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Regulation of Immunity by a Novel Population of Qa-1-Restricted CD8$\alpha\alpha^+$TCR$\alpha\beta^+$ T Cells[1]

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Regulatory mechanisms involving CD8$^+$ T cells (CD8 regulatory T cells (Tregs)) are important in the maintenance of immune homeostasis. However, the inability to generate functional CD8 Treg clones with defined Ag specificity has precluded a direct demonstration of CD8 Treg-mediated regulation. In the present study, we describe the isolation of functional lines and clones representing a novel population of TCR$\alpha\beta^+$ Tregs that control activated Vβ8.2$^+$ CD4 T cells mediating experimental autoimmune encephalomyelitis. They express exclusively the CD8$\alpha\alpha$ homodimer and recognize a peptide from a conserved region of the TCR Vβ8.2 chain in the context of the Qa-1a (CD8$\alpha\alpha$ Tregs). They secrete type 1 cytokines but not IL-2. CD8$\alpha\alpha$ Tregs kill activated Vβ8.2$^+$ but not Vβ8.2$^-$ or naive T cells. The CD8$\alpha\alpha$ Tregs prevent autoimmunity upon adoptive transfer or following in vivo activation. These findings reveal an important negative feedback regulatory mechanism targeting activated T cells and have implications in the development of therapeutic strategies for autoimmune diseases and transplantation. ***The Journal of Immunology,*** **2006, 177: 7645–7655.**

The immune system seeks to maintain a state of equilibrium while responding to microbes and self-Ags. Control mechanisms for maintaining homeostasis following an immune response to a foreign Ag and for preventing or aborting harmful responses to self-Ags include CTLA-4-mediated T cell inhibition, activation-induced cell death, IL-2-mediated regulation, and regulatory T cells (Tregs)[3] (1). Although much attention has focused recently on CD4$^+$CD25$^+$ and NK T cell suppression of the priming or expansion of T cell immunity (2, 3), less is known about the role of regulatory CD8$^+$ T cells (CD8 Tregs) in feedback regulation (4–6).

Previous data generated using various animal models have indicated an important regulatory role for CD8$^+$ lymphocytes in autoimmune diseases, transplant tolerance, neonatal tolerance, and in homeostasis of cellular and humoral immune responses (5, 7–11). CD8 Tregs have also been implicated in different conditions in humans as well, for example, their role in the survival of transplants (12), in the prevention of inflammatory bowel disease (13) and in the treatment of multiple sclerosis patients with either glatiramer acetate or following T cell vaccination (TCV) with irradiated autologous myelin basic protein (MBP)-reactive CD4$^+$ T cells (14, 15). Thus, knowledge of the phenotype, Ag specificity, and mechanisms used by CD8 Tregs is important not only for elucidating the biology of immune homeostasis but also for the development of strategies to manipulate immune responses.

Mice genetically deficient in or depleted of CD8$^+$ T cells by treatment with anti-CD8 mAb have clearly demonstrated a crucial role for CD8 T cells in regulating EAE and conferring resistance to reinduction (7, 8). Earlier studies of experimental autoimmune encephalomyelitis (EAE) in rodents, a model of Th1 CD4 T cell-mediated autoimmune disease or graft-vs-host disease and host-vs-graft disease in allotransplantation, revealed the existence of Tregs that target AgRs on T cells (16, 17). Additionally, vaccination with attenuated pathogenic CD4 T cells (TCV) or peptides derived from their AgRs provides protection from EAE and graft-vs-host disease, suggesting that the Tregs may target TCR or its peptides (18–22). Experiments using Qa-1 knockout or anti-Qa-1 sera have indicated that the Qa-1 molecules may play an important role in the function of the CD8 Tregs (23, 24). Qa-1 in mouse (HLA-E in humans), a nonclassical MHC class Ib molecule, forms a heterodimer with β_2-microglobulin (β_2m) and can present peptides from both self- and foreign Ags (25–28). Although the Qa-1 molecules and AgRs expressed on pathogenic CD4$^+$ T cells have both been proposed to play a role in CD8 Treg-mediated regulation, identification of a Qa-1-binding peptide recognized by the CD8$^+$ T cells and description of the regulatory mechanisms remain unknown.

One of the major difficulties in understanding the biology of regulatory CD8 T cells has been the inability to establish functional CD8 Treg clones in a well-defined system. We have studied Tregs in a model system in which MBP-reactive Vβ8.2$^+$ CD4 T cells mediate EAE in H-2^u mice (6). In this model, paralytic disease is generally monophasic, and once recovered, mice resist further induction of the disease (29, 30). Previous data from ourselves and others (7, 8, 31) have suggested a crucial role for the CD8 Tregs in the control of pathogenic Vβ8.2$^+$ CD4 T cells. Recent studies have further indicated a crucial role for CD8$^+$ T cells in the apoptotic depletion of MBP-reactive Vβ8.2$^+$CD4$^+$ T cells following this regulation (32). In this present study, we describe the identification of a peptide from a conserved region of the TCR Vβ8.2

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[3] Abbreviations used in this paper: Treg, regulatory T cell; TCV, T cell vaccination; MBP, myelin basic protein; EAE, experimental autoimmune encephalomyelitis; β_2m, β_2-microglobulin; Tg, transgenic; PT, Pertussis toxin; TL, thymic leukemia; TAP, transporter associated with Ag processing; IEL, intraepithelial lymphocyte.

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Table I. *Vaccination with CDR2 region peptides (9- or 10-mer) from the Vβ8.2 chain prevents EAE in B10.PL mice*

Vaccination Peptides	Size (no. amino acid residues)	Amino Acid Sequence	Incidence of EAE No. of Animals with Disease/Total No. of Animals (maximal individual disease scores)
PBS/IFA			12/14 (5,5,4,4,4,4,2,2,2,2,1,1,0,0)
p3–12	10	AVTQSPRNKV	3/3 (4,3,3)
p5–14	10	TQSPRNKVAV	3/3 (5,2,0)
p13–21	9	AVTGGKVTL	2/3 (4,4,0)
p24–33	10	NQTNNHNNMY	3/3 (4,2,1)
p26–35	10	TNNHNNMYWY	2/3 (5,3,0)
p37–45	9	QDTGHGLRL	3/4
p41–50	10	HGLRLIHYSY	1/11 (3,0,0,0,0,0,0,0,0,0,0)
p42–50	9	GLRLIHYSY	3/8 (1,1,1,0,0,0,0,0,0)
p56–64	9	EKGDIPDGY	4/4
p69–77	9	PSQENFSLI	3/3
p70–78	9	SQENFSLIL	3/3 (3,3,3)
p72–80	9	ENFSLILEL	3/3 (3,2,1)
p80–88	9	LATPSQTSV	2/3 (4,4,0)
p81–90	10	ATPSQTSVYF	3/3 (4,3,1)
p41–70	30	HGLRLIHYSYGAGSTEKGDIPDGYKASRPS	1/6 (5,0,0,0,0,0)
p41–50[a]	10	HGLRLIHYSY	11/12 (4,4,4,4,4,3,3,2,1,1,1,0)

[a] In this experiment, SJL/J mice were vaccinated with p41–50. All of the other experiments were done in B10.PL mice.

chain that is targeted by the CD8 Treg population. Vaccination with this peptide prevents MBP-induced EAE in $CD8^{+/+}$ but not in $CD8^{-/-}$ mice. We also demonstrate the Ag specificity, phenotype, MHC restriction, and regulatory mechanism used by this novel $CD8\alpha\alpha^{+}TCR\alpha\beta^{+}$ Treg (CD8αα Treg) population using functional T cell clones and lines. These findings have important implications not only for understanding immune homeostasis mediated by the CD8αα Tregs, but also for the development of therapies for T cell-mediated diseases.

Materials and Methods

Mice and cell lines

B10.PL/73NS/SnJ, PL/J, C57BL/6J (B6), BALB/cJ, NOD/Lt, NZB/BINJ, B10.BR/SgSnJ, SWR/J, and SJL/J mice were purchased from The Jackson Laboratory. MBP-specific Vβ8.2 TCR transgenic (Tg) PL/J (T/R^{+}) (33), OT-1 TCR Tg RAG-$1^{-/-}$ (34), $CD8\alpha^{-/-}$ PL/J (8), and B6.Tla[a] (B6.A-H2-T18[a]/BoyEg) (35) mice were provided by Drs. J. Lafaille (New York University Medical Center, New York, NY), S. P. Schoenberger (La Jolla Institute for Allergy and Immunology, San Diego, CA), T. Mak (University of Toronto, Toronto, Ontario, Canada), and P. E. Jensen (Emory University, Atlanta, GA), respectively. Mice were bred under specific pathogen-free conditions in our own colony at Torrey Pines Institute for Molecular Studies. Age-matched female mice (from 6 to 14 wk of age) were used in all experiments. Experiments involving animals were performed in compliance with federal and institutional guidelines and have been approved by Torrey Pines Institute for Molecular Studies Institute Animal Care and Use Committee.

