ Tregs showed superiority for suppressing T cells stimulated by plate-bound anti-CD3 ϵ (Fig. 2c, upper graphs). In contrast, Helios⁺ Tregs showed superior suppression on IL-17-production mediated by both DNBS and

plate-bound anti-CD3 ϵ (Fig. 2c, lower graphs). Our findings suggest that Helios⁺ Tregs preferably inhibit IL-17 production rather than IFN- γ production. Recent study showed that TIGIT⁺ Tregs are highly suppressive and selectively suppress Th1 and Th17 cells but not Th2 cell responses (S5). Likewise, Helios⁺ Tregs may be endowed with a function that suppress production of certain cytokines including IL-17.

In our study, Helios⁺ Tregs inhibited the production of inflammatory cytokines such as IFN- γ and IL-17 more efficiently than Helios⁻ Tregs did. Some investigators reported that heat shock protein 70 (HSP70), which is one of the inflammatory mediators of CHS, attenuates CHS by increasing TGF- β secretion and number of CD4⁺CD25⁺Foxp3⁺ Tregs (9). As Helios is effectively induced with TCR-stimulation in the presence of TGF- β (7), these findings have important implications for therapeutic strategy to attenuate CHS by deliberately increasing Helios⁺ Tregs in the skin DLNs with HSP70.

Conclusions

We generated Helios^{Venus} mice, a strain that enables us to isolate Helios⁺ cells. Using this system, we could assess the suppressive function of Helios⁺ Tregs. Our finding indicated that Helios is a new marker for isolating or preparing potent suppressive Tregs.

Helios^{Venus} mice can be applied to analyze the function and developmental process of Helios⁺ cells, which include Foxp3⁺ Tregs, Helios⁺ conventional T cells of unknown function, NK cells, and neuronal cells.

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Author contributions

KS, KK and TN designed the research study. KS, SH, TH and TN performed the experiments. KS, SH and TN analyzed the data, prepared the Figures and drafted the manuscript under the supervision of GK, YM and KK. KS, SH, KK and TN wrote the paper. SH, TH, YM, KK and TN reviewed the paper.

Conflicts of interest

The authors declare no conflicts of interest.

Figure legends

Figure 1.

(a) Targeting of murine Helios gene. A targeting vector named p88C8, containing Venus cDNA with a terminator sequence (white box) and a neomycin-resistant gene (Neo^R) cassette flanked with FLP recombinase targets (FRT), was introduced in KY1.1 ES cells by electroporation followed by selection by G418. We analyzed the clones by genomic Southern blots and found five independent clones, three of which were used for blastocyst injections. Numbered boxes indicate exons. ATG indicates the first start codon residing in exon 2. FRT indicates flippase recognition target. (b) Expression of Helios in Tregs in

various tissues. CD4⁺ T cells in Helios^{Venus}/Foxp3^{hCD2/hCD52} mice were assessed for the expression of Helios. Representative flow plots, gated on CD4⁺ T cells, show the frequency of cells expressing Foxp3 and Helios. In the thymus, most of the Foxp3⁺ cells were Helios⁺. CD4SP and LNs indicates CD4 single positive thymocytes and lymph nodes, respectively. (c and d) The percentage and number of Foxp3⁺CD4 T cells expressing Helios. (e) Mean fluorescence intensity (MFI) of CD25 and (f) CTLA-4. Statistical significance between the indicated groups was indicated by * and **** for $P < 0.05$ and $P < 0.0001$, respectively.

Figure 2.

(a) The skin DLNs of Helios^{Venus}/Foxp3^{hCD2/hCD52} mice were collected five days after sensitization with DNFB and were stained with CD4 and hCD2 mAbs. The flow cytometric plot shows Helios and hCD2/Foxp3 expression among CD4⁺ cells.

(b) Skin DLN cells of mice sensitized with DNFB were stimulated with DNBS or anti-CD3 ϵ Ab in the presence or absence of Helios⁻Tregs or Helios⁺Tregs *in vitro*.

Asterisk (*) indicates $P < 0.05$ between the indicated groups. Data are presented as means \pm SEM.

