Anti-TCR Antibody Treatment Activates a Novel Population of Nonintestinal CD8 $\alpha\alpha^+$ TCR$\alpha\beta^+$ Regulatory T Cells and Prevents Experimental Autoimmune Encephalomyelitis

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Anti-TCR Antibody Treatment Activates a Novel Population of Nonintestinal CD8αα+TCRαβ+ Regulatory T Cells and Prevents Experimental Autoimmune Encephalomyelitis

Xiaolei Tang, Igor Maricic, and Vipin Kumar

CD8αα+CD4−TCRαβ+ T cells are a special lineage of T cells found predominantly within the intestine as intraepithelial lymphocytes and have been shown to be involved in the maintenance of immune homeostasis. Although these cells are independent of classical MHC class I molecules (3–6). For these reasons CD8 concentration of agonistic self-Ags and is not dependent on clas-

Recent studies have identified a novel subset of nonintestinal CD8α T cells in mice that bear both an Ag-specific TCR and its cognate Ag (3, 7). These results suggest that CD8αα+CD4−TCRαβ+ T cells in mice (B10.PL or PL/J) with myelin basic protein (MBP) emulsi-

To date, CD8αα+CD4−TCRαβ+ T cells have been studied mainly as intestinal components, and little is known regarding their function and origin in the periphery. Although Ags recognized by the CD8αα+CD4−TCRαβ+ T cells (CD8αα T cells) are a special lineage of T cells that primarily reside among the intestine intraepithelial lymphocytes (iIELs). The most recent data suggest that CD8αα T cells in the iIELs (CD8αα iIELs) are derived from the thymus and migrate into the gut at an early age (1, 2). The selection of CD8αα iIELs requires a higher concentration of agonistic self-Ags and is not dependent on classical MHC class I molecules (3–6). For these reasons CD8αα iIELs are not subjected to negative selection in a double transgenic mouse that bears both an Ag-specific TCR and its cognate Ag (3, 7). To date, CD8αα T cells have been studied mainly as intestinal components, and little is known regarding their function and origin in the periphery.

Although Ags recognized by the CD8αα iIELs remain unknown, these cells are believed to have an important role in maintaining the integrity of the mucosal barrier (2). CD8αα iIELs appear to be conditioned during the agonistic selection in the thymus and therefore do not mount destructive immune responses to the epithelium in the presence of a myriad of foreign Ags in the gut (8). Additionally, previous data further suggest that the CD8αα iIELs can suppress colitis induced by the adoptive transfer of TCRαβ+CD4+CD45RBhigh T cells into SCID mice (9) and thus may have an important regulatory role in the control of autoimmune disease in the intestine.

Control mechanisms for maintaining immune responses following an immune response to a foreign Ag and for preventing or aborting harmful responses to self-Ags include deletion, anergy, or suppression by regulatory T cells (Tregs) (10). Among Tregs there exists several distinct lymphocyte populations including CD4+CD25+ and NK T cells. These cells are efficient in suppressing the priming or expansion of T cell immunity (11, 12). In addition, there is ample evidence for the existence of regulatory CD8 T cells (CD8 Tregs) that play a role in autoimmune diseases, tumor control, infection, transplantation tolerance, neonatal tolerance, and oral tolerance (13–19). However, characteristic surface markers for identifying these CD8 Tregs and the molecular mechanisms by which they induce regulation have yet to be thoroughly characterized.

We have studied Tregs in a model system, experimental autoimmune encephalomyelitis (EAE), induced by immunizing H-2b mice (B10.PL or PL/J) with myelin basic protein (MBP) emulsified in CFA. In this model paralytic disease is generally monophasic and, once recovered, mice resist further induction of the disease (20, 21). The MBP-reactive CD4+ pathogenic T cells predomin-

We have recently identified a novel subset of CD8 Tregs in peripheral lymphoid organs other than the gut that exclusively express the CD8αα homodimer shown by the CD8αα iIEL population. These CD8αα Tregs target activated T cells expressing only a particular TCR Vβ2 chain (in our case Vβ8.2) and are restricted by a non-

Abbreviations used in this paper: iIEL, intestine intraepithelial lymphocyte; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; PT, pertussis toxin; TL, thymic leukemia Ag; Treg, regulatory T cell.