Induction of EAE

Mice were immunized s.c. with 150 μg of MBPAc1–9 (AcASQKRPSQR) emulsified in CFA. Pertussis toxin (PT, 0.15 μg) was injected in PBS on the same day and 48 h later. Mice were observed for clinical symptoms of paralysis daily. Disease was scored on a five-point scale, as described earlier (22): 1, flaccid tail; 2, hind limb weakness; 3, hind limb paralysis; 4, whole body paralysis; and 5, death.

Proliferation assay

To examine proliferative response, draining lymph node cells from immunized mice, CD8 T cell lines or clones (50,000 cells/well) were stimulated with relevant peptides in the presence of 500,000 APCs. For the last 18 h, cultures were pulsed with [^{3}H]TdR (1 μCi/well), harvested, and counted on a Trilux Scintillation beta counter.

Cytotoxicity assay

Cytotoxicity of CD8αα Tregs was tested using a standard 4-h ^{51}Cr release assay with minor modifications. Target cells (Con A-activated blasts or Ag-activated CD4 clones) were labeled with ^{51}Cr (MP Biomedicals) at 37°C for 45 min. A total of 10,000/well ^{51}Cr-labeled targets and 200 ng/well peptides was added into round-bottom 96-well plates. Plates were then incubated at 37°C, 10% CO_2 for 1 h. Effector cells were added in triplicate to the wells at the indicated E:T cell ratios to a total volume of 200 μl. Spontaneous ^{51}Cr release was measured in control wells containing target cells with medium alone. Maximum release values were obtained by lysis of target cells with 2.5% Triton X-100 (Sigma-Aldrich). The plates were then incubated for 4 h before 50 μl of supernatants was transferred into sample plates. A total of 150 μl/well scintillation liquid (Optiphase Supermix; PerkinElmer) was added, and the plates were read on a Trilux Scintillation gamma counter. The percent-specific release was calculated as (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100%.

Generation of CD8 T cell lines and clones

PL/J mice were immunized s.c. with p42–50 in CFA at a dose of 20 μg/mouse. Ten days later, draining lymph node cells were harvested and stimulated in vitro as follows: 30×10^6 irradiated splenocytes in 5 ml of DMEM complete medium (20% FBS, 100 U/ml penicillin and streptomycin, 3.125×10^{-5} M 2-ME, 1 mM sodium pyruvate, 0.1 mM nonessential amino acid, and 10 mM HEPES) were pulsed with p42–50 (10 μg/ml) at 37°C, 10% CO_2 for 2 h. A total of 30×10^6 draining lymph node cells in a 5-ml volume was added into the flask and mixed. Cells were cultured vertically at 37°C, 10% CO_2 for 7 days. The resulting cells were then cloned at 100 or 1000 cells/well in DMEM containing 10 IU IL-2 and 1×10^6 p42–50-pulsed irradiated syngeneic splenocytes. The cells were supplemented with fresh medium (2% conditioned medium and 50 IU/ml IL-2) every 3–4 days and were restimulated with p42–50 every 2 wk. Cells from proliferating wells were then transferred to 24-well plates and expanded using the procedure as described above. To generate p42–50-reactive lines, the restimulated draining lymph node cells were expanded using 50 IU/ml IL-2 and 2% conditioned medium in 24-well plates. The lines were supplemented with fresh medium every 3–4 days and restimulated with p42–50-pulsed irradiated splenocytes every 2 wk.

To generate SIINFEKL (OVAp257–264)-specific CTL clones, splenocytes from OT-1 Tg mice were stimulated with an engineered fibroblast cell line MEC.B7.SigOVA. Briefly, the adherent fibroblast APCs were seeded at 100,000 cells/well in 24-well plates and cultured overnight. The next day, the plates were irradiated with 7000 rad and washed three times with medium to remove any nonadherent cells or cell debris. A total of 5×10^5 OT-1 Tg cells was seeded into wells and then replenished with new medium containing 50 IU/ml IL-2 and 2% conditioned medium every 3–4 days and restimulated every 2 wk. Clonality was confirmed by flow cytometry, which showed all cells expressed the Vβ5 and Vα2 Tg TCR.

Peptide binding to recombinant Qa-1a molecules

Purified Qa-1a/Qdm complexes were generated from *Escherichia coli*-derived proteins as described previously (25). Qa-1a/Qdm complexes were incubated with the indicated concentrations of biotin peptides (b-Qdm or

b-p42–50) alone or with competing unlabeled peptides (Qdm, p42–50, or QdmM2K) overnight at room temperature to allow for peptide exchange in PBS containing 0.01% Nonidet P-40 and 200 mM citrate phosphate buffer (pH 7.0). The next day, the complexes were separated from unbound peptides using G-25 Sephadex Quick Spin Protein Columns (Roche). The amount of biotin-peptide bound to Qa-1a was measured by a Europium-based fluorescence immunoassay modified for measuring MHC class I complexes using an anti-β_2m capture Ab.

Real-time PCR

Total mRNA was extracted from cells using the RNeasy mini kit (Qiagen) and was subjected to cDNA synthesis with an oligo dT_{12-18} primer. Real-time PCR was performed using Brilliant SYBR Green QPCR kit (Stratagene) on a Stratagene Mx3000p machine. The calculation of comparative mRNA expression was performed by the Stratagene software and was designated as relative quantity after normalization against internal control genes (L32 and cyclophilin) and after consideration of amplification efficiency of individual genes. The primers used are as follows (36): L32, sense, 5′-GAAACTGGCGGAAACCCA-3′, and antisense, 5′-GGATCTG GCCCTTGAACCTT-3′; cyclophilin, sense, 5′-GGCCGATGACGA GCCC-3′, and antisense, 5′-TGTCTTTGGAACTTTGTCTGCAA-3′; Qa-1a, sense, 5′-GCGGTATTTCCACACTGCCA-3′, and antisense, 5′-TCTG TGAGGCAAAGTCAGTC-3′; and Qa-1b, sense, 5′-CCTGGACCGCGA ATGACATA-3′, and Qa-1b, antisense, 5′-CACCACAGCTCCAAGGA TGAT-3′.

ELISA

IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-α, and IFN-γ levels were measured by a sandwich ELISA using supernatants obtained from peptide-stimulated CD8 T cell clone cultures as described earlier (37). Briefly, Nunc Immuno Plates MaxiSorp F96 (Roskilde) were coated with a capture Ab at 4°C overnight. After blocking with PBS containing 10% FBS, 50 μl of supernatants was added, and the plates were incubated overnight at 4°C. Plates were extensively washed with PBS 0.05% Tween 20 and incubated with a biotin-conjugated detection Ab. Finally, plates were washed and developed using avidin-peroxidase and 2-2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) substrate (Sigma-Aldrich). OD405 was measured. All cytokine capture and detection Ab pairs were purchased from BD Pharmingen.

Flow cytometry

Cell surface molecules were detected by fluorescence conjugated mAbs. Briefly, 100,000–400,000 cells/100 μl were incubated with relevant Abs at 4°C for 30 min. Cells were then washed twice with FACS buffer containing 1% FCS and 0.05% sodium azide and analyzed on a FACSCalibur (BD Biosciences). Abs purchased from BD Pharmingen include 145-2C11 (anti-CD3), 53-6-7 (anti-CD8α), 53-5.8 (anti-CD8β.2), 7D4 (anti-CD25), H1.2F3 (anti-CD69), IM7 (anti-CD44), MEL-14 (anti-CD62L), HM40-3 (anti-CD40), 37.51 (anti-CD28), PK136 (anti-NK1.1), GL3 (anti-TCR$\gamma\delta$), A1 (anti-Ly49A), Dx5 (CD49b), GL-7 (anti-GL-7), 6A8.6F10.1A6 (anti-Qa-1b), RR4-7 (anti-TCR Vβ6), and MR5-2 (anti-TCR Vβ8.1/8.2). Abs purchased from Biolegend include 18d3 (anti-CD94) and C7 (anti-NKG2D). Anti-CD122, anti-IL7R, anti-thymic leukemia (TL)-HD168, and anti-TL-18/20 were gifts from Dr. H. Cheroutre (La Jolla Institute for Allergy and Immunology, San Diego, CA).

