The Journal of Immunology

CD8$^+$CD122$^+$ Regulatory T Cells (Tregs) and CD4 $^+$ Tregs Cooperatively Prevent and Cure CD4 $^+$ Cell-Induced Colitis

Agustina Tri Endharti, Yusuke Okuno, Zhe Shi, Nobuaki Misawa, Shinya Toyokuni, Masafumi Ito, Ken-ichi Isobe and Haruhiko Suzuki

J Immunol 2011; 186:41-52; Prepublished online 22 November 2010;
doi: 10.4049/jimmunol.1000800
http://www.jimmunol.org/content/186/1/41

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

$CD8^+CD122^+$ Regulatory T Cells (Tregs) and $CD4^+$ Tregs Cooperatively Prevent and Cure $CD4^+$ Cell-Induced Colitis

Agustina Tri Endharti,*,† Yusuke Okuno,* Zhe Shi,‡ Nobuaki Misawa,§ Shinya Toyokuni,§ Masafumi Ito,¶ Ken-ichi Isobe,* and Haruhiko Suzuki*

We identified $CD8^+CD122^+$ regulatory T cells (Tregs) and demonstrated their importance in the maintenance of immune homeostasis and in the recovery from experimental autoimmune encephalomyelitis. In this paper, we show that $CD8^+CD122^+$ Tregs effectively prevent and cure colitis in a mouse model. In our experiments, colitis was induced in lymphocyte-deficient RAG-$2^{-/-}$ mice by transferring $CD4^+CD45RB^{high}$ cells that were excluded with $CD4^+$ Tregs. Cotransfer of $CD8^+CD122^+$ cells clearly suppressed the development of colitis, and this suppressive effect was similar to that of $CD4^+CD45RB^{low}$ cells that were mostly $CD4^+$ Tregs. $CD8^+CD122^+$ cells obtained from IL-$10^{-/-}$ mice were unable to suppress colitis, indicating that IL-10 is an important effect-transmitting factor in the suppression of colitis. $CD8^+CD122^+$ cells showed a suppressive effect when they were transferred 4 wk after $CD4^+CD45RB^{high}$ cells, indicating the therapeutic potential of $CD8^+CD122^+$ cells. A mixture of $CD8^+CD122^+$ cells and $CD4^+CD45RB^{low}$ cells was far more effective than single Tregs, indicating the synergistic effect of these Tregs. These overall findings demonstrate the potential role of $CD8^+$ Tregs, and possibly together with $CD4^+$ Tregs, in the medical care of inflammatory bowel disease patients. ***The Journal of Immunology*****, 2011, 186: 41–52.**

Inflammatory bowel disease (IBD) is a general term for ulcerative colitis and Crohn's disease. The prevalence of ulcerative colitis is approximately >0.3 million in the United States and 0.6 million in the world. Six to seven per 0.1 million in the United States and >0.4 million patients in world suffer from Crohn's disease. Although an immunological mechanism is thought to be involved in the progression of IBD, the pathogenesis of IBD is largely unknown (1–4).

Because murine models of human IBD have been established, they have been frequently used to study colitis development and therapeutic options (5, 6). One such colitis model is the transfer of $CD4^+CD45RB^{high}$ cells into lymphopenic mice such as RAG-deficient mice, SCID mice, or nude mice (6–8). Because $CD4^+CD45RB^{high}$ cells are excluded with naturally arising $CD4^+CD25^+Foxp3^+$ regulatory T cells (nTregs), $CD4^+$ cells that are not regulated by nTregs are thought to cause colitis (6, 9, 10). Actually, transfer of $CD4^+CD25^+$ nTregs into mice with $CD4^+CD45RB^{high}$ cell transfer-induced colitis effectively prevented and cured colitis, further confirming the importance of $CD4^+CD25^+$ nTregs in colitis (11–13). The importance of $CD4^+CD25^+Foxp3^+$ Tregs in the maintenance of immune homeostasis has been well documented by other animal disease models such as experimental autoimmune encephalomyelitis (EAE) (14). Despite the importance of $CD4^+$ Tregs in colitis, the involvement of other Tregs, especially $CD8^+$ Tregs, has not been well studied (15, 16).

T cells are largely divided into two populations of $CD4^+$ and $CD8^+$ cells, and both populations contain Tregs (17, 18). Compared with the rapid expansion of studies on $CD4^+$ Tregs, there are few studies of $CD8^+$ Tregs. We have identified $CD8^+$ Tregs that express CD122, the β-chain of IL-2R, and proved the importance of such Tregs in the maintenance of immune homeostasis (19). We also reported that such $CD8^+CD122^+$ Tregs play an essential role in the recovery phase of EAE, a mouse model of multiple sclerosis (20). Considering the beautiful harmony, coordination, and synergy between $CD4^+$ cells and $CD8^+$ cells in general, it is reasonably logical that Tregs distribute both $CD4^+$ and $CD8^+$ populations. We have clarified part of the regulatory mechanism of $CD8^+CD122^+$ Tregs that is different from that of $CD4^+CD25^+Foxp3^+$ Tregs (21). However, the shared role of these two types of Tregs remains unknown.

In this study, we first investigated the role of $CD8^+CD122^+$ Tregs in preventing and curing colitis in a mouse model. Furthermore, we studied the relationship of $CD8^+CD122^+$ Tregs and $CD4^+CD25^+Foxp3^+$ Tregs and found evidence of a synergistic effect of these Tregs.

Materials and Methods

Mice

C57BL/6 mice (6–8 wk old) were purchased from Japan SLC (Hamamatsu, Japan). Recombination activating gene (RAG)-$2^{-/-}$ mice with C57BL/6 genetic background were originally purchased from Taconic Farms (Hudson, NY) and maintained in our animal facility. IL-10–knockout mice with C57BL/6 genetic background were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in our animal facility. All mice used in this study were maintained under specific pathogen-free conditions. Animal care was conducted in accordance with the guidelines of Nagoya University (Nagoya, Japan).

*Department of Immunology and §Department of Pathology and Biological Responses, Nagoya University Graduate School of Medicine; ¶Department of Pathology, Japanese Red Cross, Nagoya Daiichi Hospital, Nagoya; †Japanese Society for Promotion of Science; and ‡Department of Pediatrics, Tokyo Medical and Dental University, Tokyo, Japan

Received for publication March 12, 2010. Accepted for publication October 16, 2010.

This work was supported by a grant-in-aid from the Japanese Ministry of Education, Culture, Science, Sports, and Technology and the Japanese Society for Promotion of Science.

Address correspondence and reprint requests to Dr. Haruhiko Suzuki, Department of Immunology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. E-mail address: harusuzu@med.nagoya-u.ac.jp

The online version of this article contains supplemental material.

Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; IBD, inflammatory bowel disease; nTreg, naturally arising regulatory T cell; RAG, recombination activating gene; SAA, serum amyloid-α; Treg, regulatory T cell.

Abs

FITC- or PE-conjugated anti-mouse CD8a (clone 53-6.7), FITC- or PE-conjugated anti-mouse CD4 (L3T4), FITC-conjugated anti-mouse CD25 (clone PC61.5), biotin-conjugated anti-mouse CD4 (clone H129.19), biotin-conjugated anti-mouse CD122 (clone 5H4), PE-conjugated anti-mouse CD45RB (clone 363-16A9), PE-conjugated anti-mouse IL-2 (clone JES6-5H4), biotin-conjugated anti-mouse IFN-γ (clone XMG1.2), FITC- or biotin-conjugated anti-mouse CD45.1 (clone A20) Abs, biotin-conjugated anti-mouse Foxp3 (clone FJK-16a) and streptavidin-PE-Cy5 conjugate were purchased from eBioscience (San Diego, CA).

Serum amyloid-α quantification by ELISA

Serum concentrations of serum amyloid-α (SAA) levels were monitored every week and determined by ELISA (BioSource International, Camarillo, CA). The plates were read at an OD of 450 nm as described previously (22).