(c) Suppressive effect of Helios⁺Tregs on cytokine production *in vitro*. Skin DLN cells were stimulated as above and cultured for 72 hours and then supernatants were prepared for cytokine production. Asterisk (*) indicates $P < 0.05$ between the indicated groups. Data are presented as means \pm SEM.

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E-supplement

Experimental design

Targeted KY1.1 clones were injected in Blastocysts, obtained from F₁ of C57BL/6CrSlc females and DBA/2CrSlc males, were injected with Helios-targeted KY1.1 clones and transferred into pseudopregnant ICR female mice to obtain chimaera mice, which were mated with C57BL/6J to generate Helios^{Venus} reporter mouse strain that expresses Venus under the control of Helios promoter. The strain was backcrossed to C57BL/6 mice for at least 8 generations.

To evaluate the role of Helios⁺ Tregs, we generated Helios^{Venus}/Foxp3^{hCD2/hCD52} mice by inter-crossing Helios^{Venus} mice with Foxp3^{hCD2/hCD52}, in which Foxp3⁺ Tregs express human CD2/human CD52 fusion protein on the surface (S1). These mice were bred in specific pathogen-free facilities at Kyoto University. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Faculty of Medicine. Fluorochrome-conjugated anti-mouse CD3 ϵ , CD4, CD8a, CD25, CD45.2, 7-AAD, Helios, Foxp3, human CD2, CTLA-4 and Helios mAbs were obtained from BD Bioscience (San Jose, CA), eBiosciences (San Diego, CA), or Biolegend (San

Diego, CA). Data were acquired using LSRFortessa (BD Biosciences) and analyzed with FlowJo software (TreeStar, San Carlos, CA). For cell-sorting, Helios⁺ Tregs or Helios⁻ Tregs were purified from inguinal and axillary lymph nodes (LNs) of Helios^{Venus}/Foxp3^{hCD2/hCD52} mice by the FACS Aria II flow cytometry system (BD Biosciences). For 2,4-dinitrobenzene sulfonic acid (DNBS)-dependent cell proliferation, mice were sensitized with 50 μ l of 0.5% (wt/vol) 2,4-dinitro-1-fluorobenzene (DNFB) (Nacalai Tesque, Kyoto, Japan) in acetone/olive oil (4:1) on the dorsal skin. Five days later, single-cell suspensions were prepared from inguinal and axillary LNs and were cultured in 96-well plate at densities of 7×10^5 LN cells/well in RPMI 1640 containing 10% fetal bovine serum (FBS) with or without 50 μ g/ml DNBS for 72 hours. IFN- γ and IL-17 in the culture supernatants were measured by ELISA (R&D systems, Minneapolis, MN). Otherwise plate-bound anti-CD3 ϵ antibody (immobilized at 1 μ g/ml in PBS without Ca²⁺/Mg²⁺ for 15 min at room temperature) was used to stimulate LN cells.

Figure legends

Figure S1.

Kinetics of Helios⁺ or Helios⁻ Tregs in the DLNs after the DNFB challenge. The number of Helios⁺ Tregs in the DLNs was much higher than Helios⁻ Tregs at each time point tested. Asterisk (*) indicates $P < 0.05$ between the indicated groups. Data are presented as means \pm SEM.