TL-tetramer staining of the CD8 T cell clones

TL monomers were provided by Dr. H. Cheroutre's laboratory. To conjugate the monomers, streptavidin-PE was added into biotinylated TL-monomer at 1:1 ratio. Mixtures were incubated on ice for 15 min in dark. To stain the CD8 clones, 1 μl of the above conjugated TL-tetramer was added into 100,000 cells/100 μl in FACS buffer. Cells were incubated for 30 min at 4°C and were then washed twice with buffer before being analyzed on the FACSCalibur (BD Biosciences).

Statistical analysis

Data are expressed as mean ± SEM for each group. Statistical analyses were performed using SPSS software. Two independent samples were tested by independent *t* test. Otherwise, the ANOVA test was used. A value of $p < 0.05$ was considered statistically significant.

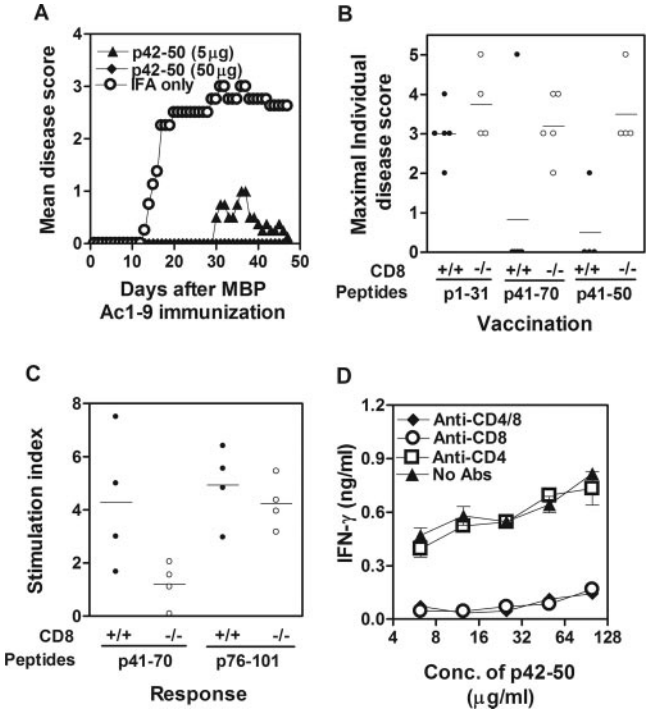

FIGURE 1. A nonamer peptide p42–50 derived from a conserved region of the TCR Vβ8.2 chain induces a CD8$^+$ T cell response and prevents EAE. *A*, Mice vaccinated i.p. with a titrated dose of p42–50 in IFA are protected from EAE. Paralytic disease was monitored daily and scored on a five-point scale as shown in *Materials and Methods*. Data are representative of three independent experiments. *B*, The protective effect of the TCR peptide is CD8 dependent. Maximal disease scores in CD8$\alpha^{+/+}$ and CD8$\alpha^{-/-}$ mice vaccinated with p41–50, p41–70, or a control p1–31 are shown. These data were combined from two independent experiments. *C*, Loss of a proliferative response to p41–70 in CD8α-deficient mice. Stimulation indices of a 4-day culture of draining lymph node cells from TCR p41–70- or p76–101-immunized mice in the presence of corresponding peptides are shown. The stimulation indices, counts per minute in the presence of stimulating peptides/counts per minute in the absence of stimulating peptides. Thymidine incorporation (counts per minute) in the absence of peptides in CD8$\alpha^{+/+}$ and CD8$\alpha^{-/-}$ PL/J mice are 7,000 ± 2,309 and 12,500 ± 1732, respectively. Data are representative of three independent experiments. *D*, The cytokine (IFN-γ) response to p42–50 in draining lymph node cells isolated from p42–50-immunized mice is blocked in the presence of anti-CD8α but not anti-CD4 mAb. The data are representative of two independent experiments.

Results

Identification of antigenic determinants recognized by the CD8 Tregs

In this study, we sought to characterize the Ag-specificity of the CD8 Tregs that control MBP-reactive Vβ8.2$^+$ T cells in a well-defined model of T cell-mediated autoimmune disease in B10.PL mice. Helper CD4 Tregs in this system recognize a determinant from the conserved framework 3 region of the Vβ8.2 TCR, and because, in general, helper determinants are in close proximity to the determinants for CD8$^+$ T cells (22), we focused on the TCR Vβ8.2 chain as the target for the CD8 Treg response. We synthesized a series of 9 or 10-mer peptides (Table I) from the entire sequence of the TCR Vβ8.2 chain (29) ending at hydrophobic residues, a requirement for binding to the transporter associated with Ag processing (TAP) (38). Groups of B10.PL mice were vaccinated with individual peptides and 1 wk later challenged with MBP/CFA/PT for the induction of EAE. Data in Table I show that animals vaccinated with p42–50 or longer peptides containing

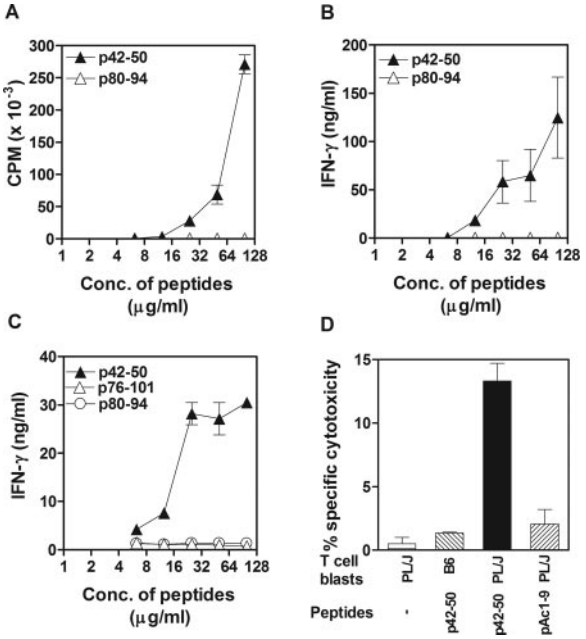

FIGURE 2. Treg clones and lines are TCR$\alpha\beta^+$CD8$\alpha\alpha^+$ and recognize p42–50 peptide from the conserved region of the TCR Vβ8.2 chain. Proliferative (*A*) and IFN-γ secretion (*B*) of a representative CD8 Treg clone, 2D11 and a CD8 T cell line (line 2) (*C*) are shown. CD8 T cells (50,000) were incubated with p42–50 or control peptide p80–94 at titrated concentrations in the presence of 500,000 irradiated syngenic APCs, and thymidine incorporation was assayed at 72 h. Cytokine secretion was determined by a standard sandwich ELISA in 48-h culture supernatants. *D*, Specific cytotoxicity of the 2D11 CD8 T cell clone toward p42–50-pulsed targets. ^{51}Cr-labeled blasts (10,000) from the syngenic PL/J or C57BL/6 mice pulsed with 10 μg/ml p42–50 or irrelevant peptide Ac1–9 were incubated with the 2D11 at an E:T ratio of 30:1 for 4 h. Supernatants were collected, and chromium release was determined using a Trilux gamma counter. These data are representative of four independent experiments.

these residues (p41–70) but not with others are significantly protected from disease. To determine an appropriate dose, mice were vaccinated with 5 or 50 μg of p42–50 before EAE induction. As shown in Fig. 1*A*, although mice vaccinated with the lower dose contracted a much milder paralytic disease with delayed onset followed by a quick spontaneous recovery, the higher dose provided complete protection from EAE. To determine whether the protective effect is dependent upon the presence of CD8$^+$ cells in vivo, CD8$\alpha^{+/+}$ or CD8$\alpha^{-/-}$ PL/J mice were vaccinated with p41–50, p41–70, or an irrelevant TCR peptide before the induction of disease. The data in Fig. 1*B* show that p41–50, as well as the longer peptide p41–70, provides significant protection from EAE in CD8$^{+/+}$ but not in CD8$^{-/-}$ mice, clearly demonstrating that the p42–50-induced regulation requires CD8$^+$ T cells. In addition, PLP139–151-induced disease in SJL/J mice, in which the pathogenic T cells do not predominantly use the TCR Vβ 8 gene segment, is not prevented following vaccination with the p41–50 (Table I).