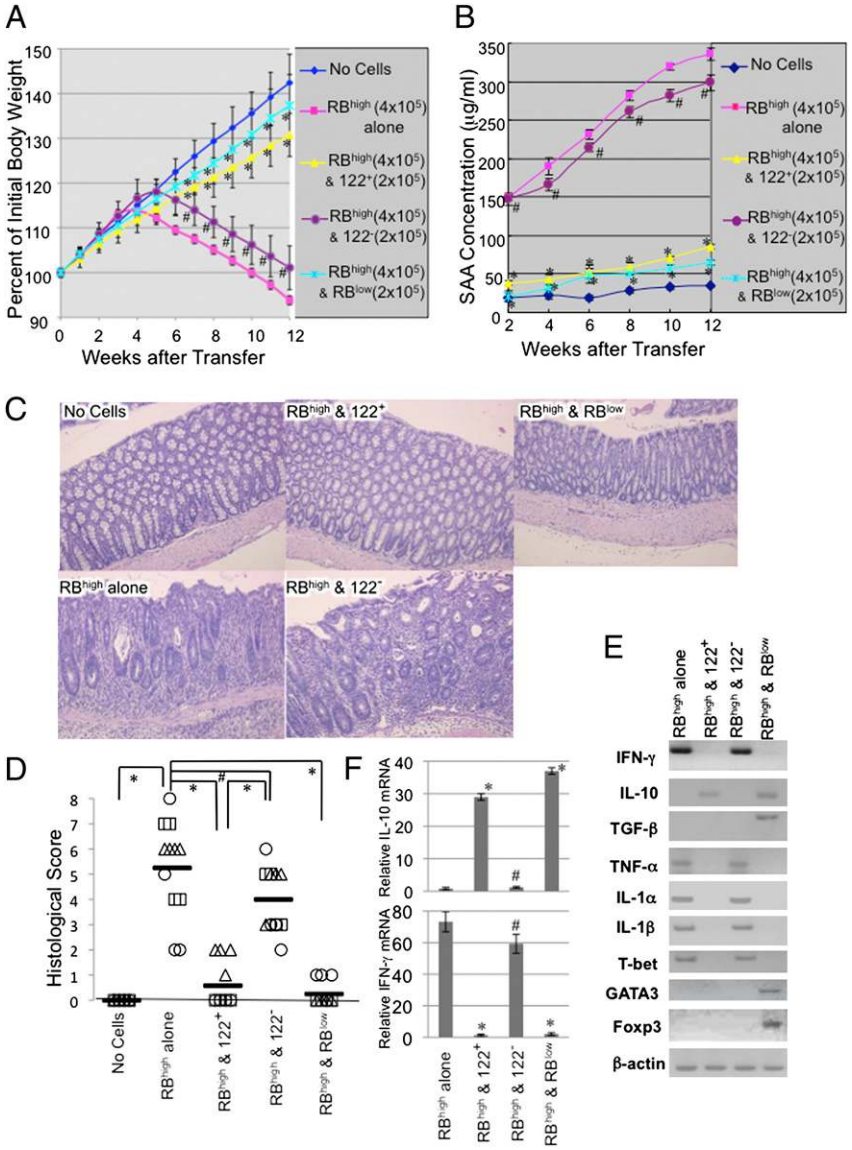

FIGURE 1. Prevention of colitis by CD8$^+$CD122$^+$ Tregs. *A*, D4$^+$CD45RB^{high} cells (4×10^5) in combination with either 2×10^5 CD4$^+$CD45RB^{low} cells, 2×10^5 CD8$^+$CD122$^+$ cells, or 2×10^5 CD8$^+$CD122$^-$ cells were transferred into RAG-2$^{-/-}$ mice. Change of body weight was monitored until 12 wk after T cell transfer. Data were obtained from three independent experiments ($n = 3$), each of which contained four mice ($n = 4$ each and $n = 12$ total; error bar = SD). Data of transfer of CD4$^+$CD45RB^{low} cells, CD8$^+$CD122$^+$ cells, or CD8$^+$CD122$^-$ cells mixed with CD4$^+$CD45RB^{high} cells were compared with that of transfer of CD4$^+$CD45RB^{high} cells alone; data with statistically significant differences (*$p < 0.005$; Student *t* test and Bonferroni correction) and data without significant differences (#$p > 0.005$; Student *t* test and Bonferroni correction). *B*, SAA was monitored ($n = 4$). Data of transfer of CD4$^+$CD45RB^{low} cells, CD8$^+$CD122$^+$ cells, or CD8$^+$CD122$^-$ cells mixed with CD4$^+$CD45RB^{high} cells were compared with that of transfer of CD4$^+$CD45RB^{high} cells alone; data with statistically significant differences (*$p < 0.005$; Student *t* test and Bonferroni correction) and data without significant difference (#$p > 0.005$; Student *t* test and Bonferroni correction). *C*, Mice were sacrificed at 12 wk after T cell transfer, and the dissected colon was analyzed by H&E staining (original magnification ×100). Representative pictures of each group are shown. *D*, Histological scores of individual mice are shown as ○ (first experiment, $n = 4$), △ (second experiment, $n = 4$), and □ (third experiment, $n = 4$), and the averages of each group are shown as horizontal bars (total, $n = 12$). Statistical analysis was performed. Data with significant differences between two groups (*$p < 0.005$; Student *t* test and Bonferroni correction) and data without significant differences (#$p > 0.005$; Student *t* test and Bonferroni correction). *E*, Colon tissue was obtained from RAG-2$^{-/-}$ mice treated as in *A*, and RT-PCR was performed to evaluate the expression of cytokines and transcription factors. Representative results obtained from four mice in each group are shown. *F*, Results of real-time PCR analysis are shown as bar graphs. Data obtained from four mice were analyzed, and the averages with SD (error bars) are shown. Significant differences compared with control (CD45RB^{high} alone) (*$p < 0.00825$; Student *t* test and Bonferroni correction) and not significant differences (#$p > 0.00825$; Student *t* test and Bonferroni correction).

Table I. *Fecal blood of $CD4^+CD45RB^{high}$ cell-induced colitis is prevented by cotransfer of $CD8^+CD122^+$ cells*

Transferred Cells	Weeks after T Cell Transfer									
	3	4	5	6	7	8	9	10	11	12
No cells	0/4	0/4	0/4	0/4	0/4	04	0/4	0/4	0/4	0/4
4^+45RB^{high} alone (4×10^5)	0/4	1/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
4^+45RB^{high} and 8^+122^+ (2×10^5)	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
4^+45RB^{high} and 8^+122^- (2×10^5)	0/4	0/4	1/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4
4^+45RB^{high} and 4^+45RB^{low} (2×10^5)	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

Rag-$2^{-/-}$ mice that received 4×10^5 $CD4^+CD45RB^{high}$ cells with or without 2×10^5 of indicated cells in the table were analyzed for their fecal blood every week. Data indicate fecal blood-positive mice in total mice used for each experimental group.

RT-PCR

Total RNA was extracted from the descending colon with TRIzol (Invitrogen, Carlsbad, CA). The cDNA reaction was conducted using Superscript II reverse transcriptase (Invitrogen). PCR (25–40 cycles) was performed in a thermal cycler using the following conditions: denaturation, 45 s at 94°C; annealing, 1 min at 60°C; and extension, 90 s at 72°C. The PCR products were electrophoresed through a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide and visualized under UV light. The sequences of the primer are shown in Supplemental Table I.

Real-time RT PCR

Real-time PCR was performed using reaction mixture of SYBR Premix Ex Taq II (Takara, Ohtsu, Japan) and ROX Reference Dye II (Takara). Initial denaturation at 95°C for 10 s, 40 cycles of amplication with denaturation at 95°C for 5 s, followed by annealing at 60°C for 20 s and dissociation at 95°C for 60 s, 55°C for 30 s, and 95°C for 30 s. Product quantity was subtracted using hypoxanthine phosphoribosyltransferase as an internal housekeeping gene control. PCRs were performed in triplicate. Expression of IL-10 and hypoxanthine phosphoribosyltransferase was detected by using Mx3000P QPCR system (Stratagene, La Jolla, CA) and analyzed by MxPro QPCR Software.

Activation of $CD8^+122^+$ T cells in vitro

Freshly isolated $CD8^+CD122^+$ cells (5×10^5) obtained from C57BL/$6^{CD45.2}$ mice were cultured in anti-CD3 mAb-coated 96-well plates with a medium containing recombinant human IL-2 (10 ng/ml). Twenty-four hours later, these activated $CD8^+CD122^+$ cells (1×10^6) were transferred into Rag-$2^{-/-}$ mice that had received $CD4^+CD45RB^{high}$ cells 4 wk before.

Cell preparation

Spleen cells were incubated with anti-mouse CD8- or CD4-coated magnetic beads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), and the CD8 or CD4 cell-enriched fraction was recovered from the magnetic column, according to the manufacturer's protocol. Then, cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD45RB or FITC-conjugated anti-CD8 and biotin-conjugated anti-CD122, followed by staining with streptavidin-PECy5. Stained cells were subjected to the FACSVantage cell sorter (BD Biosciences, San Jose, CA), and $CD8^+$ $CD122^+$, $CD8^+CD122^-$, $CD4^+CD45RB^{high}$, and $CD4^+CD45RB^{low}$ cells were obtained.

T cell transfer for therapeutic effect

A total of 4×10^5 $CD4^+CD45RB^{high}$ T cells obtained from C57BL/$6^{CD45.1}$ mice were i.p. injected into Rag-2–deficient mice of C57BL/6 genetic background. When the mice developed colitis 4–5 wk after transfer of $CD4^+CD45RB^{high}$ cells, mice secondarily received 1×10^6 naive $CD8^+$ $CD122^+$ cells, preactivated $CD8^+CD122^+$ cells, or $CD4^+25^+$ cells. These adoptively transferred mice were monitored for 14 wk.

Induction of experimental colitis

Splenic $CD4^+CD45RB^{high}$ cells from C57BL/$6^{CD45.1}$ mice were isolated by FACS (FACSVantage; BD Biosciences). Sorted cells were >98% pure, and 4×10^5 cells of $CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ at a 2:1 or 4:1 ratio were coinjected with $CD8^+CD122^+$ T cells i.p. into recipient RAG-2–deficient mice. Body weight, fecal blood, and SAA levels were monitored weekly. The adoptively transferred mice were monitored for 12 wk.

T cell culture and cytokine assay

For detection of cytoplasmic cytokines, the indicated cells were stimulated on 96-well flat-bottom plates coated with anti CD3 mAb (145-2C11; BD Biosciences) at a density of 5×10^5 cells/well. $CD4^+$cells were cocultured with cells obtained from mesenteric lymph nodes of cell-transferred mice at

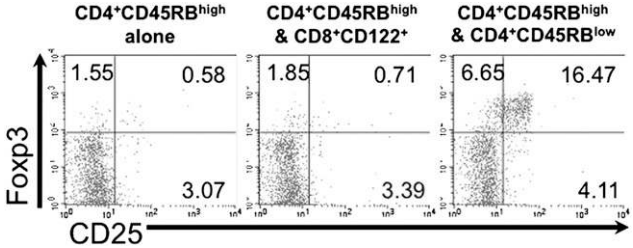

FIGURE 2. Foxp3 expression in colitis-induced mice. Mesenteric lymph nodes were obtained from Rag-$2^{-/-}$ mice that received 4×10^5 $CD4^+$ $CD45RB^{high}$ cells alone, 4×10^5 $CD4^+CD45RB^{high}$ cells plus 2×10^5 $CD8^+CD122^+$ cells, or 4×10^5 $CD4^+CD45RB^{high}$ cells plus 2×10^5 $CD4^+$ $CD45RB^{low}$ cells. Cells were first stained with anti-CD4 and anti-CD25 Abs, fixed, and permeabilized using the solution provided by eBioscience and stained by anti-Foxp3 Ab. Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences), and expression of CD25 and Foxp3 in cells gated to $CD4^+$ is shown. Percentages of cells in each quadrant are shown inside the panels. Representative results of four mice in each group are shown.

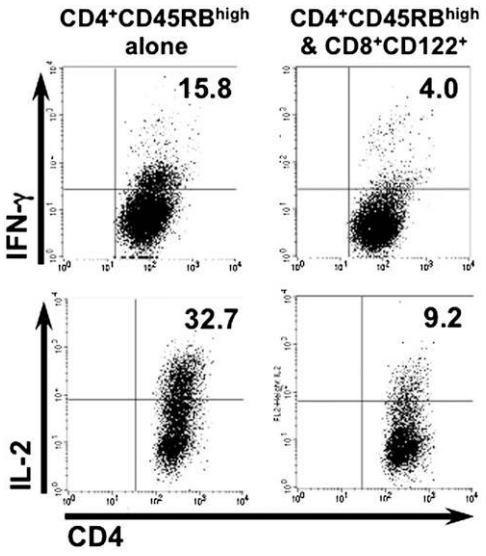

FIGURE 3. Cells with suppressive activity exist in mesenteric lymph nodes of mice that received $CD8^+CD122^+$ cells. Rag-$2^{-/-}$ mice that received $CD4^+CD45RB^{high}$ cells ($CD45.1^+$) alone or $CD4^+CD45RB^{high}$ cells ($CD45.1^+$) plus $CD8^+CD122^+$ cells were sacrificed at 12 wk after T cell transfer, and the cells recovered from mesenteric lymph nodes were cocultured with $CD4^+$ cells freshly isolated from C57BL/6 mice ($CD45.1^-$). After culture for 48 h, cells were stained with anti-CD45.1 and anti-CD4 Abs and then fixed, permeabilized, and stained with anti–IFN-γ or anti–IL-2 Ab. Cells were analyzed by flow cytometry, and expression of IFN-γ and IL-2 in $CD4^+CD45.1^-$ cells was assessed. Percentages of cells expressing the indicated cytokines in total $CD4^+$ cells are shown in each panel. Representative results of three independent experiments are shown.

a ratio of 1:1. Cells were cultured for 48 h and treated with PMA (50 ng/ml)-ionomycin (1 μg/ml) and GolgiPlug (1 μg/ml) for the final 4 h. The stimulated cells were subjected to intracellular cytokine analysis using the BD Cytofix/Cytoperm kit per the manufacturer's instructions (BD Biosciences). Cytokine expression was analyzed with a FACSCalibur flow cytometer (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

Histology

Descending colons of mice were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E. Histological assessment of colon inflammation was performed by a single pathologist in a blinded fashion using a standardized scoring system, as described previously (23, 24).