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3 Abbreviations used in this paper: iIEL, intestine intraepithelial lymphocyte; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; PT, pertussis toxin; TL, thymic leukemia Ag; Treg, regulatory T cell.

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CD8α homodimer may serve as a specific marker for TCR Vβ-reactive CD8α Tregs. In order to further investigate the in vivo stimulation of the CD8α Tregs in the control of EAE, using clones of TCR Vβ8.2-reactive CD8α Tregs, we have examined their TCR repertoire and found that they are oligoclonal with respect to their TCR Vβ gene usage. Surprisingly we found that the CD8α Tregs were resistant to deletion and instead were activated by a TCR Vβ-specific mAb in vivo. These data underscore the unique properties of these self-reactive MHC class I-b-restricted CD8α Tregs in vivo and the importance of CD8α T cells in the maintenance not only of gut homeostasis but also of immune tolerance in the periphery.

Materials and Methods

**Mice and cell lines**

B10.PL and PL/J mice were purchased from The Jackson Laboratory and bred under specific pathogen-free conditions in our own colony at Torrey Pines Institute for Molecular Studies (San Diego, CA). Aged-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 the Institutional Animal Care and Use Committee of the Torrey Pines Institute for Molecular Studies.

**Induction of EAE**

Mice were immunized s.c. with 150 μg of MBPAc1–9 (AcASQKRPSQR) (where Ac indicates N-terminal acetylation) emulsified in CFA. Pertussis toxin (PT; 0.15 μg) in PBS was injected on the same day and 48 h later. Mice were monitored for clinical symptoms of paralysis during disease. Disease was scored on a five point scale as described earlier (28): 1, flaccid tail; 2, hind limb weakness; 3, hind limb paralysis; 4, whole body paralysis; 5, death.

**Proliferation assay**

To examine the proliferation response of draining lymph node cells derived from immunized mice and CD8 T cell lines and clones, 5 × 10⁶ cells were stimulated with relevant peptides in the presence of 5 × 10⁵ syngeneic APCs. Eighteen hours before harvest, the cells were pulsed with [H]ThDr (1 μCi/well), and counts per minute were counted on a Trilux scintillation beta counter.

**Generation of CD8 T cell lines and clones**

PL/J mice were immunized s.c. with the peptide p42–50 at a dose of 20 μg/mouse. Ten days later draining lymph node cells were harvested and stimulated in vitro as follows: 30 × 10⁶ irradiated splenocytes in 5 ml of DMEM complete medium (20% FBS, 100 U/ml penicillin and streptomycin, 3.125 × 10⁻⁴ 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 and 10% CO₂ for 2 h. Draining lymph node cells (30 × 10⁶) in a 5-ml volume were added into the flask and mixed. Cells were cultured vertically at 37°C and 10% CO₂ for 7 days. The resulting cells were then cloned at 100 or 1000 cells/well in DMEM medium containing 10 IU of IL-2 and 1 × 10⁶ p42–50 pulsed irradiated syngeneic splenocytes. The cells were supplemented with fresh medium (2% conditioned medium and 50 IU/ml IL-2) every 3 or 4 days and 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 stimulated 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 to 4 days and restimulated with p42–50-pulsed irradiated splenocytes every two weeks.

To generate SINFEKL (OVAp257–264)-specific CTL clones, splenocytes from OT-1 transgenic mice were stimulated with an engineered fibroblast cell line, MEC.B7.SigOVA. Briefly, the adherent fibroblast APCs were seeded at 100,000 cells per 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. OT-1 transgenic cells (5 × 10⁶) were seeded into wells and then replenished with new medium containing 50 IU/ml IL-2 and 2% conditioned medium every 3 or 4 days and restimulated every 2 wk. Clonality was confirmed by flow cytometry, which showed that all cells expressed the Vβ5 and Vα2 transgenic TCR.