To further determine whether the CDR2 region peptides induce a CD4 or a CD8 response in vivo, proliferation and cytokine responses of draining lymph node cells were examined in the presence of anti-CD4- or anti-CD8-blocking Abs or in CD8$\alpha^{+/+}$ or CD8$\alpha^{-/-}$ mice. As shown in Fig. 1*C*, p41–70 induces a proliferative response in CD8$\alpha^{+/+}$ but not in CD8$\alpha^{-/-}$ PL/J mice. In contrast, another TCR peptide p76–101, containing a determinant for the CD4 Tregs, induces a similar response in both CD8$\alpha^{+/+}$ and CD8$\alpha^{-/-}$ mice. Similarly, anti-CD8 Abs are able to completely block an in vitro recall response to p42–50 in the draining lymph node cells of mice immunized with p42–50 (Fig. 1*D*). In contrast, anti-CD4 Abs had no effect on the response to p42–50. These data collectively indicate that p42–50-reactive CD8 Tregs are important in the control of Vβ8.2$^+$ T cell-mediated disease.

Generation of p42–50-specific CD8 Treg lines and clones

To further characterize the CD8 Treg response, CD8$^+$ T cell clones were generated from p42–50-immunized mice using the limiting dilution cloning technique. Despite the difficulty of maintaining long-term in vitro culture of the p42–50-reactive CD8 T cells, three CD8 Treg clones from separate animals were generated. A representative clone, 2D11, was selected for detailed characterization. To eliminate possible artifacts resulting from long-term in vitro culture, properties of these clones were verified at the bulk level using freshly generated p42–50-reactive CD8 T cell lines. OVA-specific CD8 T cell lines and clones were generated and cultured in parallel as controls.

To examine Ag specificity, the CD8 Treg clones were assayed for proliferation and cytokine secretion in response to in vitro challenge with a panel of peptides derived from the TCR Vβ8.2 chain in the presence of irradiated syngeneic APCs. Fig. 2, *A* and *B*, shows that 2D11 proliferates and secretes IFN-γ in response to p42–50 but not to another peptide, p80–94, derived from the TCR Vβ8.2 chain. Similarly a short-term CD8 T cell line responds to p42–50 but not to other Vβ8.2 chain-derived peptides (Fig. 2*C*). The cytotoxic capacity of the CD8 Treg clones was determined using a standard 4-h ^{51}Cr release assay. Fig. 2*D* shows the killing of syngeneic Con A blasts pulsed with p42–50 but not an irrelevant peptide. The killing of p42–50-pulsed targets by CD8 Treg clones and lines is MHC-restricted as it does not occur when allogeneic (BL/6) blasts are used as targets (Fig. 2*D* and data not shown).

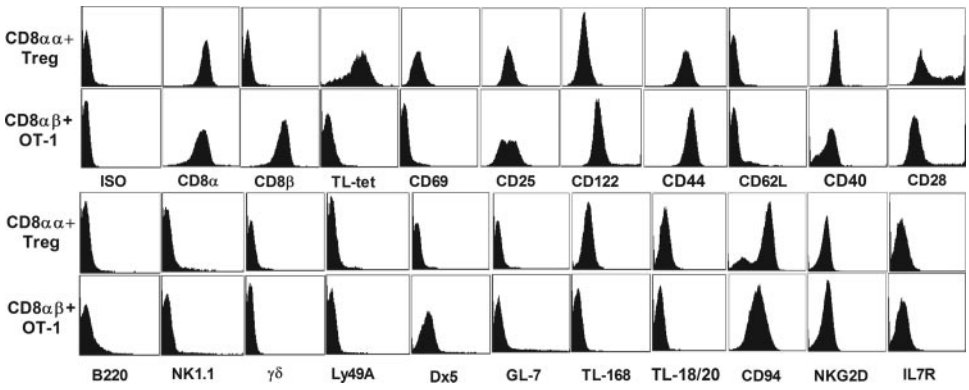

FIGURE 3. CD8 Tregs are TCR$\alpha\beta^+$ and express the CD8$\alpha\alpha$ homodimer. Six days after in vitro restimulation, CD8 Treg clone 2D11 and an irrelevant CD8 T cell clone were stained with the Abs indicated in the figure. Staining was analyzed by flow cytometry. These data are representative of three independent experiments. TL-tet, TL tetramer; HD168 and 18/20, biotinylated Abs specific for two different epitopes on TL Ags.

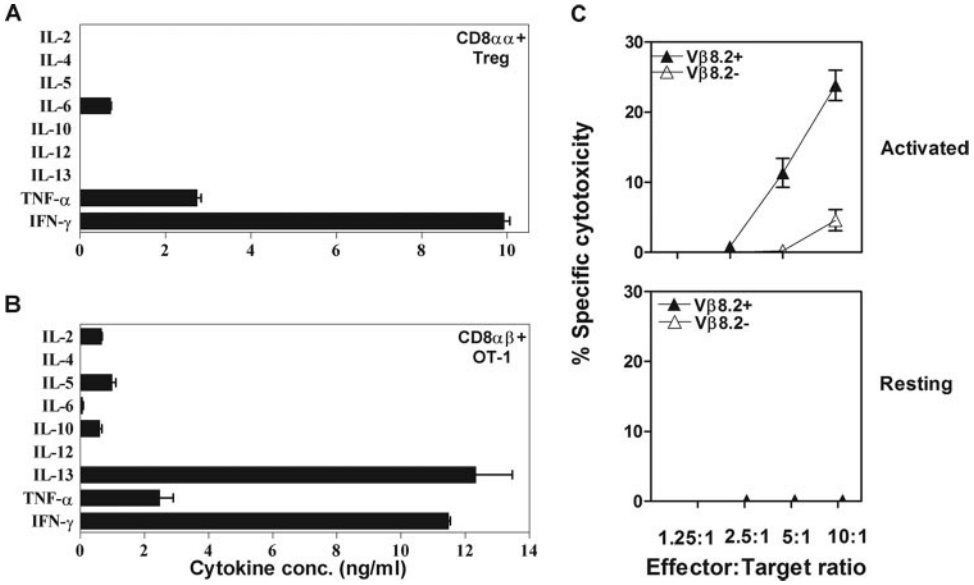

FIGURE 4. CD8 Treg clones secrete Tc-1-like cytokines and kill activated Vβ8.2$^+$CD4$^+$ T cells. Culture supernatants from 2D11 (*A*) and an OVA-reactive CD8 clone (*B*) were assayed for cytokines using sandwich ELISA. These data are representative of at least three independent experiments. *C*, Activated or resting Vβ8.2$^+$ or Vβ14$^+$ CD4 T cells were labeled with ^{51}Cr (10,000 target cells) and incubated with 2D11 at the indicated E:T ratio. These data are representative of three independent experiments.

CD8 Tregs are TCR$\alpha\beta^+$ and express the CD8$\alpha\alpha$ homodimer (CD8$\alpha\alpha$ Treg)

The cell surface phenotype of the CD8 Treg clones was determined by staining 2D11 with a panel of mAbs and analyzed by flow cytometry. In parallel, a conventional CD8 T cell clone (OT-1) specific for a peptide of OVA (SIINFEKL) and propagated under equivalent conditions was used as a control. As shown in Fig. 3, the CD8 Treg clone is TCR$\alpha\beta^+$, $\gamma\delta^-$, NK1.1$^-$, and B220$^-$. Both clones express CD25 (IL-2Rα chain), CD122 (IL-2Rβ chain), and IL-7R, suggesting an activated/memory phenotype. Interestingly, the CD8 Tregs maintain a low-level expression of CD69 even after a prolonged resting period in vitro in the absence of exogenous TCR peptide. This may indicate low-level cross-presentation of p42–50 because of the presence of Vβ8.2$^+$ T cells in the irradiated splenocytes that were used as APCs. The CD8 Treg clones are negative for CD62L, Ly49A, and GL-7 expression and positive for CD28, NKG2, and TL Ag expression.

It has been shown that CD8 T cells expressing a high level of CD94/NKG2 are relatively resistant to apoptosis compared with those with a null or intermediate level (39). Additionally, CD94/NKG2 receptors can interact with Qa-1/Qdm and provide survival signals for CD8 T cell maintenance in vivo (39). Therefore, we examined CD94 (paired with NKG2A, B, C, and E to form a heterodimer) and NKG2D (40) (homodimer) expression on 2D11 and the OT-1 clone. As shown in Fig. 3, although both clones are NKG2D$^+$ and CD94$^+$, 2D11 expresses higher level of CD94, which may explain its relative resistance to apoptosis in vivo (see below).