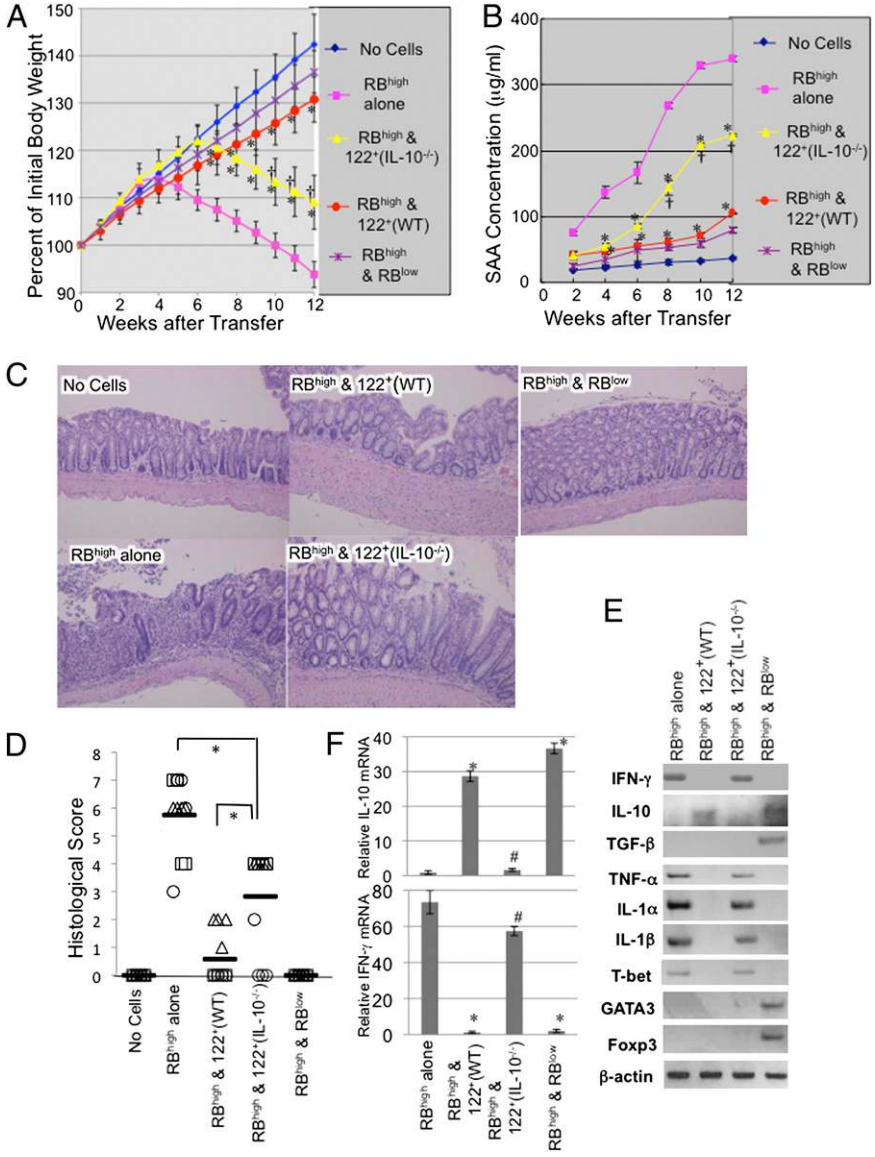

FIGURE 4. The importance of IL-10 in the suppression of colitis. *A*, $CD4^+CD45RB^{high}$ cells (4×10^5) in combination with either 2×10^5 $CD4^+$ $CD45RB^{low}$ cells, 2×10^5 $CD8^+CD122^+$ cells obtained from wild-type (WT) C57BL/6 mice, or 2×10^5 $CD8^+CD122^+$ cells obtained from IL-$10^{-/-}$ mice were transferred into RAG-$2^{-/-}$ mice. Change of body weight was monitored until 12 wk after T cell transfer. Data were obtained from three independent experiments ($n = 3$), each of which contained four mice (total, $n = 12$; error bar = SD). Data of transfer of $CD122^+$ cells derived from WT mice or $CD122^+$ cells derived from IL-10–deficient mice mixed with $CD45RB^{high}$ cells were compared with that of transfer of $CD45RB^{high}$ cells alone; data with statistically significant differences ($*p < 0.005$; Student *t* test and Bonferroni correction) and data of $CD122^+$ cells derived from IL-10–deficient mice with significant differences compared with that of $CD122^+$ cells derived from wild-type mice ($\dagger p < 0.005$; Student *t* test and Bonferroni correction). *B*, SAA was monitored ($n = 4$). Data of transfer of $CD122^+$ cells derived from WT mice or $CD122^+$ cells derived from IL-10–deficient mice mixed with $CD45RB^{high}$ cells were compared with that of transfer of $CD45RB^{high}$ cells alone; data with statistically significant differences ($*p < 0.005$; Student *t* test and Bonferroni correction) and data of $CD122^+$ cells derived from IL-10–deficient mice with significant differences compared with that of $CD122^+$ cells derived from WT mice ($\dagger p < 0.005$; Student *t* test and Bonferroni correction). *C*, Dissected colon was analyzed by H&E staining (original magnification ×100). Representative pictures of each group are shown. *D*, Histological scores of colitis of individual mice are shown as ○ (first experiment, $n = 4$), △ (second experiment, $n = 4$), and □ (third experiment, $n = 4$). Data with statistically significant differences between two groups ($*p < 0.005$; Student *t* test and Bonferroni correction). *E*, Colon tissue was analyzed by RT-PCR to evaluate the expression of cytokines and transcription factors. Representative results obtained from four mice in each group are shown. *F*, Results of real-time PCR analysis are shown as bar graphs. Data obtained from four mice were analyzed, and the averages with SD (error bars) are shown. Significantly different values compared with control ($CD45RB^{high}$ alone) ($*p < 0.00825$; Student *t* test and Bonferroni correction) and not significantly different values ($\#p > 0.00825$; Student *t* test and Bonferroni correction).

Statistical analysis

For the analysis of body weight change of mice and histological scores of colitis, we combined the data obtained from three independent experiments ($n = 3$), each of which contained 4 mice ($n = 4$). A total of 12 mice were analyzed in each group. For the analysis of SAA and fecal blood tests, we performed an initial experiment ($n = 1$) containing four mice ($n = 4$). Judgment of significant difference was based on Student t test, and Bonferroni corrections were applied to determine the p value for each experiment (25).

Results

$CD8^+CD122^+$ cells but not $CD8^+CD122^-$ cells suppress the development of $CD4^+CD45RB^{high}$ cell-induced colitis

First, we examined the simple preventive effect of $CD8^+CD122^+$ Tregs on colitis. We induced colitis by transferring 4×10^5 FACS-purified $CD4^+CD45RB^{high}$ cells into lymphocyte-deficient RAG-$2^{-/-}$ mice. As we had previously shown that $CD8^+CD122^+$ Tregs suppressed not only $CD8^+$ cells but also $CD4^+$ cells in vitro (19), it was anticipated that the transfer of $CD8^+CD122^+$ Tregs would suppress $CD4^+CD45RB^{high}$ cell transfer-induced colitis. RAG-2–deficient mice that received $CD8^+CD122^+$ Tregs and $CD4^+CD45RB^{high}$ cells did not develop colitis signs, whereas mice that received $CD8^+CD122^-$ cells and $CD4^+CD45RB^{high}$ cells or $CD4^+CD45RB^{high}$ cells alone developed colitis. Colitis was indicated by weight loss (Fig. 1*A*), fecal blood (Table I), and SAA (Fig. 1*B*) and then terminally confirmed by histological analysis of the colon (Fig. 1*C*, 1*D*). There are almost no abnormal changes including infiltration of inflammatory cells into the colon mucosa of mice that received $CD4^+CD45RB^{high}$ cells plus $CD8^+CD122^+$ cells or $CD4^+CD45RB^{high}$ cells plus $CD4^+CD45RB^{low}$ cells similarly to those of mice without cell transfer, compared with massive infiltration of inflammatory cells including lymphocytes and neutrophils into the lamina propria of the colon in mice that received $CD4^+CD45RB^{high}$ cells alone. The transfer of 2×10^5 $CD8^+CD122^+$ cells had a similar effect on colitis as the same number of $CD4^+CD45RB^{low}$ cells that included $CD4^+CD25^+Foxp3^+$ Tregs. RT-PCR analysis of colon tissue indicated that inflammatory cytokines (TNF-α and IL-1s) and IFN-γ are expressed in colons of mice with colitis that received $CD4^+CD45RB^{high}$ cells alone or $CD4^+CD45RB^{high}$ cells plus $CD8^+CD122^-$ cells but not in the colons of mice without colitis that received $CD4^+CD45RB^{high}$ cells plus $CD8^+CD122^+$ cells or $CD4^+CD45RB^{high}$ cells plus $CD4^+CD45RB^{low}$ cells (Fig. 1*E*). Rather, expression of immunosuppressive cytokines including IL-10 and TGF-β was not observed in colons with colitis, but IL-10 was observed in colons free of colitis in mice that received $CD4^+CD45RB^{high}$ cells plus $CD8^+CD122^+$ cells and those that received $CD4^+CD45RB^{high}$ cells plus $CD4^+CD45RB^{low}$ cells. These RT-PCR and gel electrophoresis findings were confirmed by real-time PCR analysis (Fig. 1*F*). Expression of TGF-β was also observed in the colons of mice that received $CD4^+CD45RB^{high}$ cells plus $CD4^+CD45RB^{low}$ cells (Fig. 1*E*). Expression of T-bet had a similar pattern to that of IFN-γ and other inflammatory cytokines, whereas the expression pattern of GATA3 was similar to that of TGF-β. Transfer of $CD8^+CD122^+$ cells resulted in induction of IL-10 but no detectable induction of TGF-β and GATA3, all of which were induced by transfer of $CD4^+CD45RB^{low}$ cells. This suggested a different mechanism of colitis prevention between $CD8^+CD122^+$ cells and $CD4^+CD45RB^{low}$ cells that contain $CD4^+CD25^+Foxp3^+$ Tregs. Another interesting finding observed in the mixed transfer of effector cells ($CD4^+CD45RB^{high}$) and Tregs ($CD4^+CD45RB^{low}$ or $CD8^+CD122^+$ cells) is that reversal of cell number between effector cells and regulatory cells occurs during 12 wk of experimental period (Supplemental Fig. 1*A*). The ratio of cell number between effector cells and regulatory cells at the time of cell transfer was 2:1, whereas the ratio became 1:2∼1:5 after 12 wk. This phenomenon is supposed to be caused by expansion of regulatory cells and/or decrease of effector cells.