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Figure 1

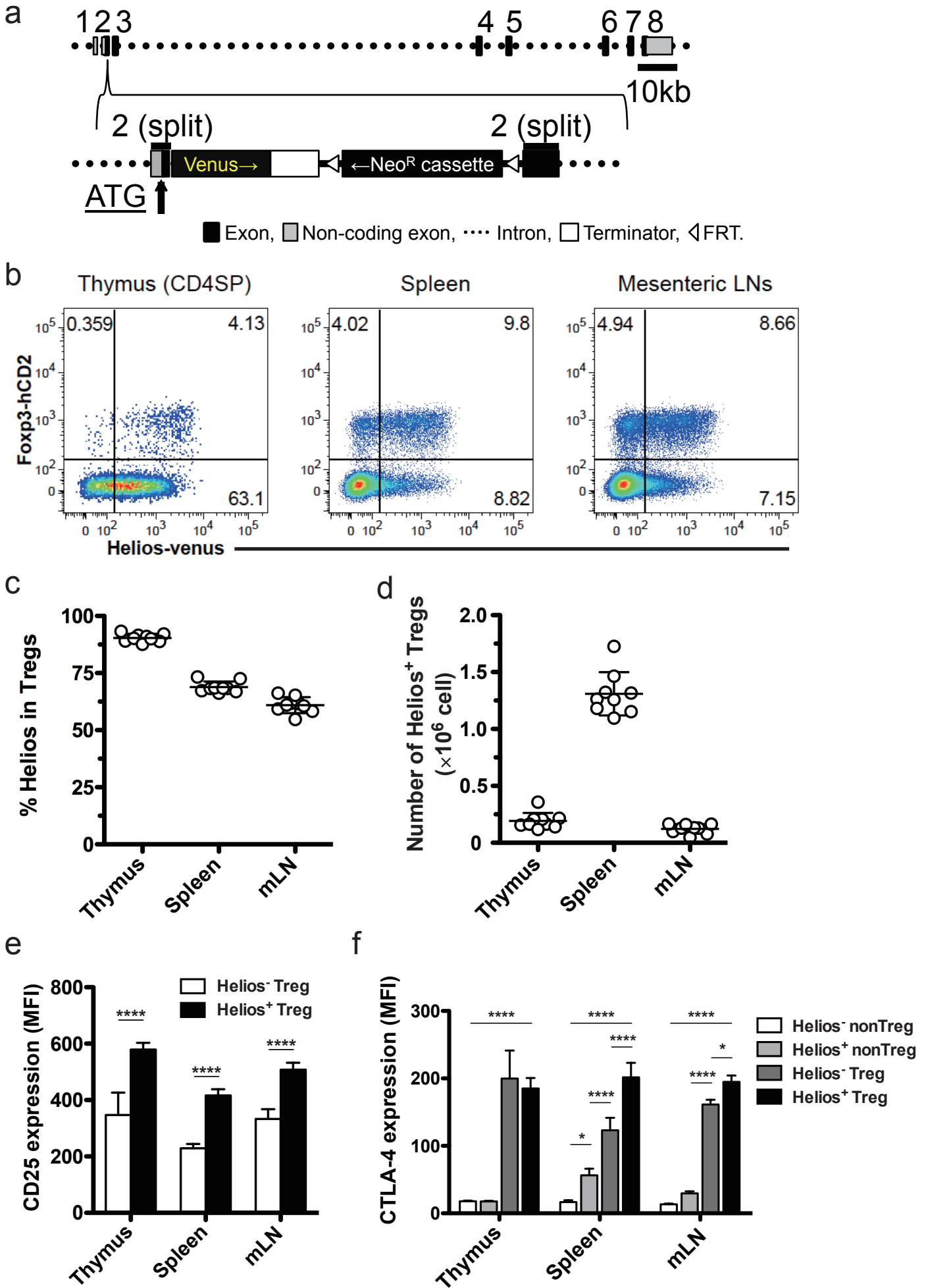
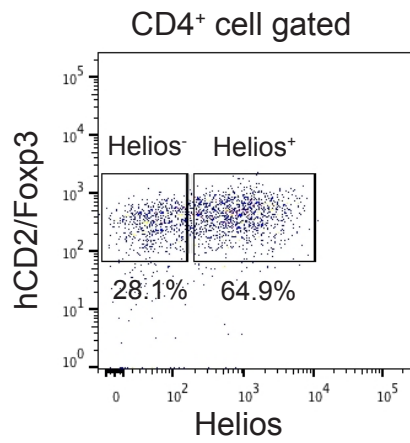
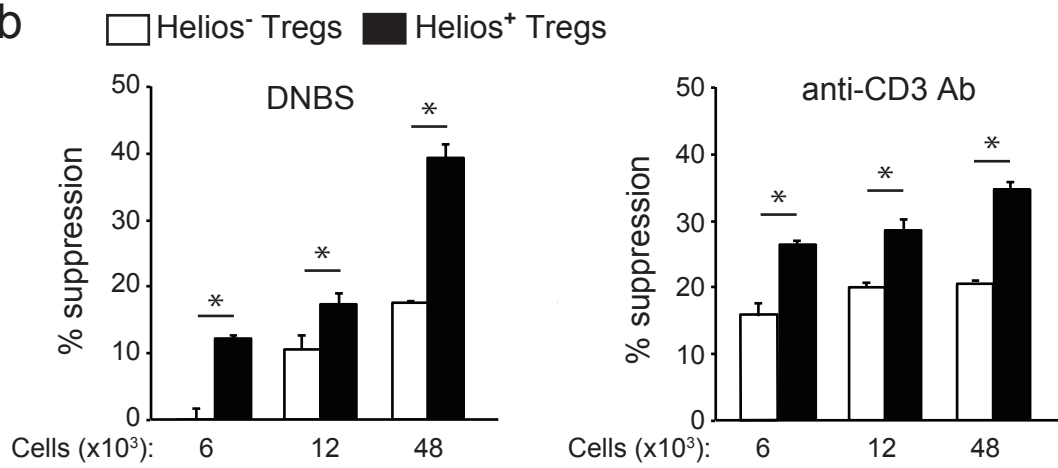