**RT-PCR, real-time PCR, and gene sequencing**

Total mRNA was extracted from cells using the RNeasy mini kit (Qiagen) and subjected to cDNA synthesis with an oligo d(T)₁₇₋₁₈ primer. For RT-PCR analysis, each PCR contained 2 μl of cDNA, 0.5 μl of dNTP (20 mM; Amersham Biosciences), 0.4 μl of AmpliTaq polymerase (5 U/μl; Applied Biosystems), 6 μl of MgCl₂ (25 mM, Applied Biosystems), 1 μl of forward and reverse primers (10 μM), and 5 μl of 10× PCR buffer (Applied Biosystems). The reaction volume was 50 μl. A typical cycle for TCR Vβ6 or Vβ8.2-CH145 was as follows: denatured at 95°C for 1 min, 40 × 22.5-min cycles (45 s at 95°C, 45 s at 60°C, and 45 s at 72°C), and 10 min of incubation at 72°C. The Mg²⁺ concentration and cycles were varied depending on the primers used.

Real-time PCR was performed using the Brilliant SYBR Green quantitative PCR 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 the amplification efficiency of individual genes.

To sequence the TCR Vβ gene from the p42–50-reactive CD8 Tregs clones, TCR Vβ6 and CH145 primers were used to amplify the Vβ6-CH145 gene products. Vβ6-CH145 PCR products were cloned into a pCR2.1-TOPO vector using a TOPO TA cloning kit for sequencing (Invitrogen Life Technologies). The pCR2.1-TOPO constructs were transformed into competent Escherichia coli and positive clones were then sequenced using a BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems) on an automated ABI PRISM 3100 genetic analyzer with a POP-4 polymer and a 5–47 cm × 50-μm capillary (Applied Biosystem).

Major primers used in the above studies: L32 sense (5’-GAAACTCGTG CGGAAACCCCA-3’) and L32 antisense (5’-GGAATCTGGCCCTGAAGCC TT-3’); cyclophilin sense (5’-GCGCCGACGGCCCCCC-3’) and cyclophilin antisense (5’-GTCTTTGGAATCTTGCTGCAAA-3’); TCR-Vβ6 sense (5’-CTCTCTACCTGCTGACATCTGGCC-3’); TCR-Vβ8.2 sense (5’-CA TTATCATATGCTGGTGCCG-3’); CH145 antisense (5’-CAGCTGATGT CTTGCTGACA-3’) and CHβ5 antisense (5’-CTTGCCGGTGGACTCACAT TTCC-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 clone cultures as described earlier (29). Briefly, Nunc MaxiSorp F96 immunoplates 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 plus 0.05% Tween 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). OD₄₅₀ was measured. All cytokine capture and detection Abs were purchased from BD Pharmingen.

**Cellular ELISPOT analysis**

IFN-γ- and IL-4–producing cells were enumerated in restimulated splenocytes from MBPAc1–9-immunized mice by cellular ELISPOT assay as described earlier (30). Briefly, splenocytes (5 × 10⁶ cells/ml) were cultured for 48 h in 24-well plates either with medium alone or with MBPAc1–9 (40 μg/ml). Millititer HA nitrocellulose plates (Millipore) were coated overnight at 4°C with anti-IFN-γ or anti-IL-4 Abs. After blocking the coated plates, Ag-stimulated cells were added at graded concentrations for 24 h at 37°C. The wells were then incubated with biotin-conjugated anti-IFN-γ or anti-IL-4 mAbs followed by incubation with avidin peroxidase (Vector Laboratories). Spots were developed by the addition of 3-aminio-9-ethylcarbazole substrate (Sigma-Aldrich) and counted using a computerized image analysis system (LighTools Research) and the image analyzer program NIH Image 1.61.