One of the most striking features of the CD8 Treg clones is the absence of surface CD8β chain expression (see Fig. 3). As TL Ag displays a 10-fold higher binding affinity for CD8$\alpha\alpha$ homodimers compared with CD8$\alpha\beta$ heterodimers (41), we examined the binding of TL tetramers to 2D11 cells. As shown in Fig. 3, only the CD8 Treg clone binds the TL-tetramer, confirming the expression of CD8$\alpha\alpha$ by the Treg clone. In fact, cloning of CD8$\alpha\alpha^+$TCR$\alpha\beta^+$ intraepithelial lymphocytes (IELs) (CD8$\alpha\alpha$ IELs) have not been reported so far and which may explain the difficulty in generating CD8$\alpha\alpha^+$ T cell clones. The CD8$\alpha\alpha$ Tregs also express TL Ags that can be recognized by two different Abs, HD-168 and 18/20, specific for two different epitopes on TL Ags (42, 43).

CD8$\alpha\alpha$ Tregs secrete Tc1-like cytokines

The cytokine secretion profile of 2D11, as well as an OT-1 CD8 clone, was examined in cell cultures after stimulation with peptide-pulsed APCs. As shown in Fig. 4*A*, 2D11 secretes IFN-γ and TNF-α (Tc1-like) but no detectable levels of IL-2, IL-4, IL-5, IL-10, IL-12, and IL-13. A very low level of IL-6 secretion was detected. In contrast, the OT-1 clone secretes IFN-γ, TNF-α, IL-2, IL-5, IL-10, and IL-13 (Fig. 4*B*). It is interesting that, like CD25$^+$CD4$^+$ Tregs, CD8$\alpha\alpha$ Tregs do not secrete detectable amounts of IL-2. The Tc1 phenotype of the CD8$\alpha\alpha$ Treg clone is not an artifact of long-term culture because short-term p42–50-reactive T cell lines also secrete IFN-γ but not Tc2-like cytokines (X. Tang and V. Kumar, data not shown).

CD8$\alpha\alpha$ Tregs specifically kill activated Vβ8.2$^+$ but not Vβ8.2$^-$ cells

We have demonstrated recently the CD8 T cell-dependent depletion of activated but not resting MBP-reactive Vβ8.2$^+$ CD4 T cells following induction of regulation in vivo (32). To determine whether CD8$\alpha\alpha$ Treg clones can specifically kill Vβ8.2$^+$ CD4 T cells, a MBPAc1–9-reactive pathogenic Vβ8.2$^+$ T cell clone (33)

Table II. *CD8$\alpha\alpha$ Tregs specifically kill TCR Vβ8$^+$ but not Vβ8$^-$ T cell targets*

	MBP-Reactive CD4$^+$ T Cell Lines		MBP-Reactive CD4$^+$ T Cell Clones		
	PL4/Vβ8	PL9/non-Vβ8	3C10/Vβ8.2Vα4.2	2C6/Vβ8.2Vα2.3	A25/Vβ13Vα2.3
Percentage of specific cytotoxicity	13 ± 1.2[a]	1.1 ± 0.6	12.8 ± 3.1	14.2 ± 2.5	0.9 ± 0.3

[a] The data are means ± SEs and are combined from four independent experiments.

Table III. *Cytotoxicity of CD8αα Tregs toward MBPAc1–9-reactive CD4$^+$ T cell hybridomas*[a]

					I-12/Vβ14 pulsed with[b]		
E:T = 7.35:1	172.10/Vβ8	1934.4/Vβ8	L-12/Vβ4	L-15/Vβ3	None	p42–50	Ac1–9
	2.34 ± 4.05[c]	0.13 ± 0.23	1.81 ± 0.67	2.13 ± 1.38	2.05 ± 2.96	13.6 ± 5.72	2.63 ± 0.98
	172.10/Vβ8 pulsed with				1934.4/Vβ8 pulsed with		
E:T = 36:1	None	p42–50	Ac1–9		None	p42–50	Ac1–9
	0.49 ± 0.5	25.73 ± 1.68	0.37 ± 0.54		0.92 ± 0.74	28.87 ± 0.87	0.71 ± 1.17

[a] The data are combined from two independent experiments.
[b] Targets were loaded with indicated peptides (20 μg/ml).
[c] The data are means ± SEs of specific cytotoxicity.

was used as a target in an in vitro cytotoxicity assay. In parallel, a Vβ14$^+$ CD4 T cell clone was used as a Vβ8.2$^-$ target (22). As shown in Fig. 4*C*, 2D11 kills Ag-activated Vβ8.2$^+$ but not Vβ14$^+$ T cells (*upper panel*). To determine whether Vβ8.2$^+$ T cell activation was required for the killing, we rested the CD4 T cell clones until they had the small rounded appearance of naive T cells. No detectable cytotoxicity toward the resting CD4 T cell targets was found (*lower panel* in Fig. 4*C*).

To further define Ag specificity of CD8αα Tregs, a number of Vβ8.2$^+$ and Vβ8.2$^-$ T cell lines, clones, and hybridomas were used as targets in cytotoxicity assays. CD8αα Tregs showed specific cytotoxicity toward MBPAc1–9-reactive TCR Vβ8$^+$ CD4 T cell line (PL4/Vβ8$^+$) or clones (3C10/Vβ8.2Vα4.2 and 2C6/Vβ8.2Vα2.3) but not TCR Vβ8$^-$ CD4 T cell line (PL9/non-Vβ8) or clone (A25/Vβ13Vα2.3) (Table II). It is clear that Vβ8.2$^+$ T cells using different Vα chains are efficiently killed by the CD8αα Tregs. We also examined the ability of the CD8αα Tregs to kill MBPAc1–9-reactive TCR Vβ8$^+$ or Vβ8$^-$ CD4$^+$ T cell hybridomas. Interestingly, CD8αα Tregs did not show detectable cytotoxicity toward either TCR Vβ8$^+$ (172.10 generated from the clone 2C6) or TCR Vβ8- (I-12, L-12, and L-15) hybridomas. However, CD8αα Tregs do kill p42–50-pulsed TCR Vβ8$^-$ (I-12) or Vβ8$^+$ (172.10 or 1934.4) hybridomas (Table III), suggesting poor display of the TCR-derived peptide Qa-1a complexes on the target T cell hybridomas (see below).

CD8αα Tregs are restricted by a class Ib MHC molecule, Qa-1a

Genetic, biochemical, and immunological approaches were used to determine the MHC restriction of the CD8 Treg clones. First, to determine whether presentation of the p42–50 requires β_2m, which pairs with a H chain to form MHC class I molecules, we compared the ability of APCs from $\beta_2m^{+/+}$ and $\beta_2m^{-/-}$ mice to stimulate 2D11 in vitro. As shown in the *upper panel* of Fig. 5*A*, $\beta_2m^{+/+}$ but not $\beta_2m^{-/-}$ APCs are able to present p42–50 and stimulate the 2D11 clone. These data clearly suggest that 2D11 clone is restricted by a class I MHC molecule. Next, in vitro proliferation assays were performed to examine the response of 2D11 to