$CD8^+CD122^+$Tregs suppress $CD45RB^{high}$ cell-induced colitis without inducing $CD4^+CD25^+Foxp3^+$ Tregs

$CD8^+CD122^+$ cells suppressed the development of colitis (Fig. 1). There were some possibilities for this experimental result. $CD8^+CD122^+$ cells may have directly suppressed the activity of $CD4^+$ effector T cells or $CD8^+CD122^+$ T cells may induce Tregs from $CD4^+CD45RB^{high}$ cells. To distinguish these possibilities, we analyzed Foxp3 expression. In the RT-PCR analysis of colon tissue, we observed Foxp3 expression in the colons of mice that received $CD4^+CD45RB^{high}$ cells plus $CD4^+CD45RB^{low}$ cells but not in the colons of mice that received $CD4^+CD45RB^{high}$ cells plus $CD8^+CD122^+$ cells (Fig. 1*E*). In the flow cytometric analysis, mesenteric lymph node cells derived from mice that received $CD4^+CD45RB^{high}$ cells plus $CD4^+CD45RB^{low}$ cells clearly had a remarkable number of $CD4^+Foxp3^+$ cells but those derived from mice that received $CD4^+CD45RB^{high}$ cells plus $CD8^+CD122^+$ cells did not (Fig. 2). These results indicate that $CD8^+CD122^+$ cells do not induce $CD4^+Foxp3^+$ Tregs but likely suppress $CD4^+CD45RB^{high}$ cells directly. Another possible mechanism of suppression is that $CD8^+CD122^+$ Tregs induce IL-10–producing Tr1 cells. We isolated $CD4^+$ cells from mice that had received $CD4^+CD45RB^{high}$ cells plus $CD8^+CD122^+$ cells and examined the expression of IL-10 by real-time RT-PCR analysis. The result showed that $CD4^+$ cells did not express IL-10, indicating that Tr1 cells were not induced (Supplemental Fig. 1*B*).

Tregs that suppress the activity of colitis-promoting $CD4^+$ $CD45RB^{high}$ cells exist in colitis-free mice

We examined the suppressive activity of cells in mesenteric lymph nodes when the development of colitis was suppressed by transferring $CD8^+CD122^+$ Tregs. $CD4^+$ cells obtained from wild-type

Table II. *Fecal blood of $CD4^+CD45RB^{high}$ cell-induced colitis is only partially prevented by cotransfer of IL-10–deficient $CD8^+$ $CD122^+$ cells*

Transferred Cells	Weeks after T Cell Transfer									
	3	4	5	6	7	8	9	10	11	12
No cells	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
4^+45RB^{high} alone	0/4	1/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
4^+45RB^{high} and 8^+122^+ (WT)	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
4^+45RB^{high} and 8^+122^- (IL-$10^{-/-}$)	0/4	0/4	0/4	1/4	2/4	3/4	4/4	4/4	4/4	4/4
4^+45RB^{high} and 4^+45RB^{low}	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

Rag-$2^{-/-}$ mice that received 4×10^5 $CD4^+CD45RB^{high}$ cells with/without 2×10^5 of indicated cells obtained from wild-type (WT) mice or IL-$10^{-/-}$ mice were analyzed for their fecal blood every week. Data indicate fecal blood-positive mice in total mice used for each experimental group.

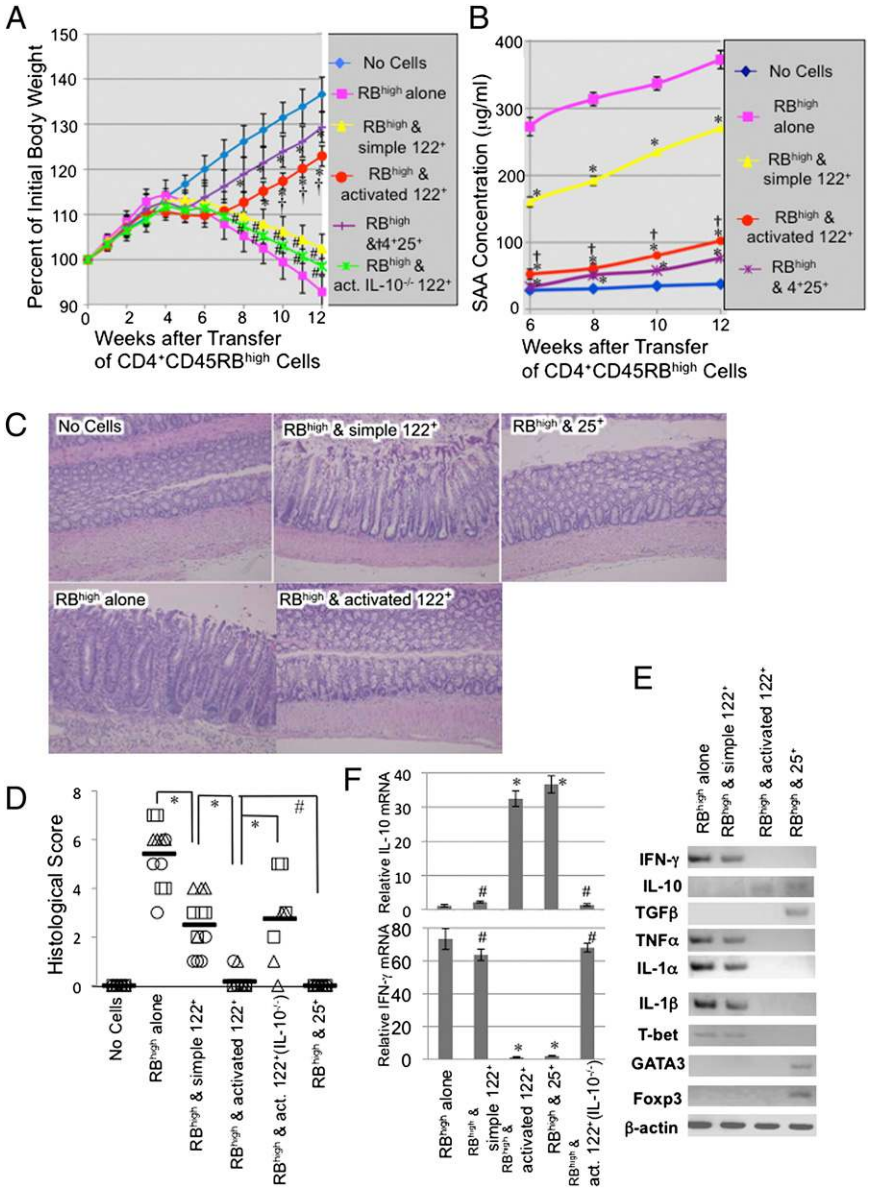

FIGURE 5. Therapeutic effect of activated $CD8^+CD122^+$ cells for colitis. *A*, $CD4^+CD45RB^{high}$ cells (4×10^5) were transferred into RAG-$2^{-/-}$ mice at week 0, and 2×10^5 $CD4^+CD25^+$ cells, 2×10^5 $CD8^+CD122^+$ cells, 2×10^5 in vitro-activated $CD8^+CD122^+$ cells, or in vitro-activated $CD8^+CD122^+$ cells derived from IL-$10^{-/-}$ mice were transferred at week 4. Change of body weight was monitored until 12 wk after the initial $CD4^+CD45RB^{high}$ cell transfer. The average data of 12 mice (three independent experiments of four mice each) in each group are demonstrated with error bars of SD. Data of transfer of $CD4^+CD25^+$ cells, wild-type (WT) mice-derived $CD122^+$ cells (simple $CD122^+$), in vitro-activated $CD122^+$ cells, or in vitro-activated $CD122^+$ cells derived from IL-$10^{-/-}$ mice were compared with that of transfer of $CD45RB^{high}$ cells alone; data with statistically significant differences ($*p < 0.0033$; Student *t* test and Bonferroni correction) and data without a significant difference ($\#p > 0.0033$; Student *t* test and Bonferroni correction). Data of in vitro-activated $CD122^+$ cells with significant differences compared with that of non-in vitro–activated $CD122^+$ cells (simple 122^+) ($\dagger p < 0.0033$; Student *t* test and Bonferroni correction). *B*, SAA was monitored until 12 wk after the initial $CD4^+CD45RB^{high}$ cell transfer. The average data of four mice in each group are demonstrated with error bars of SD. Data of transfer of $CD4^+CD25^+$ cells, non-in vitro–activated $CD122^+$ cells (simple 122^+), or in vitro-activated $CD122^+$ cells were compared with that of transfer of $CD45RB^{high}$ cells alone; data with statistically significant difference ($*p < 0.0033$; Student *t* test and Bonferroni correction). Data of in vitro-activated $CD122^+$ cells with significant difference compared with that of non-in vitro–activated $CD122^+$ cells (simple 122^+) ($\dagger p < 0.0033$; Student *t* test and Bonferroni correction). *C*, Mice were sacrificed 12 wk after T cell transfer, and dissected colon was analyzed by H&E staining (original magnification ×100). Representative pictures of each group are shown. *D*, Histological scores of colitis of individual mice are shown as ○ (first experiment, $n = 4$), △ (second experiment, $n = 4$), and □ (third experiment, $n = 4$), and the averages of each group are shown as horizontal bars. Data with statistically significant differences between the two groups ($*p < 0.0033$; Student *t* test and Bonferroni correction); data without statistically significant differences between the two groups ($\#p > 0.0033$; Student *t* test and Bonferroni correction). *E*, Colon tissue was obtained from RAG-$2^{-/-}$ mice treated as in *A*, and RT-PCR was performed to evaluate the expression of cytokines and transcription factors. Representative results obtained from four mice in each group are shown. *F*, Results of real-time PCR analysis are shown as bar graphs. Data obtained from four mice were analyzed, and the averages with SD (error bars) are shown. Significantly different values compared with control ($CD45RB^{high}$ alone) ($*p < 0.005$; Student *t* test and Bonferroni correction) and not significantly different values ($\#p > 0.00825$; Student *t* test and Bonferroni correction).

C57BL/6 mice were cocultured with cells obtained from mesenteric lymph nodes of mice that received $CD4^+CD45RB^{high}$ cells alone or a mixture of $CD4^+CD45RB^{high}$ cells and $CD8^+CD122^+$ cells, and then, expression of IFN-γ and IL-2 was measured by intracellular staining with Abs and flow cytometric analysis. Cells obtained from mesenteric lymph nodes of mice that received $CD4^+CD45RB^{high}$ cells and $CD8^+CD122^+$ cells were able to suppress expression of IFN-γ and IL-2 in cocultured $CD4^+$ cells compared with those of mice that received $CD4^+CD45RB^{high}$ cells alone (Fig. 3).