**Flow cytometry**

Cell surface molecules were detected by fluorescence-conjugated mAbs. Briefly, 1 × 10⁵ cells per 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 BD Biosciences FACScan/Calibur flow cytometer. Abs purchased from BD Pharmingen include 145-2C11 (anti-CD3), 53-6-7 (anti-CD8α), 53-5-8 (anti-CD8β2), H1.2F3 (anti-CD69), RR4-7 (anti-TCR Vβ) and MRS-2 (anti-TCR Vβ1/8.2).

Thymic leukemia Ag (TL) tetramer staining of the CD8 T cell clones was conducted. TL monomers were provided by Dr. H. Cheroutre’s laboratory (La Jolla Institute for Allergy and Immunology, San Diego, CA).
mRNA was extracted from the CD8 Tregs clones by using limiting dilution techniques as described in Materials and Methods. A, mRNA was extracted from the CD8 Tregs clones and examined for TCR Vβ and Jβ usage using gene spectratyping. Data are the TCR Vβ-Jβ profiles of a predominant CD8αα Tregs clone. B, CDR3 regions of the CD8αα Tregs clones were further analyzed by gene sequencing. Data show the CDR3 sequence of a representative CDR3 T cell clone, 2D11. C, CD8αα Tregs clones were analyzed for the surface expression of specific TCR and CD8αα homodimers by flow cytometry. Shown are flow cytometry profiles of the 2D11 clones stained with anti-TCR Vβ6 and anti-CD8α (53-6-7) or TL tetramers (TL-tet).

To conjugate the monomers, streptavidin-PE was added into a TL monomer at 1:1 ratio. Mixtures were incubated on ice for 15 min in the dark. To stain the CD8 clones, 1 μl of the above-conjugated TL-tetramer was added into 100,000 cells per 100 μl in FACScan buffer. Cells were incubated for 30 min at 4°C and were then washed twice with buffer before being analyzed on the Becton Dickinson FACSCalibur.

**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. p < 0.05 was considered statistically significant.

**Results**

**Analysis of TCR Vβ usage by the CD8αα Tregs clones**

Recently we have cloned a novel population of CD8 Tregs from draining lymph nodes of mice immunized with a peptide, p42–50, derived from the conserved CDR2 region of the TCR Vβ8.2-chain (27). The CD8 Tregs express exclusively CD8αα homodimers (CD8αα Tregs) and protect mice from EAE either by adoptive transfer or upon in vivo activation by their cognate Ag p42–50 (27). To analyze the TCR repertoire of the p42–50-reactive CD8αα Tregs, we determined the TCR Vβ usage of three independently generated CD8αα Tregs clones using gene spectratyping. The data revealed that two of the three CD8αα Tregs clones used the TCR Vβ6 gene segment. A representative spectratyping analysis of a CD8αα Tregs clone, 2D11, that uses TCR Vβ6 and Jβ2.4 gene segments is shown in Fig. 1A. To further confirm the TCR Vβ6 usage, we have also sequenced the TCR Vβ6+ CD8αα Tregs clone and determined its CDR3 region sequence (Fig. 1B). To verify the CD8αα homodimer expression by the CD8αα Tregs, the dominant Vβ6+ CD8αα Tregs were stained with anti-TCR Vβ6 and either anti-CD8α or a TL tetramer that selectively binds to CD8αα homodimers with high affinity (31). Fig. 1C clearly shows and confirms our earlier finding that the CD8αα Tregs are CD8α8 TCRVβ6 TL-tetramer".

**CD8αα Tregs predominantly use the TCR Vβ6 gene segment**

Due to the difficulty in generating and maintaining long-term CD8αα Tregs clones, we also generated a total of 14 short-term p42–50-reactive CD8 T cell lines (CD8 Tregs lines) to further analyze the apparent oligoclonal TCR Vβ6 gene usage by the CD8αα Tregs. We have previously demonstrated that T cell lines raised in response to p42–50 possess the same potent regulatory properties as our three characterized CD8 Tregs clones and that the regulatory property of these lines depends on the presence of CD8αα T cell (27). As shown in Fig. 2A, compared with naive spleen cells the p42–50-reactive CD8 Tregs lines have a significant expansion of both TCRVβ6 CD8αα (upper panel) and Vβ6 TL-tetramer T cells (lower panel). A typical dot plot analysis of a CD8αα Tregs line is shown in Fig. 2B, which demonstrates the expansion of CD8α8 TCRVβ6 TL-tetramer T cells following in vitro culture.