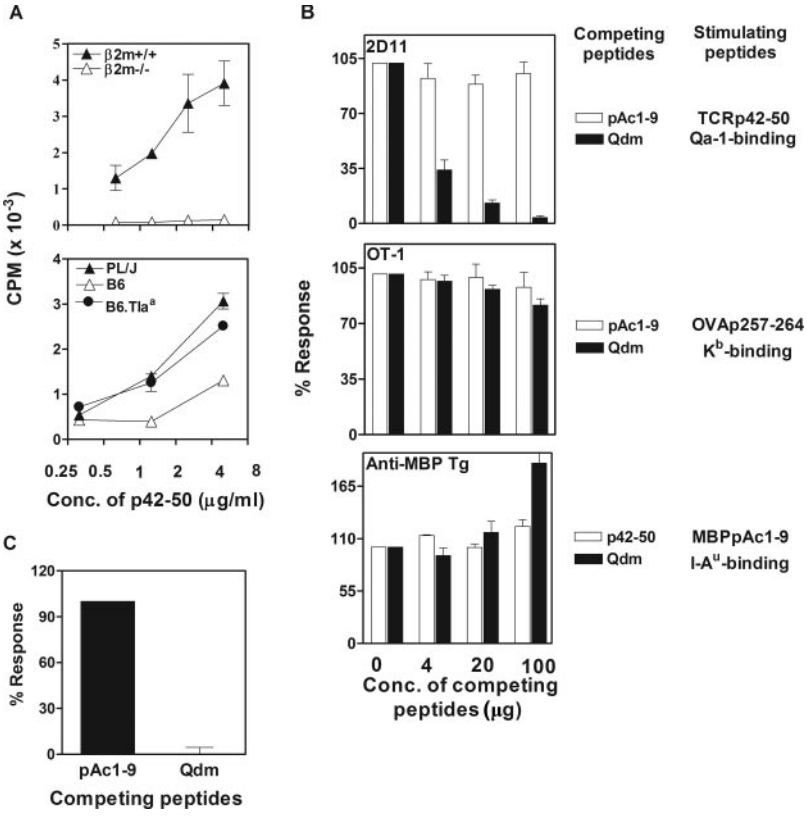

FIGURE 5. The CD8αα Tregs are restricted by MHC class Ib, Qa-1a molecules. *A*, *Upper panel*, Proliferative response of the CD8 Treg clone 2D11 in the presence of p42–50-pulsed irradiated APCs from $\beta_2m^{+/+}$ or $\beta_2m^{-/-}$ mice. Data are representative of two independent experiments. *Lower panel*, Proliferative response of 2D11 to p42–50 at titrated doses in the presence of APCs derived from syngenic PL/J, allogeneic C57BL/6, or congenic B6.Tla^a mice. Data are representative of three independent experiments. *B*, *Upper panel*, 2D11 cells were cultured at an optimal concentration of p42–50 (0.625 μg/ml) in the presence of increasing concentrations of blocking peptide Qdm or a control peptide Ac1–9. The proliferative response of 2D11 was blocked by Qdm but not Ac1–9. *Middle* and *lower panels*, Proliferative responses of OVA-reactive CD8 T cell clone (*middle panel*) or Ac1–9-reactive CD4 T cell clone (*lower panel*) to their respective peptides were not blocked in the presence of Qdm peptide. Background counts per minute in the absence of stimulating peptides were <500. Data are representative of three independent experiments. *C*, IFN-γ secretion in response to p42–50 (3.125 μM) in draining lymph node cells isolated from p42–50-immunized mice is inhibited in the presence of Qdm (100 μM) but not in the presence of an irrelevant peptide, Ac1–9 (100 μM). IFN-γ secretion in the absence of competing peptide is 4.0 ± 1.6 ng/ml. Data are representative of two independent experiments.

Table IV. *Response profile of the CD8αα Treg clone*[a]

	H-2K	H-2D	H-2L	Qa-1	Qa-2	Response[b]
PL/J $^{(u)c}$	u	d	d	a	?	+
B10.PL/73NS/SnJ $^{(u)}$	u	d	d	a	?	+
NZB/BINJ $^{(d)}$	d	d	−	a	a^+	+
B10.BR/SgSnJ $^{(k)}$	k	k	k	a		+
SWR/J $^{(q)}$	q	q	q	a	a^+	+
NOD/Lt $^{(g7)}$	d	b		a	?	+
C57BL/6J $^{(b)}$	b	b	−	b	a^{high}	−
BALB/cJ $^{(d)}$	d	d	d	b	a^{low}	−
SJL/J $^{(s)}$	s	s		b	a	−

[a] The CD8αα Treg clone 2D11 was examined for proliferation or cytokine secretion in response to p42–50 in the presence of APCs derived from mice of different H-2 haplotypes. The H-2K, D, L, Qa-1, and Qa-2 haplotypes are shown.

[b] "+" and "−" in the last column refer to a positive response and no response to p42–50, respectively. Positive response is defined as stimulating indices (SI) > 3 (7.2 ± 1.9 to 58.0 ± 18.6). Background counts in the absence of stimulating peptides were <200.

[c] H-2 haplotypes.

p42–50-pulsed APCs derived from mice of several MHC haplotypes. As shown in Table IV, 2D11 responds to p42–50-pulsed APCs from PL/J (H-2^u), B10.PL (H-2^u), NZB (H-2^d), B10.BR (H-2^k), SWR/J (H-2^q), and NOD (H-2^{g7}) mice but not to p42–50-pulsed APCs from C57BL/6 (H-2^b), BALB/c (H-2^d), and SJL/J (H-2^s) mice. Since a particular MHC class Ia gene did not correlate with the 2D11 response, we reasoned that MHC class Ib molecules might be involved in the presentation of p42–50. Indeed, those APCs expressing various class Ia alleles capable of stimulating 2D11 all express Qa-1a whereas those incapable of presentation express Qa-1b. These data indicate that Qa-1a molecules may present p42–50 to the CD8αα Tregs. This is consistent with the finding that B10.PL and PL/J mice from which the CD8αα Treg clones were isolated express Qa-1a but not Qa-1b molecules (data not shown). To further validate Qa-1a presentation, we next compared presentation of p42–50 by Qa-1 congenic mice B6.Tla^a (Qa-1a) and B6 (Qa-1b). Data in the *lower panel* of Fig. 5*A* show that APCs from B6.Tla^a, but not B6, can present peptide p42–50. Likewise, the 2D11 clone showed specific cytotoxicity toward p42–50-pulsed T2 Qa-1a transfectants but not T2 cells (data not shown).

The canonic Qa-1-binding peptide Qdm (Qa-1 determinant modifier) binds with high affinity to both Qa-1a and Qa-1b molecules (44). Therefore, we examined whether Qdm could compete with p42–50 in stimulation of the 2D11 clone. The *upper panel* of Fig. 5*B* shows a dose-dependent inhibition of 2D11 proliferation in the presence of Qdm peptide (■) but not in the presence of an irrelevant class II-binding peptide MBPAc1–9 (□) or a class Ia-binding OVA peptide (data not shown). In contrast, responses of the OT-1 clone (class Ia restricted, *middle panel*) and an MBPAc1–9-reactive CD4 T cell clone (class II restricted, *lower panel*) are not blocked by the Qdm peptide. To exclude the possibility that Qa-1 restriction was not just a property of this particular 2D11 clone, we examined whether the Qdm peptide could competitively inhibit a polyclonal response to p42–50. As shown in Fig. 5*C*, Qdm, but not an irrelevant peptide, blocks the p42–50 response of draining lymph node cells derived from p42–50-immunized mice.

The above experiments strongly indicate that p42–50 is presented by the Qa-1a molecule to the CD8αα Tregs. To directly demonstrate binding of p42–50 to Qa-1a molecules, a binding assay was performed using purified recombinant Qa-1a molecules. Both peptides p42–50 and Qdm were biotinylated (b-p42–50 and b-Qdm), and a fluorescence tag was added for their visualization and quantification in an in vitro binding assay as described earlier (25). As shown in the *upper panel* of Fig. 6, both b-Qdm and b-p42–50 bind to Qa-1a, and their binding is competed by unlabeled Qdm (100 μM). Interestingly, a 10-fold higher concentration (10 μM) of p42–50, compared with Qdm, was required for comparable binding to Qa-1a. This suggests that p42–50 may have either a lower binding affinity or a higher off-rate compared with Qdm. Accordingly, a 2-fold higher concentration (200 μM) of unlabeled p42–50 was necessary for blocking the binding of b-p42–50 to Qa-1a molecules (*lower panel* of the Fig. 6).