IL-10 is an important effect-transmitting factor produced by $CD8^+CD122^+$ Tregs that suppress colitis

Because IL-10 is the most important effect-transmitting factor for $CD8^+CD122^+$ Tregs (26), we next investigated the role of IL-10 in the prevention of colitis by $CD8^+CD122^+$ Tregs. $CD8^+CD122^+$ cells were collected from IL-$10^{-/-}$ mice, and their ability to suppress colitis was compared with that of wild-type mice. $CD8^+CD122^+$ cells derived from IL-$10^{-/-}$ mice were partially able to suppress $CD4^+CD45RB^{high}$ cell-induced colitis. Compared with no addition of Tregs to $CD4^+CD45RB^{high}$ cells, IL-10–deficient $CD8^+CD122^+$ cells partially reduced weight loss (Fig. 4*A*), reduced the SAA level (Fig. 4*B*), delayed the appearance of fecal blood (Table II), and improved histological changes in the colon (Fig. 4*C*, 4*D*). Compared with adding $CD8^+CD122^+$ cells derived from wild-type mice to $CD4^+CD45RB^{high}$ cells, the effect of IL-10–deficient $CD8^+CD122^+$ cells was significantly insufficient to normalize colitis induced by $CD4^+CD45RB^{high}$ cells (Fig. 4, Table II). These results correspond to the experiment using $CD8^+CD122^-$ cells as effector cells for $CD8^+CD122^+$ cells in our previous study (26), which showed insufficient suppressive activity of IL-10–deficient $CD8^+CD122^+$ Tregs compared with wild-type $CD8^+CD122^+$ cells but significant suppressive effect compared with no addition of $CD8^+CD122^+$ Tregs. The present study further suggested the presence of some compensatory factor(s) that suppresses effector T cells in the absence of IL-10. However, RT-PCR analysis and real-time PCR analysis revealed that the gene expression pattern of cytokines in IL-$10^{-/-}CD8^+CD122^+$ cell-transferred mice is similar to the pattern transferred with $CD4^+CD45RB^{high}$ cells alone rather than the pattern transferred with $CD4^+CD45RB^{high}$ cells plus wild-type mice-derived $CD8^+CD122^+$ cells (Fig. 4*E*, 4*F*), indicating the importance of IL-10 to suppress colitis produced by $CD8^+CD122^+$ cells.

$CD8^+CD122^+$ Tregs not only prevent the development of colitis but also work therapeutically on established colitis

We also examined whether $CD8^+CD122^+$ Tregs possessed therapeutic potential for established colitis. Purified $CD4^+CD45RB^{high}$ cells were transferred into RAG-$2^{-/-}$ mice, and 4 wk later, $CD4^+CD25^+$ cells, $CD8^+CD122^+$ cells, or $CD8^+CD122^+$ cells that had been activated by in vitro culture with stimulation by plate-bound anti-CD3 Ab were transferred into the colitis-induced mice. IL-10–deficient $CD8^+CD122^+$ cells that had been activated by anti-CD3 Ab were also examined for their suppressive effect on colitis. In this experiment, $CD4^+CD25^+$ cells were used as a positive control instead of $CD4^+CD45RB^{low}$ cells because $CD4^+CD25^+$ cells showed a clearer therapeutic effect than $CD4^+CD45RB^{low}$ cells (data not shown). Simple $CD8^+CD122^+$ cells were only partially able to improve once-established colitis, whereas in vitro-activated $CD8^+CD122^+$ cells obviously improved body weight (Fig. 5*A*), fecal blood (Table III), SAA (Fig. 5*B*), and histological changes of the colon (Fig. 5*C*, 5*D*), as such improvement was also observed by the transfer of $CD4^+CD25^+$ Tregs. RT-PCR analysis and real-time PCR analysis revealed that the expression pattern of cytokines in $CD8^+CD122^+$ cell-transferred mice is similar to that transferred with $CD4^+CD45RB^{high}$ cells alone rather than that transferred with $CD4^+CD45RB^{high}$ cells plus in vitro-activated $CD8^+CD122^+$ cells (Fig. 5*E*, 5*F*), indicating the importance of activation of $CD8^+CD122^+$ cells to perform their suppressive activity. Anti-CD3 Ab-activated $CD8^+CD122^+$ cells derived from IL-10–deficient mice failed to suppress colitis (Fig. 5), emphasizing the importance of IL-10 in suppression of colitis.

$CD4^+CD45RB^{low}$ cells and $CD8^+CD122^+$ Tregs synergistically work to suppress colitis

Next, we investigated whether $CD8^+CD122^+$ Tregs and $CD4^+CD45RB^{low}$ cells synergistically prevent colitis. To examine this hypothesis, we first transferred half the number (1×10^5) of Tregs with clear regulatory activity when 2×10^5 of either $CD8^+CD122^+$ cells or $CD4^+CD45RB^{low}$ cells were transferred with 4×10^5 $CD4^+CD45RB^{high}$ cells in the former experiment (Fig. 1). In both cases of $CD8^+CD122^+$ cells and $CD4^+CD45RB^{low}$ cells, transfer of half the number (1×10^5) of Treg-containing cells mixed with $CD4^+CD45RB^{high}$ cells was unable to prevent colitis (Fig. 6, Table IV). However, 1×10^5 of a mixture of 0.5×10^5 each of $CD4^+CD45RB^{low}$ cells and $CD8^+CD122^+$ cells effectively prevented colitis (Fig. 6, Table IV). Again, RT-PCR analysis and real-time PCR analysis revealed that the gene expression pattern of cytokines in mice transferred with a small number of either $CD45RB^{low}$ or $CD8^+CD122^+$ cells is similar to the gene expression of mice transferred with $CD4^+CD45RB^{high}$ cells alone. In contrast, the gene expression pattern in mice transferred with a small number of both $CD45RB^{low}$ and $CD8^+CD122^+$ cells is rather similar to that of mice transferred with a sufficient number of $CD45RB^{low}$ cells (Figs. 1, 6*E*, 6*F*). These results indicated the synergistic effect of $CD8^+$ Tregs in the $CD8^+CD122^+$ population and $CD4^+$ Tregs in the $CD4^+CD45RB^{low}$ population.

Effect of a combination of $CD8^+CD122^+$ Tregs and $CD4^+CD25^+$ Tregs

In Fig. 6 and Table IV, we showed a synergistic effect of $CD8^+CD122^+$ Tregs and $CD4^+$ Tregs to prevent colitis induced by transfer of $CD4^+CD45RB^{high}$ cells into RAG-$2^{-/-}$ mice. To further confirm the synergistic effect of $CD8^+CD122^+$ Tregs and

Table III. *Fecal blood of $CD4^+CD45RB^{high}$ cell-induced colitis is cured by cotransfer of in vitro-activated $CD8^+CD122^+$ cells*

Transferred Cells	Weeks after T Cell Transfer									
	3	4	5	6	7	8	9	10	11	12
No cells	0/4	0/4	0/4	0/4	0/4	094	0/4	0/4	0/4	0/4
4^+45RB^{high} alone	0/4	2/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
4^+45RB^{high} and 8^+122^+ (naive)	0/4	1/4	2/4	3/4	3/4	4/4	4/4	4/4	4/4	4/4
4^+45RB^{high} and 8^+122^+ (activated)	0/4	2/4	3/4	4/4	3/4	2/4	0/4	0/4	0/4	0/4
4^+45RB^{high} and 4^+25^+	0/4	3/4	4/4	3/4	2/4	1/4	0/4	0/4	0/4	0/4

Rag-$2^{-/-}$ mice that received 4×10^5 $CD4^+CD45RB^{high}$ cells at week 0 further received 2×10^5 of indicated cells at week 4. Fecal blood was checked every week. Data indicate fecal blood-positive mice in total mice used for each experimental group.

CD4$^+$ Tregs, we performed another experiment using mature RAG-2$^{-/-}$ mice (>10 wk old) instead of young mice (5–7 wk old) that were still growing and gaining weight (Figs. 1, 4–6*A*). RAG-2$^{-/-}$ mice that received CD8$^+$CD122$^-$ cells became ill and died within 10 wk of the T cell transfer (19). This surprising phenomenon is explained by the deficiency of CD8$^+$CD122$^+$ Tregs that control CD8$^+$CD122$^-$ cells and maintain T cell homeostasis. Without CD8$^+$CD122$^+$ Tregs, the remaining CD8$^+$CD122$^-$ T cells are violently out of control and kill the host mouse. These outrageous CD8$^+$CD122$^-$ T cells cannot be controlled by CD4$^+$ CD25$^+$ Tregs because RAG-2$^{-/-}$ mice that received CD8$^+$ CD122$^-$ cells together with CD4$^+$CD25$^+$ Tregs also die similarly to RAG-2$^{-/-}$ mice that received only CD8$^+$CD122$^-$ cells (19). These experimental results indicate that CD8$^+$CD122$^+$ Tregs exclusively work to control CD8$^+$ cells but CD4$^+$CD25$^+$ Tregs do not. Then what about the control of CD4$^+$ cells? We also found