These data confirm that the CD8αα Tregs preferentially use the TCR Vβ6 gene segment. To further explore the predominance of Vβ6 T cells among the p42–50-reactive CD8 Tregs lines, we have asked whether an Ab directed toward this TCR could block an in vitro response to its cognate Ag. Groups of PL/J mice were s.c immunized with the TCR peptide p42–50, and draining lymph node cells were analyzed for recall response to p42–50 in the presence of isotype control (IgG), anti-TCRVβ6, or anti-TCRVβ6 mAbs. As shown in the lower panel of Fig. 2C, anti-TCRVβ6, but not the IgG isotype control or the anti-TCR Vβ11 mAb, inhibits the response of the draining lymph node cells to p42–50 (the percentage of inhibition in the presence of the anti-TCR Vβ6 mAb ranged from 35.48 ± 22.04% to 78.59 ± 6.89%); the inhibitory effect of the anti-TCR Vβ6 mAb was specific, because the same Ab did not block a recall response of draining lymph node cells to MBPac1–9 whose response does not predominantly use the TCR Vβ6 gene segment (Fig. 2C, upper panel). Fig. 2D shows a dose-dependent inhibition of the above p42–50-mediated in vitro recall response of lymph node cells in the presence of the anti-TCR Vβ6 mAb, but not in the presence of an isotype-matched control Ab. Table I shows combined data from three independent experiments. Overall, the data demonstrate that the anti-TCRVβ6 mAb significantly blocks the response of the CD8αα Tregs to their cognate ligand, confirming their functional oligoclonality with respect to the TCR Vβ gene usage.

Administration of anti-TCR Vβ6 mAb leads to activation of TCR Vβ6+ lymphocytes and down-modulation of their receptors

Predominant usage of the TCR Vβ6 gene segment by CD8αα Tregs prompted us to ask how the treatment of mice with the anti-TCR Vβ6 mAb could affect the CD8αα Treg repertoire in vivo. B10.PL mice were injected i.v. with the anti-TCR Vβ6 mAb (RR4-7, 300 μg/mouse), and splenocytes were examined for the presence of Vβ6+ and Vβ8+ T cells by flow cytometry on days 0, 1, and 3. As shown in Fig. 3A, injection of the anti-TCR Vβ6 mAb leads to activation of TCR Vβ6+ T cells in vivo as determined by expression of the early activation marker CD69 (Fig. 3A, second column from left) and an apparent down-regulation of their receptors in both CD8αα (Fig. 3A, left column) and CD4+ T cells (data not shown). The effect of the anti-TCR Vβ6 mAb is TCR specific, because TCR Vβ8+ T cells are not affected under the identical conditions (Fig. 3, third and fourth columns from the left).

**FIGURE 1.** CD8αα Tregs clones predominantly use the TCR Vβ6 gene segment. Peptide p42–50-reactive CD8αα Tregs clones were generated from the draining lymph nodes of p42–50-immunized PL/J mice by using limiting dilution techniques as described in Materials and Methods. A, mRNA was extracted from the CD8αα Tregs clones and examined for TCR Vβ and Jβ usage using gene spectratyping. Data are the TCR Vβ-Jβ profiles of a predominant CD8αα Tregs clone. B, CDR3 regions of the CD8αα Tregs clones were further analyzed by gene sequencing. Data show the CDR3 sequence of a representative CDR3 T cell clone, 2D11. C, CD8αα Tregs clones were analyzed for the surface expression of specific TCR and CD8αα homodimers by flow cytometry. Shown are flow cytometry profiles of the 2D11 clone stained with anti-TCR Vβ6 and anti-CD8α (53-6-7) or TL tetramers (TL-tet).
The data are representative of three independent experiments. The data are representative of three independent experiments. The data are combined from at least four independent experiments. The data are representative of two independent experiments. The data are representative of two independent experiments.