Qa-1 expression on target cells is important for the killing by CD8αα Tregs

Inability of CD8αα Tregs to kill TCR Vβ8.2$^+$ T cell hybridomas that were generated by fusion of MBPAc-1–9-reactive CD4$^+$ T cells (Qa-1a$^+$) with a tumor cell line BW5147 (Qa-1b$^+$) led us to investigate whether this is due to an inefficient expression of Qa-1 on T cell hybridomas in comparison to freshly activated T cells or clones. Therefore, we examined the expression of Qa-1a and Qa-1b in the hybridomas by real-time PCR (Qa-1a and Qa-1b) and FACS analysis (Qa-1b only). Although the TCR Vβ8.2$^+$ CD4

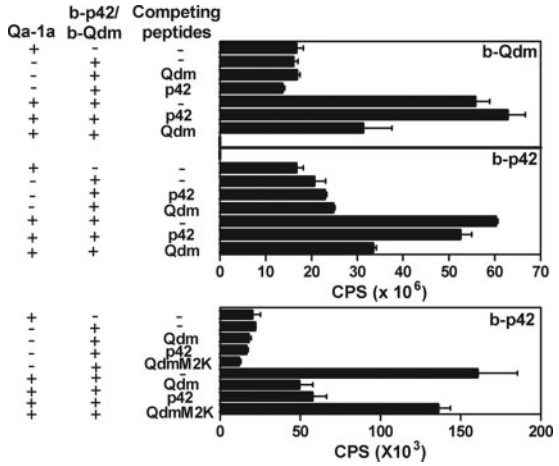

FIGURE 6. Binding of TCR peptide p42–50 to purified Qa-1a molecules. Purified Qa-1a/Qdm complexes were incubated overnight with 1 μM biotin-Qdm4C (b-Qdm) or 10 μM biotin-p42–50-4C (b-p42–50) in the presence or absence of 100 μM (*upper panel*) or 200 μM (*lower panel*) unlabeled competing peptides (p42–50, Qdm, or QdmM2K). Complexes were then separated from unbound peptides, and the amount of biotinylated peptide bound to Qa-1a was measured by europium-based fluorescence immunoassay using an anti-β_2m capture Ab. QdmM2K is a negative control peptide with a substitution of methionine with lysine at P2, leading to the loss of binding to Qa-1a. Data are representative of at least three independent experiments.

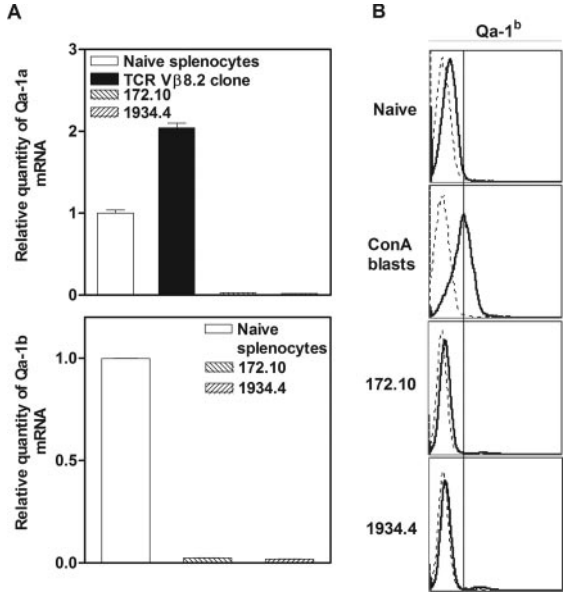

FIGURE 7. Expression of Qa-1 in MBPAc1–9-reactive CD4$^+$ T cells. *A*, mRNAs were extracted from naive splenocytes (B10.PL for Qa-1a and or C57BL/6 for Qa-1b), MBPAc1–9-reactive TCR Vβ8.2$^+$ CD4 T cell clone (Qa-1a), and MBPAc1–9-reactive TCR Vβ8.2$^+$ CD4 T cell hybridomas (172.10 and 1934.4) (Qa-1a and Qa-1b). Expression of mRNA for the Qa-1a or Qa-1b was examined by real-time PCR. The data are representative of two independent experiments and are presented as relative quantity normalized to two internal control genes L32 and cyclophilin. *B*, The indicated cells were stained with biotin-anti-Qa-1b mAb, followed by streptavidin-FITC and analyzed by flow cytometry. The data are representative of two independent experiments.

hybridomas (172.10 and 1934.4) express mRNA for both Qa-1a and Qa-1b as expected, the expression is decreased greatly compared with freshly activated polyclonal splenocytes or Vβ8.2$^+$ CD4 T cell clone (Fig. 7*A*). Using anti-Qa-1b mAb, we further analyzed the surface Qa-1b expression. As shown in Fig. 7*B*, the Qa-1b expression on the two TCR Vβ8.2$^+$ hybridomas (172.10 and 1934.4) was reduced greatly compared with naive splenocytes and Con A blasts.

Adoptive transfer of CD8αα Treg clones provides protection from EAE

To determine the in vivo regulatory potential of the CD8αα Tregs, 2D11 cells were adoptively transferred i.v. into syngeneic mice.

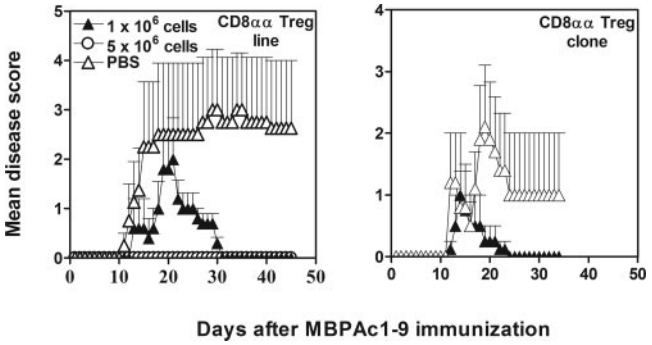

FIGURE 8. Adoptive transfer of the CD8αα$^+$ Treg lines and clones results in quick recovery and protection from EAE. CD8αα$^+$ Treg lines (*left panel*) or clones (*right panel*) were adoptively transferred into syngenic recipients i.v. 24 h before the induction of EAE with MBPAc1–9/CFA/PT. Paralytic disease was monitored daily for 35–45 days. Disease was scored on a five-point scale as described in *Materials and Methods*. Data are representative of two independent experiments.

The following day recipients were immunized with MBPAc1–9/CFA/PT for the induction of EAE, and clinical symptoms were monitored daily and scored on a scale from 1 to 5 as described previously (22). As shown in the *right panel* of Fig. 8, mice injected with 1 million 2D11 cells recover more rapidly from a milder disease than those in the control group. To rule out the possibility that the ability to control disease is an artifact of the long-term culture of a particular clone, short-term p42–50-reactive T cell lines were generated and used in similar adoptive transfer experiments. As shown in the *left panel* of the Fig. 8, mice that receive 5 million cells of a p42–50-reactive T cell line are completely protected from MBP-induced EAE, and transfer of only 1 million cells enables a more rapid recovery from a milder paralysis in comparison to animals in the control group. Adoptive transfer of short-term T cell lines raised against two irrelevant TCR peptides had no effect on EAE (Ref. 22 and data not shown).

Discussion

In this study, for the first time, we present a detailed characterization of a novel clonal population of regulatory CD8αα$^+$TCR$\alpha\beta^+$ T cells (CD8αα Tregs) that are involved in a negative feedback mechanism for the regulation of pathogenic CD4$^+$ T cells reactive to a self-Ag. Our findings suggest a mechanism of active immune regulation wherein CD8αα Tregs kill only activated T lymphocytes through specific recognition of a TCR-derived peptide bound to class Ib MHC molecules. The CD8αα Tregs described in this study are CD8αα$^+$ and TCR $\alpha\beta^+$ and recognize a TCR peptide from the conserved CDR2 region of the Vβ8.2 chain in the context of the class Ib molecule Qa-1a. They are cytotoxic, secrete Tc1-like cytokines, and are able to kill activated but not resting Vβ8.2$^+$ CD4 T cells. Adoptive transfer of p42–50-reactive CD8αα Tregs, as well as in vivo activation of the CD8αα Tregs following immunization with p42–50, protects mice from Ag-induced EAE. Since this immune regulatory mechanism operates following activation and expansion of CD4$^+$ T cells, it is likely that CD8αα Tregs of the type characterized in this study participate in the contraction phase following a productive immune response to a self- or foreign Ag, thereby contributing to immune homeostasis.

The degradation of a cytosolic protein into peptides for MHC class I presentation is a process that includes cleavage of the protein by proteosomes to produce a series of peptides that can be transported into the endoplasmic reticulum by TAP. Mouse TAP preferably transports peptides with hydrophobic carboxy termini (38). These peptides are further trimmed by aminopeptidase in the endoplasmic reticulum before binding to the MHC class I molecules (45, 46). Upon stimulation with IFN-γ or TNF-α, cells synthesize three proteosome subunits, low-molecular-mass polypepticle2 (β1i), low-molecular-mass polypepticle7 (β5i), and multicatalytic endopeptidase complex-like 1 (β2i), inducing the assembly of immunoproteosomes that generate new antigenic determinant (47). We have shown earlier that IFN-γ secretion by CD4 Tregs is required for the recruitment and expansion of the CD8αα Tregs that ultimately kill the Vβ8.2$^+$ pathogenic T cell population and protect mice from EAE (48). It is noteworthy that one of the optimal cleavage sites within the TCR Vβ8.2 chain for immunoproteosomes (49) lies immediately following the tyrosine at position 9 in p42–50. We postulate that IFN-γ secreted by the CD4 Tregs would enhance the presentation of p42–50 in both the target Vβ8.2$^+$ T cells, increasing their sensitivity to killing by the CD8αα Tregs, as well as in dendritic cells promoting Ag cross-presentation.