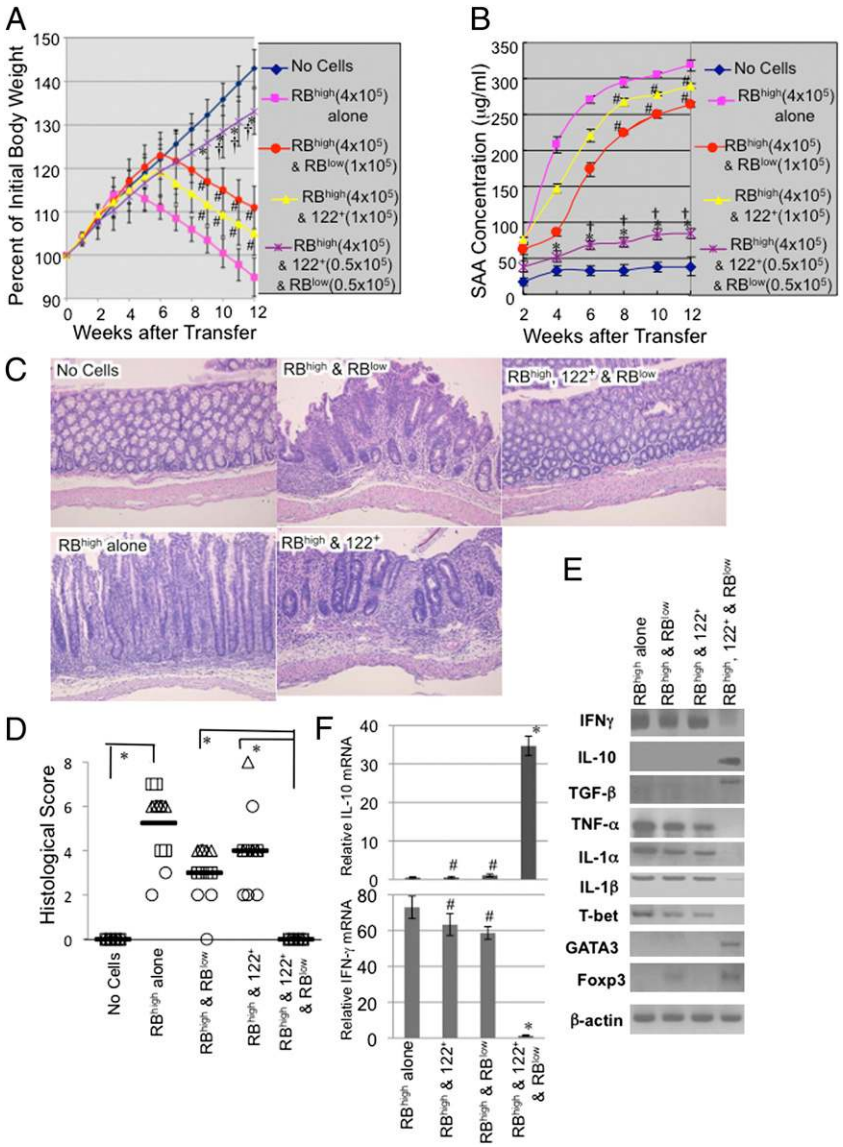

FIGURE 6. Synergistic effect of CD8$^+$CD122$^+$ cells and CD4$^+$CD45RB^{low} cells in the prevention of colitis. *A*, CD4$^+$CD45RB^{high} cells (4×10^5) in combination with either 1×10^5 CD4$^+$CD45RB^{low} cells, 1×10^5 CD8$^+$CD122$^+$ cells, or a mixture of 0.5×10^5 CD8$^+$CD122$^-$ cells and 0.5×10^5 CD4$^+$ CD45RB^{low} cells were transferred into RAG-2$^{-/-}$ mice. Change of body weight was monitored. The average data of 12 mice (three independent experiments of four mice each) are shown. Data of transfer of CD45RB^{low} cells, CD122$^+$ cells, and both CD45RB^{low} cells and CD122$^+$ cells were compared with that of transfer of CD45RB^{high} cells alone; data with statistically significant differences (*$p < 0.005$; Student *t* test and Bonferroni correction) and data without significant difference (#$p > 0.005$; Student *t* test and Bonferroni correction). Data from the transfer of both CD122$^+$ cells and CD45RB^{low} cells with significant differences compared with that of the transfer of CD45RB^{low} cells but no CD122$^+$ cells (†$p < 0.005$; Student *t* test and Bonferroni correction). *B*, SAA was monitored ($n = 4$; error bar = SD). Data of transfer of CD4$^+$CD45RB^{low} cells, CD8$^+$CD122$^+$ cells, or both CD8$^+$CD122$^-$ cells and CD4$^+$CD45RB^{low} cells were compared with that of transfer of CD4$^+$CD45RB^{high} cells alone; data with statistically significant differences (*$p < 0.005$; Student *t* test and Bonferroni correction) and data without significant differences (#$p > 0.005$; Student *t* test and Bonferroni correction). *C*, The dissected colon was analyzed 12 wk after T cell transfer (original magnification ×100). Representative pictures in each group are shown. *D*, Histological scores of colitis of individual mice are shown as ○ (first experiment, $n = 4$), △ (second experiment, $n = 4$), and □ (third experiment, $n = 4$). Data with statistically significant difference between the two groups (*$p < 0.005$; Student *t* test and Bonferroni correction). *E*, Colon tissue was analyzed by RT-PCR to evaluate the expression of cytokines and transcription factors. Representative results obtained from four mice in each group are shown. *F*, Results of real-time PCR analysis are shown as bar graphs. Data obtained from four mice were analyzed, and the average with SD (error bars) is shown. Significantly different values compared with control (CD45RB^{high} alone) (*$p < 0.00825$; Student *t* test and Bonferroni correction) and not significantly different values (#$p > 0.00825$; Student *t* test and Bonferroni correction).

Table IV. *Fecal blood of $CD4^+CD45RB^{high}$ cell-induced colitis is more effectively prevented by cotransfer of $CD8^+CD122^+$ cells and $CD4^+CD45RB^{low}$ cells*

Transferred Cells	Weeks after T Cell Transfer									
	3	4	5	6	7	8	9	10	11	12
No cells	0/4	0/4	0/4	0/4	0/4	094	0/4	0/4	0/4	0/4
4^+45RB^{high} alone (4×10^5)	0/4	1/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
4^+45RB^{high} and 8^+122^+ (1×10^5)	0/4	0/4	0/4	0/4	2/4	4/4	4/4	4/4	4/4	4/4
4^+45RB^{high} and 4^+45RB^{low} (1×10^5)	0/4	0/4	0/4	0/4	1/4	3/4	4/4	4/4	4/4	4/4
4^+45RB^{high}, 8^+122^+(0.5×10^5), 4^+45RB^{low} (0.5×10^5)	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

Rag-$2^{-/-}$ mice that received 4×10^5 $CD4^+CD45RB^{high}$ cells with or without 1×10^5 $CD8^+122^+$cells, 1×10^5 $CD4^+CD45RB^{low}$ cells, or a mixture of 0.5×10^5 $CD8^+122^+$cells and 0.5×10^5 $CD4^+CD45RB^{low}$ cells were analyzed for their fecal blood every week. Data indicate fecal blood-positive mice in total mice used for each experimental group.

that $CD8^+CD122^+$ Tregs control $CD4^+$ cells (19). To clarify the role of $CD8^+CD122^+$ Tregs in possible coordination with $CD4^+CD25^+$ Tregs, we set up a similar experiment of T cell transfer into RAG-$2^{-/-}$ mice in which $CD4^+CD25^-$ cells work as effector cells and become target cells for the Tregs. First, we show the survival of mice and their change in body weight (Fig. 7). In this experiment, we injected 1.2×10^5 $CD4^+CD25^-$ cells mixed or unmixed with possible combinations of 0.6×10^5 $CD4^+CD25^+$ Tregs and/or 0.6×10^5 $CD8^+CD122^+$ Tregs into RAG-$2^{-/-}$ mice. As shown in Fig. 7, mice that received only $CD4^+CD25^-$ cells began to die ~6 wk after cell transfer, and all the mice died within 10 wk. This result is similar to that of an experiment in which $CD8^+CD122^-$ T cells were transferred into RAG-$2^{-/-}$ mice (19), further confirming the dangerous action of unregulated T cells. When we transferred $CD8^+CD122^+$ Tregs and $CD4^+CD25^-$ cells, the survival of mice was slightly increased, and this improvement in survival was much more obvious when $CD4^+CD25^+$ Tregs were transferred together with $CD4^+CD25^-$ cells. However, mice transferred with $CD4^+CD25^-$ cells and $CD4^+CD25^+$ cells still died within 18 wk after cell transfer. This suggested that $CD4^+$

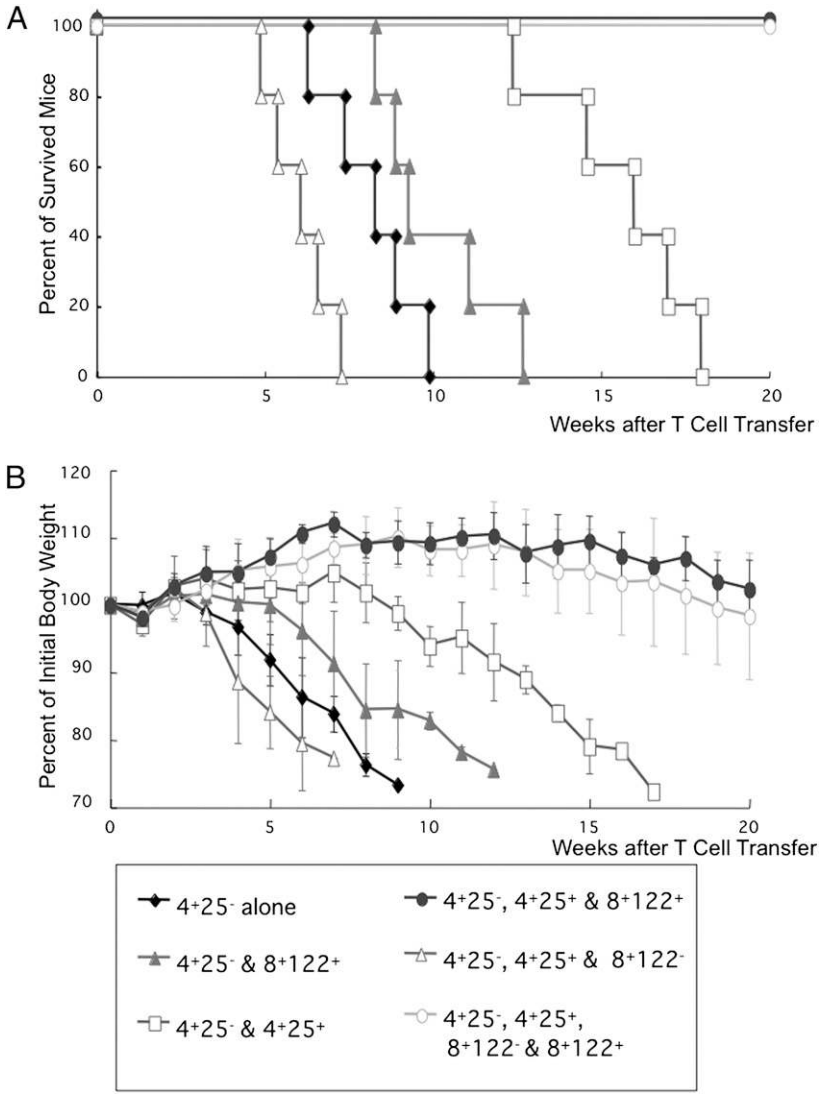

FIGURE 7. Synergistic effect of $CD8^+CD122^+$ Tregs and $CD4^+$ Tregs in another experimental system. *A*, The survival of T cell-transferred RAG-$2^{-/-}$ mice was chased until 20 wk after the T cell transfer. The survival rate is presented as a Kaplan-Meier curve. Five mice in each group were analyzed. *B*, The body weight of T cell-transferred RAG-$2^{-/-}$ mice was measured until 20 wk after T cell transfer. Body weight change was monitored for five mice in each group. The average data of five mice in each group are demonstrated with error bars of SD.

CD25$^+$ cells alone are not perfect for the control of CD4$^+$CD25$^-$ cells because we transferred more CD4$^+$CD25$^+$ Tregs than the natural ratio of CD4$^+$CD25$^+$ cells to CD4$^+$CD25$^-$ cells. A significant result was obtained when we mixed both CD4$^+$CD25$^+$ cells and CD8$^+$CD122$^+$ cells with CD4$^+$CD25$^-$ cells. All the mice (five of five) that received CD4$^+$CD25$^-$ cells together with both CD8$^+$CD122$^+$ cells and CD4$^+$CD25$^+$ cells survived >20 wk after T cell transfer without any signs of illness. The efficacy of the combination of two Tregs was also confirmed by chasing body weight change of RAG-2$^{-/-}$ mice that received a combination of CD4$^+$CD25$^+$ Tregs and CD8$^+$CD122$^+$ Tregs. The change of body weight apparently indicates the health of mice and perfectly parallels the survival of mice (Fig. 7).