FIGURE 2. p42–50-reactive CD8αα Tregs dominantly use the TCR Vβ6 gene segment in vivo. A, p42–50-reactive CD8 Treg lines were generated from the draining lymph nodes of p42–50-immunized mice as described in Materials and Methods. The expression of CD8αα homodimers and TCR Vβ6 was analyzed by FACS using anti-TCR Vβ6 mAb and either the anti-CD8α (53-6-7) mAb or TL tetramers. The data show the percentage of TCR Vβ6+CD8+ (upper panel) or the TCR Vβ6+TL-tetramer+ (lower panel) T cells in both naive spleens and p42–50-reactive CD8 Treg lines. The data are combined from at least four independent experiments. *, p < 0.001; **, p < 0.05; ANOVA test. B, Dot plot analysis of a p42–50-reactive CD8αα T cell line for the expression of CD8, TCRVβ6, PL/J mice were immunized with p42–50 and the draining lymph node cells were examined for cytokine response (IFN-γ) in the presence of an isotype control, anti-TCR Vβ6 11, or anti-TCR 16 mAb or TL tetramers. The data show the percent- age of CD8, TCRVβ8, or homodimers (TL tetramer−). The data are representative of two independent experiments. C, PL/J mice were immunized with either p42–50 (lower panel) or MBPac1–9 (upper panel). The draining lymph node cells were examined for recall response (IFN-γ) to p42–50 (lower panel) or MBPac1–9 (upper panel) in the presence of an isotype control, anti-TCR Vβ11, or anti-TCR Vβ6 mAbs. The results are shown as percentages relative to the response in the presence of IgG isotype control (100%). The data are representative of two independent experiments. *, p < 0.01 for anti-TCR Vβ6 vs IgG or anti-TCR Vβ11. D, PL/J mice were immunized with p42–50 and the draining lymph node cells were examined for cytokine response (IFN-γ) upon re-stimulation with p42–50 in vitro in the presence of the indicated mAbs. The data are representative of three independent experiments.

To further evaluate the effect of the anti-TCR Vβ6 mAb on TCR Vβ6+ T cells in vivo, we analyzed the TCR Vβ6 mRNA in splenocytes of injected mice. As shown in the upper panel of Fig. 3B, injection of the anti-TCR Vβ6 mAb leads to a brief increase followed by a significantly reduced level of TCR Vβ6 mRNA (day 0 vs day 3; p < 0.0001). The level of TCR Vβ8.2 mRNA, however, is slightly decreased followed by an increase on day 3 (day 0 vs day 3; p < 0.05). However, the increase in Vβ8 mRNA is more variable from mouse to mouse compared with the TCR Vβ6 mRNA level (Fig. 3B, lower panel). Furthermore, the transfer of CFSE-labeled TCR Vβ6+ cells into syngeneic mice followed by the injection of the anti-TCRVβ6 mAb yielded a reduced number of Vβ6+ T cells (data not shown). These data collectively suggest that in vivo injection of the anti-TCR Ab results in partial deletion of the TCR Vβ6+ T cells following an initial activation.

Injection of anti-TCR Vβ6 mAb does not delete the 2D11 clone or CD8αα T cells in the periphery

CD8αα iIELs have been shown to use different CD3 signaling molecules than conventional CD8αβ+CD4− TCRαβ+ T cells (CD8αβ T cells) and to express high levels of anti-apoptotic molecules of the Bcl-2 family (32). We therefore planned to determine whether an in vivo injection of the anti-TCR Vβ6 mAb depletes the CD8αα Tregs population or not by using TL-tetramers and primers derived from the TCR Vβ6+ CD8αα Tregs clone 2D11. As shown in Fig. 4A, these primers specifically amplify an mRNA sequence from the CD8αα Tregs clone, but not from an irrelevant OT-1 clone or from naive splenocytes. Fig. 4B shows an analysis of mRNA amplification in the CD8αα Treg clone, the OT-1 clone, and naive splenocytes by real time PCR using the CD8αα Treg-specific primers.