Although earlier studies have implicated either class Ia or Ib MHC restriction of the CD8αα Tregs, they have remained inconclusive because of the lack of a defined peptide and nonavailability

of functional CD8αα Treg clones (15, 23, 24). Our data clearly indicate that a TCR-derived peptide p42–50 (GLRLIHYSY) binds to the Qa-1a class Ib molecule and is recognized by the CD8αα Tregs. Although p42–50 does not possess the anchor residues suggested by the Qdm peptide (AMAPRTLLL) for binding to Qa-1a or b (50), it contains a number of residues present in other Qa-1-binding peptides (25). The leucine and histidine residues in p42–50 at P2 and P6, respectively, are also the predominant residues at the same positions in Qdm variant pools (25). Similarly, consistent with other Qa-1-binding peptides (28), hydrophilic and nonpolar or hydrophobic residues, at P3 (arginine) and P7/P9 (tyrosine), respectively, are present in p42–50. It is clear from our data (Fig. 6) that p42–50 binds with lower affinity to Qa-1a than the Qdm peptide. This relatively poor but still functional binding property of p42–50 is reflected in the dose needed to stimulate CD8αα Treg clones, which is similar to a Qa-1b-restricted clone specific for an insulin peptide (51).

Besides the Qa-1a-restriction, another important feature of the CD8αα Tregs described in this study is the expression of the CD8αα homodimer as demonstrated by the binding of TL-tetramer (Fig. 3). Most $CD8\alpha\alpha^+TCR\alpha\beta^+$ T cells reside in IELs (CD8αα IELs) and are present in mice genetically deficient in class Ia MHC molecules (52, 53), suggesting their potential restriction by class Ib MHC. Our p42–50-reactive CD8αα Tregs share several characteristics with CD8αα IELs (54). First, our CD8αα Tregs expand poorly in response to Ag stimulation both in vitro and in vivo (X. Tang and V. Kumar, unpublished observation), comparable with the poor response of CD8αα IELs to self-Ags (55). Second, both CD8αα Tregs and CD8αα IELs display regulatory function (54). Experiments are in progress to examine whether the CD8αα Tregs described here represent circulating CD8αα IELs or are part of a separate lineage.

Activation-induced cell death and/or passive cell death of activated T cells in the periphery are important mechanisms for maintaining homeostasis in the immune system and peripheral tolerance (1). Fas and/or Fas ligand knockout mice have a normal contraction phase in $CD8^+$ T cells following their activation, whereas in perforin knockout mice, a large number of activated T cells remain (56). Therefore, perforin is likely to be involved in the death of activated T cells. In fact, a perforin gene defect has been associated with familial hemophagocytic lymphohistiocytosis, which is characterized by uncontrolled activation of T cells (57), and our own preliminary experiments indicate that CD8αα Treg-mediated killing of activated $V\beta8.2^+$ CD4 T cells involves the perforin but not the Fas-Fas ligand pathway (T. Beeston, I. Maricic, X. Tang, and V. Kumgr, manuscript in preparation). Taken together, these observations suggest that, following the expansion phase of a dominant T cell clone(s), a CD8αα Treg response is cross-primed by dendritic cells that have captured apoptotic T cells and present TCR-peptides to both CD4 and CD8 T cells. Thus, primed TCR-peptide-reactive CD8αα Tregs will contribute to the clearance of the activated effectors and further recruitment of Tregs (58). This is consistent with the depletion of Ag-reactive T cells and the control of autoimmunity and transplant rejection following TCV with irradiated autologous T cells (15, 17, 18). Thus, CD8αα Tregs may control effector lymphocytes through both direct killing (6) or by modulation of APCs (59). Killing of CD4 T cells by the CD8αα Tregs is restricted to activated but not resting $V\beta8.2^+$ or $V\beta8.2^-$ CD4 T cells (Fig. 4*C*). Since both the $V\beta8.2^+$ and $V\beta8.2^-$ CD4 T cells have identical Qa-1a alleles, it is likely that the CD8αα Tregs discriminate among cells by differential recognition of TCR peptide/Qa-1a complexes on the cell surfaces. In contrast to MHC class Ia molecules, Qa-1b expression is low on the surface of resting T cells and up-regulated substantially upon activation (60) (Fig. 7*B*). Resting T cells may not be targeted due to poor display of TCR-peptide/Qa-1 complexes on the cell surface (Fig. 4*C*). There also exists a correlation between the ability of CD8αα Tregs to kill and the level of expression of Qa-1 molecules on target T cells. Thus, CD8αα Tregs can kill the T cell clones but not the hybridomas expressing the same TCR Vβ/Vα chains (Tables II and III) but having much reduced expression of Qa-1 on their cell surface (Table III and Fig. 7). Similarly, naive $V\beta8.2^+$ T cells that express very low levels of Qa-1 are not subjected to killing by the CD8αα Tregs. Thus, a dynamic expression of TCR peptide/Qa-1 complexes ensures that T cells are only targeted for killing by the CD8αα Tregs within a narrow window of time.

Why are p42–50-reactive CD8αα Tregs not negatively selected in the thymus? There are at least four possibilities. First, Qa-1 expression is generally much lower in comparison to class Ia molecules. Second, it appears that p42–50 can only be processed when immunoproteosomes are present (X. Tang and V. Kumar, unpublished observation). Third, we have found that cross-presentation of TCR-peptide to CD8αα Tregs by immature DC is very inefficient (T. R. F. Smith, X. Tang, and V. Kumar, unpublished data) (58). Fourth, the CD8αα Treg TCR has a low avidity for p42–50/Qa-1a complexes as manifested by a requirement for relatively higher concentrations of stimulating peptide and the relative instability of peptide MHC-Ib complexes (61). It has been shown that positive selection of MHC class Ib-restricted CD8 T cells reactive to insulin or CD8αα IELs can be mediated by recognition of agonistic ligands on thymic epithelial or hemopoietic cells (51). Therefore, it will be interesting to investigate whether TCR peptides act as agonistic ligands for positive selection of the CD8αα Tregs in the thymus similar to the positive selection of $CD4^+CD25^+$ Tregs following recognition of high-affinity self-ligands (2).

Since CD8αα Tregs recognize a determinant from a conserved region of the TCRVβ8.2 chain rather than a clone-specific variable CDR3 region, this enables them to control activated T cells that use the same TCRVβ chain but may have different Ag specificity. Therefore, this regulation is not anti-idiotypic in the classical sense. A single CD8αα Treg population could potentially regulate activated T cells of differing Ag specificities as long as they express the same TCR (the p42–50 sequence of Vβ8.1, Vβ8.2, or Vβ8.3 is identical). Thus, clonal dominance among responding CD4 effectors and specificity of the CD8αα Tregs for a conserved region of TCRs make these regulators highly effective and independent of minor TCR sequence variations on the target $CD4^+$ T cells (6, 58, 62). We suggest that CD8αα Tregs of the type described here may represent part of the CD8 Treg population involved in the prevention of autoimmune diseases, transplant tolerance, neonatal tolerance, oral tolerance, and homeostasis of immune responses in general (9–11, 59, 63, 64).

It is interesting to note that although CD8αα Tregs secrete Tc-1-like cytokines, they do not secrete detectable level of IL-2 and thus may be dependent upon the provision of IL-2 from other cells, including activated pathogenic CD4 T cells. This dependency of the CD8αα Tregs on IL-2 is reminiscent of $CD25^+CD4^+$ Tregs (2). Thus, IL-2 secreted by conventional CD4 T cells upon exposure to Ags serves to attract or even expand both Treg subsets that in turn inhibit the proliferative response of ($CD25^+CD4^+$ Tregs) or kill (CD8αα Tregs) activated effector T cells in a negative feedback loop. Since the priming and expansion of the CD8αα Tregs depend upon the

cross-presentation of TCR peptide by activated dendritic cells and the armed CD8αα Tregs act only on activated T cells, it is a perfect mechanism to limit uncontrolled T cell expansion following a productive immune response.

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Disclosures

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