The number of CD69$^+$ activated T cells was increased in sick mice with uncontrolled T cells

Five weeks after T cell transfer, spleen cells were analyzed for their origin (Supplemental Fig. 2). In this experiment, Tregs were derived from a C57BL/6$^{CD45.1}$ congeneic strain and could be distinguished from CD4$^+$CD25$^-$ effector cells that were derived from normal C57BL/6$^{CD45.2}$ mice. Transferred cells and their derivatives were clearly observed (Supplemental Fig. 2). When we analyze the total number of spleen cells, they were normally conserved in RAG-2$^{-/-}$ mice that received CD4$^+$CD25$^-$ cells together with both CD8$^+$CD122$^+$ cells and CD4$^+$CD25$^+$ cells but were increased in other mice that received CD4$^+$CD25$^-$ cells with a single Treg population.

The ratio of CD69$^+$ activated T cells in total CD4$^+$ cells was examined 5 wk after T cell transfer (Fig. 8*A*). Apparently, the number of CD4$^+$CD69$^+$ cells was higher in RAG-2$^{-/-}$ mice that received only CD4$^+$CD25$^-$ cells than in mice that received CD4$^+$CD25$^-$ cells with either CD8$^+$CD122$^+$ cells or CD4$^+$CD25$^+$ cells (Fig. 8*B*). The number of CD4$^+$CD69$^+$ cells was further decreased when RAG-2$^{-/-}$ mice received a mixture of CD4$^+$CD25$^-$ cells, CD4$^+$CD25$^+$ cells, and CD8$^+$CD122$^+$ cells. This decrease of CD69$^+$ activated T cells was also observed in the presence of CD8$^+$CD122$^-$ effector T cells (Fig. 8). Thus, the synergistic effect of CD4$^+$CD25$^+$ Tregs and CD8$^+$CD122$^+$ Tregs was also confirmed in this experimental system.

Discussion

In this study, we demonstrated that CD8$^+$CD122$^+$ Tregs have a capacity to suppress colitis induced by CD4$^+$CD45RB^{high} cells. One may wonder whether CD8$^+$ Tregs suppress not only CD8$^+$ cells but also CD4$^+$ cells. This question may be answered by the following experimental results. First, CD8$^+$CD122$^+$ Tregs suppress IFN-γ production from CD4$^+$CD25$^-$ T cells and IL-2 production from CD4$^+$ Th1 clones in the in vitro coculture systems (19). Second, CD8$^+$CD122$^+$ Tregs improve the signs of EAE induced by immunizing mice with a peptide that is presented on MHC class II and stimulates CD4$^+$ cells (20). CD8$^+$CD122$^+$ Tregs recognize activated T cells in an MHC class I-restricted manner (21), indicating that these Tregs suppress both CD8$^+$ cells and CD4$^+$ cells that express MHC class I molecules.

Some CD8$^+$ Tregs other than CD8$^+$CD122$^+$ cells suppress colitis (27). CD8$^+$CD28$^-$ cells may be the best-described candidate among such CD8$^+$ Tregs (28–30) and raise a hypothesis that CD8$^+$CD28$^-$ cells and CD8$^+$CD122$^+$ cells overlap. However, we have clearly shown that there are no CD122$^+$ cells in the CD8$^+$CD28$^-$ cell population, and CD8$^+$CD122$^+$ cells are mostly CD28$^+$ (Y. Okuno, unpublished observations), indicating that CD8$^+$CD28$^{-/-}$ cells and CD8$^+$CD122$^+$ cells are completely different. The functional and developmental relationships between these CD8$^+$ Tregs remain unknown at present.

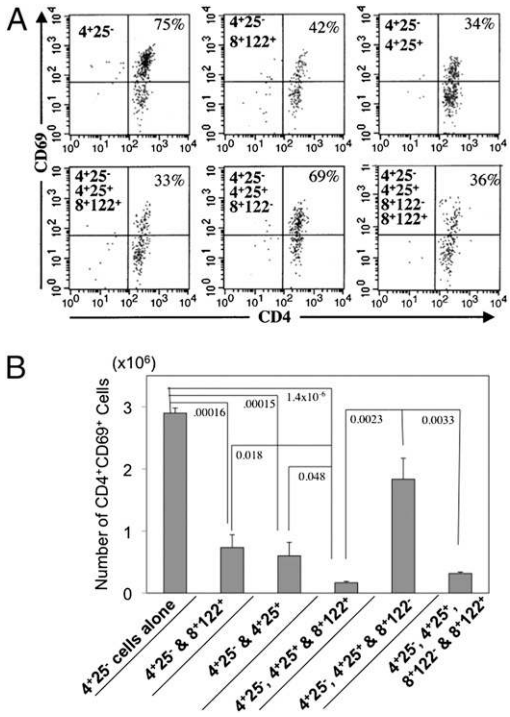

FIGURE 8. Synergistic effect of CD8$^+$CD122$^+$ Tregs and CD4$^+$ Tregs to reduce the number of activated T cells. *A*, Spleen cells gated to CD4$^+$ CD45.1$^+$ (originally CD4$^+$CD25$^-$ cells) were analyzed for the expression of CD69 to determine the activation status 5 wk after transfer. Percentages of CD69$^+$ cells in total CD4$^+$ cells are shown inside the panels. Representative results obtained from two experiments containing five mice each are shown. *B*, Total of CD4$^+$CD69$^+$ cells 5 wk after cell transfer. Data are obtained from two experiments containing five mice in each group and are shown as averages with error bars of SD. Statistical analysis was performed, and *p* values comparing the two groups are shown in the graph (Student *t* test).

IL-10 is supposed to be the main effect-transmitting molecule of CD8$^+$CD122$^+$ Tregs (26). In this study, CD8$^+$CD122$^+$ Tregs derived from IL-10$^{-/-}$ mice did not show sufficient regulatory activity to suppress colitis (Fig. 4). However, RAG-2$^{-/-}$ mice transferred with CD8$^+$CD122$^+$ cells derived from IL-10$^{-/-}$ mice were much better than mice transferred with CD4$^+$CD45RB^{high} cells alone, indicating that such non–IL-10–producing cells still maintain some capacity to suppress colitis induced by CD4$^+$ CD45RB^{high} cells. This result corresponds to the capacity of CD8$^+$CD122$^+$ cells to suppress CD8$^+$ cells (26). By considering these similar results, we can conclude that IL-10 is the primary effector molecule that is produced by CD8$^+$CD122$^+$ cells, whereas these cells may be able to use another effect-transmitting mechanism in the absence of IL-10. Again, these similar results of suppression of CD8$^+$ cells and CD4$^+$ cells by CD8$^+$CD122$^+$ Tregs further support that these regulatory cells use the same mechanism to suppress both CD8$^+$ cells and CD4$^+$ cells. Compared with the current study examining the role of IL-10 for regulation of colitis by CD8$^+$ Tregs, numerous studies have already been performed on CD4$^+$ Tregs (22, 31–38). Although most of the studies support an essential role of IL-10 for control of colitis by CD4$^+$ Tregs, IL-10–independent regulation of colitis by CD4$^+$ Tregs has not been excluded. TGF-β does not seem to be involved in the prevention and cure of colitis; however, this finding remains controversial (39–41). Our experimental result of a lack of TGF-β expression in healthy mice that received CD8$^+$ CD122$^+$ cells (Fig. 1*E*) may further support the negative role of TGF-β.

When transferring $CD8^+CD122^+$ cells after the transfer of $CD4^+CD45RB^+$ cells, only activated $CD8^+CD122^+$ cells are effective. $CD8^+CD122^+$ cells are supposed to be partially activated and respond quickly to stimulation. However, $CD8^+CD122^+$ cells that are not activated ex vivo may need further stimulation, and it may require more time for nonactivated $CD8^+CD122^+$ Tregs to become fully active Tregs. As reported (11, 12) and confirmed by our experiment (Fig. 5), $CD4^+CD25^+$ Tregs have the capacity to cure already-developed colitis. Compared with the effective capacity of $CD4^+$ Tregs to cure colitis, the effect of $CD8^+CD122^+$ Tregs for colitis seems weak. However, ex vivo-activated $CD8^+CD122^+$ Tregs showed sufficient regulatory activity for already-developed colitis (Fig. 5), suggesting that it may take a little longer time for regulation by $CD8^+CD122^+$ Tregs, and there may be a different working phase for $CD4^+$ Tregs and $CD8^+CD122^+$ Tregs.

In this paper, we also demonstrated a possible synergistic action of $CD4^+$ Tregs and $CD8^+$ Tregs. In the current study, we observed that $CD8^+CD122^+$ Tregs have the capacity to suppress the development of colitis induced by $CD4^+CD45RB^{high}$ cells, and the capacity to suppress colitis by $CD4^+$ Treg has been reported (8–10). These effects are obvious by introducing either type of Tregs, but considerable numbers of regulatory cells are necessary for complete suppression. The minimal ratio of Tregs and effector cells ($CD4^+CD45RB^{high}$ cells) is 1:2 (2×10^5 Tregs/4×10^5 effector T cells) when using a single type of Tregs. We cannot directly compare the potential of $CD8^+$ Tregs and $CD4^+$ Tregs to suppress colitis because the real action of these regulatory cells is biased by their capacity for homeostatic proliferation of transferred cells, and we can only evaluate the suppressing capacity of these Tregs after homeostatic proliferation. Actually, $CD8^+$ cells proliferate more than $CD4^+$ cells, and the $CD4^+/CD8^+$ ratio after homeostatic proliferation was rather $CD8^+$ cell dominant in the case of transfer of $CD4^+CD45RB^{high}$ cells and $CD8^+CD122^+$ cells. Nevertheless, when the ratio of regulatory cells and effector cells was 1:4 (1×10^5 Tregs/4×10^5 effector T cells), both types of Tregs were not sufficient to suppress colitis. In contrast, a 1:4 ratio of total Tregs, which consists of 1:8 of both types of Tregs (0.5×10^5 each of $CD4^+CD45RB^{low}$ cells and $CD8^+CD122^+$ cells) effectively suppressed colitis (Fig. 6), indicating a synergistic effect of these completely different types of Tregs. In this report, we show a synergistic effect of $CD4^+$ Tregs and $CD8^+$ Tregs and provide a potential application of Tregs to the medical field. The combination of both $CD4^+$ and $CD8^+$ Tregs is much better than a single type of Treg. However, the mechanism of the synergistic effect of $CD4^+$ Tregs and $CD8^+$ Tregs is unknown. We do not have an answer for this question yet, but it is unlikely that $CD8^+$ Tregs induce $CD4^+$ induced-type Tregs (Fig. 2).

In this study, we used a mouse system to demonstrate that $CD8^+CD122^+$ Tregs have the potential to prevent and cure colitis. Next, it will be most important to determine whether the regulatory potential of $CD8^+CD122^+$ cells can be applied to actual human diseases. This application is based on the existence of human $CD8^+$ Tregs that correspond to murine $CD8^+CD122^+$ Tregs. In our previous study, we identified $CD8^+CXCR3^+$ Tregs in humans that are functionally similar to murine $CD8^+CD122^+$ Tregs, although there are no $CD8^+CD122^+$ cells in human peripheral blood (42). Thus, human $CD8^+$ Tregs, possibly in combination with $CD4^+$ Tregs, may open the door to treatment of human IBD.