Figure 2

a



b



c

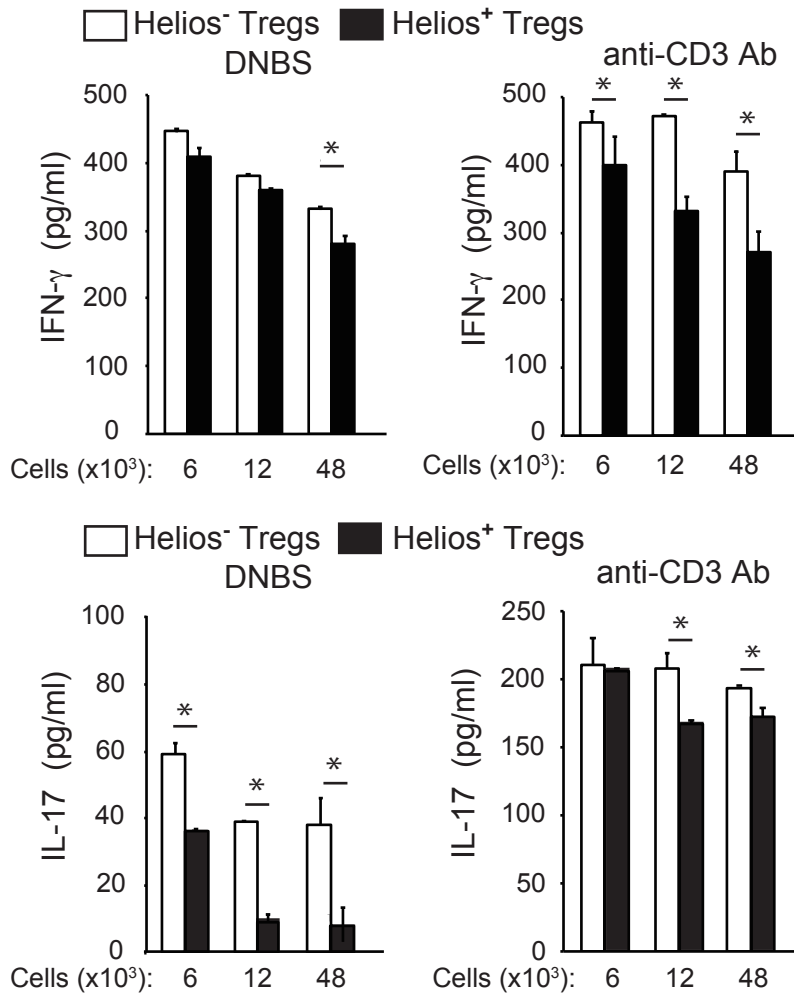


Figure S1