Acknowledgments

We thank Ayako Murakoshi for experimental help.

Disclosures

The authors have no financial conflicts of interest.

References

1. Groux, H., and F. Powrie. 1999. Regulatory T cells and inflammatory bowel disease. *Immunol. Today* 20: 442–445.
2. Singh, B., S. Read, C. Asseman, V. Malmström, C. Mottet, L. A. Stephens, R. Stepankova, H. Tlaskalova, and F. Powrie. 2001. Control of intestinal inflammation by regulatory T cells. *Immunol. Rev.* 182: 190–200.
3. Allez, M., and L. Mayer. 2004. Regulatory T cells: peace keepers in the gut. *Inflamm. Bowel Dis.* 10: 666–676.
4. Huibregtse, I. L., A. U. van Lent, and S. J. van Deventer. 2007. Immunopathogenesis of IBD: insufficient suppressor function in the gut? *Gut* 56: 584–592.
5. Elson, C. O., Y. Cong, V. J. McCracken, R. A. Dimmitt, R. G. Lorenz, and C. T. Weaver. 2005. Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunol. Rev.* 206: 260–276.
6. Coombes, J. L., N. J. Robinson, K. J. Maloy, H. H. Uhlig, and F. Powrie. 2005. Regulatory T cells and intestinal homeostasis. *Immunol. Rev.* 204: 184–194.
7. Kanai, T., T. Kawamura, T. Dohi, S. Makita, Y. Nemoto, T. Totsuka, and M. Watanabe. 2006. TH1/TH2-mediated colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T lymphocytes into nude mice. *Inflamm. Bowel Dis.* 12: 89–99.
8. Hyjek, E. M., J. Bartkowiak, R. Drozdz, T. J. Wasik, M. Jasinski, Y. Kaneko, H. W. Lischner, and D. Kozbor. 1997. Evidence for B cell-mediated activation of $V\delta1^+$ T lymphocytes during progression of HIV infection. *J. Immunol.* 158: 464–474.
9. Yu, Q. T., M. Saruta, A. Avanesyan, P. R. Fleshner, A. H. Banham, and K. A. Papadakis. 2007. Expression and functional characterization of $FOXP3^+$ $CD4^+$ regulatory T cells in ulcerative colitis. *Inflamm. Bowel Dis.* 13: 191–199.
10. Reardon, C., A. Wang, and D. M. McKay. 2008. Transient local depletion of $Foxp3^+$ regulatory T cells during recovery from colitis via Fas/Fas ligand-induced death. *J. Immunol.* 180: 8316–8326.
11. Mottet, C., H. H. Uhlig, and F. Powrie. 2003. Cutting edge: cure of colitis by $CD4^+CD25^+$ regulatory T cells. *J. Immunol.* 170: 3939–3943.
12. Liu, H., B. Hu, D. Xu, and F. Y. Liew. 2003. $CD4^+CD25^+$ regulatory T cells cure murine colitis: the role of IL-10, TGF-β, and CTLA4. *J. Immunol.* 171: 5012–5017.
13. Denning, T. L., G. Kim, and M. Kronenberg. 2005. Cutting edge: $CD4^+CD25^+$ regulatory T cells impaired for intestinal homing can prevent colitis. *J. Immunol.* 174: 7487–7491.
14. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 101: 455–458.
15. Vlad, G., R. Cortesini, and N. Suciu-Foca. 2005. License to heal: bidirectional interaction of antigen-specific regulatory T cells and tolerogenic APC. *J. Immunol.* 174: 5907–5914.
16. Najafian, N., T. Chitnis, A. D. Salama, B. Zhu, C. Benou, X. Yuan, M. R. Clarkson, M. H. Sayegh, and S. J. Khoury. 2003. Regulatory functions of $CD8^+CD28^-$ T cells in an autoimmune disease model. *J. Clin. Invest.* 112: 1037–1048.
17. Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775–787.
18. Smith, T. R., and V. Kumar. 2008. Revival of $CD8^+$ Treg-mediated suppression. *Trends Immunol.* 29: 337–342.
19. Rifa'i, M., Y. Kawamoto, I. Nakashima, and H. Suzuki. 2004. Essential roles of $CD8^+CD122^+$ regulatory T cells in the maintenance of T cell homeostasis. *J. Exp. Med.* 200: 1123–1134.
20. Lee, Y. H., Y. Ishida, M. Rifa'i, Z. Shi, K. Isobe, and H. Suzuki. 2008. Essential role of $CD8^+CD122^+$ regulatory T cells in the recovery from experimental autoimmune encephalomyelitis. *J. Immunol.* 180: 825–832.
21. Rifa'i, M., Z. Shi, S. Y. Zhang, Y. H. Lee, H. Shiku, K. Isobe, and H. Suzuki. 2008. $CD8^+CD122^+$ regulatory T cells recognize activated T cells via conventional MHC class I-αβTCR interaction and become IL-10–producing active regulatory cells. *Int. Immunol.* 20: 937–947.
22. Singh, U. P., S. Singh, R. Singh, Y. Cong, D. D. Taub, and J. W. Lillard, Jr. 2008. CXCL10-producing mucosal $CD4^+$ T cells, NK cells, and NKT cells are associated with chronic colitis in $IL\text{-}10^{-/-}$ mice, which can be abrogated by anti-CXCL10 antibody inhibition. *J. Interferon Cytokine Res.* 28: 31–43.
23. Ameho, C. K., A. A. Adjei, E. K. Harrison, K. Takeshita, T. Morioka, Y. Arakaki, E. Ito, I. Suzuki, A. D. Kulkarni, A. Kawajiri, and S. Yamamoto. 1997. Prophylactic effect of dietary glutamine supplementation on interleukin 8 and tumour necrosis factor α production in trinitrobenzene sulphonic acid induced colitis. *Gut* 41: 487–493.
24. Kjellev, S., D. Lundsgaard, S. S. Poulsen, and H. Markholst. 2006. Reconstitution of Scid mice with $CD4^+CD25^-$ T cells leads to rapid colitis: an improved model for pharmacologic testing. *Int. Immunopharmacol.* 6: 1341–1354.
25. Olsen, C. H. 2003. Review of the use of statistics in infection and immunity. *Infect. Immun.* 71: 6689–6692.
26. Endharti, A. T., M. Rifa'I, Z. Shi, Y. Fukuoka, Y. Nakahara, Y. Kawamoto, K. Takeda, K. Isobe, and H. Suzuki. 2005. Cutting edge: $CD8^+CD122^+$ regulatory T cells produce IL-10 to suppress IFN-γ production and proliferation of $CD8^+$ T cells. *J. Immunol.* 175: 7093–7097.
27. Das, G., M. M. Augustine, J. Das, K. Bottomly, P. Ray, and A. Ray. 2003. An important regulatory role for $CD4^+CD8\alpha\alpha$ T cells in the intestinal epithelial layer in the prevention of inflammatory bowel disease. *Proc. Natl. Acad. Sci. USA* 100: 5324–5329.

28. Ménager-Marcq, I., C. Pomié, P. Romagnoli, and J. P. van Meerwijk. 2006. CD8$^+$ CD28$^-$ regulatory T lymphocytes prevent experimental inflammatory bowel disease in mice. *Gastroenterology* 131: 1775–1785.
29. Pomié, C., I. Ménager-Marcq, and J. P. van Meerwijk. 2008. Murine CD8$^+$ regulatory T lymphocytes: the new era. *Hum. Immunol.* 69: 708–714.
30. Ben-David, H., A. Sharabi, M. Dayan, M. Sela, and E. Mozes. 2007. The role of CD8$^+$CD28 regulatory cells in suppressing myasthenia gravis-associated responses by a dual altered peptide ligand. *Proc. Natl. Acad. Sci. USA* 104: 17459–17464.
31. Singh, U. P., S. Singh, D. D. Taub, and J. W. Lillard, Jr. 2003. Inhibition of IFN-γ–inducible protein-10 abrogates colitis in IL-10$^{-/-}$ mice. *J. Immunol.* 171: 1401–1406.
32. Foussat, A., F. Cottrez, V. Brun, N. Fournier, J. P. Breittmayer, and H. Groux. 2003. A comparative study between T regulatory type 1 and CD4$^+$CD25$^+$ T cells in the control of inflammation. *J. Immunol.* 171: 5018–5026.
33. Annacker, O., R. Pimenta-Araujo, O. Burlen-Defranoux, T. C. Barbosa, A. Cumano, and A. Bandeira. 2001. CD25$^+$CD4$^+$ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J. Immunol.* 166: 3008–3018.
34. Ikenoue, Y., T. Tagami, and M. Murata. 2005. Development and validation of a novel IL-10 deficient cell transfer model for colitis. *Int. Immunopharmacol.* 5: 993–1006.
35. Autenrieth, I. B., N. Bucheler, E. Bohn, G. Heinze, and I. Horak. 1997. Cytokine mRNA expression in intestinal tissue of interleukin-2 deficient mice with bowel inflammation. *Gut* 41: 793–800.
36. Asseman, C., S. Mauze, M. W. Leach, R. L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* 190: 995–1004.
37. Maloy, K. J., L. Salaun, R. Cahill, G. Dougan, N. J. Saunders, and F. Powrie. 2003. CD4$^+$CD25$^+$ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. *J. Exp. Med.* 197: 111–119.
38. Asseman, C., S. Read, and F. Powrie. 2003. Colitogenic Th1 cells are present in the antigen-experienced T cell pool in normal mice: control by CD4$^+$ regulatory T cells and IL-10. *J. Immunol.* 171: 971–978.
39. Kullberg, M. C., V. Hay, A. W. Cheever, M. Mamura, A. Sher, J. J. Letterio, E. M. Shevach, and C. A. Piccirillo. 2005. TGF-β1 production by CD4$^+$CD25$^+$ regulatory T cells is not essential for suppression of intestinal inflammation. *Eur. J. Immunol.* 35: 2886–2895.
40. Piccirillo, C. A., J. J. Letterio, A. M. Thornton, R. S. McHugh, M. Mamura, H. Mizuhara, and E. M. Shevach. 2002. CD4$^+$CD25$^+$ regulatory T cells can mediate suppressor function in the absence of transforming growth factor β1 production and responsiveness. *J. Exp. Med.* 196: 237–246.
41. Powrie, F., J. Carlino, M. W. Leach, S. Mauze, and R. L. Coffman. 1996. A critical role for transforming growth factor-β but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB^{low}CD4$^+$ T cells. *J. Exp. Med.* 183: 2669–2674.
42. Shi, Z., Y. Okuno, M. Rifa'i, A. T. Endharti, K. Akane, K. Isobe, and H. Suzuki. 2009. Human CD8$^+$CXCR3$^+$ T cells have the same function as murine CD8$^+$ CD122$^+$ Treg. *Eur. J. Immunol.* 39: 2106–2119.