Using the CD8αα Treg clone-specific primers and TL tetramers, we then further examined the repertoire change in the splenocytes of mice injected with the anti-TCR Vβ6 mAb. As shown in the upper panel of Fig. 4C, TL-tetramer+CD8+ T cells are not deleted following the Ab injection. Indeed, the percentage TL tetramer+ CD8+ T cells was increased (p < 0.05) following the Ab injection and maintained for at least 7 days. Similarly, the lower panel of Fig. 4C shows that the mRNA specific for the CD8αα
Tregs clone, 2D11, was not decreased but rather increased significantly \((p < 0.05)\) in a similar pattern to that of the TL-tetramer \(\text{CD8}^+\) T cells. The increased expression in both TL-tetramer \(\text{CD8}^+\) T cells and mRNA specific for the CD8\(\alpha\alpha\) Tregs clone is consistent with the activation of TCR V\(\beta\)6 cells as mentioned above and may further suggest a moderate in vivo expansion of the CD8\(\alpha\alpha\) Tregs following anti-TCR V\(\beta\)6 mAb injection. These data are similar to findings in the CD8\(\alpha\alpha\) iIELs that show poor but definite responses to their cognate Ags or to TCR cross-linking (7).

**FIGURE 3.** In vivo injection of the anti-TCR V\(\beta\)6 mAb leads to activation of TCR V\(\beta\)6\(^+\) lymphocytes and down-modulation of their receptors. **A**, The anti-TCR V\(\beta\)6 mAb was injected i.v. (300 \(\mu\)g/mouse) at days 0, 1, and 3 and splenocytes were stained with TCR V\(\beta\)6-FITC/CD8\(\alpha\)-PE/CD69-PerCP (first and second columns from the left) or TCR V\(\beta\)8-FITC/CD8\(\alpha\)-PE/CD69-PerCP (third and fourth columns from the left) to analyze the expression of the early activation marker CD69. Data are representative of two independent experiments. **B**, mRNA was extracted from the splenocytes of the above anti-TCR V\(\beta\)6 mAb-injected mice and examined for the expression of TCR V\(\beta\)6 (upper panel) or TCR V\(\beta\)8.2 (lower panel) gene expression using real-time PCR. Expression of the specific transcripts is presented as relative quantity after normalization against two internal control genes (L32 and cyclophilin). Data are representative of two independent experiments. Statistical analysis from combined data of two independent experiments: \#, \(p < 0.05\) for day 0 vs day 1; \#\#, \(p < 0.0001\) for day 0 vs day 3 and day 1 vs day 3; \*, \(p < 0.05\) for day 0 vs day 1 and day 0 vs day 3; \#\#, \(p = 0.01\) for day 1 vs day 3.

**FIGURE 4.** In vivo injection of the anti-TCR V\(\beta\)6 mAb does not delete CD8\(\alpha\alpha\) or the dominant p42–50-reactive CD8\(\alpha\alpha\) Tregs clone 2D11. **A**, Primers specific for the CDR3 region of the dominant CD8\(\alpha\alpha\) Tregs clone (2D11) were designed and used to amplify the clone-specific transcripts from the 2D11 clone, the OT-1 clone, and naive splenocytes using RT-PCR. Data are representative of three independent experiments. **B**, The amplification of the 2D11 specific transcripts by the above designed primers was further confirmed by real-time PCR. Expression of the 2D11-specific transcripts is presented as the relative quantity after normalization against two internal control genes (L32 and cyclophilin). Data are representative of three independent experiments. **C**, the anti-TCR V\(\beta\)6 mAb was injected into mice i.v. The TL tetramer was used to detect CD8\(\alpha\alpha\) T cells by FACS analysis (upper panel) and 2D11-specific primers were used to detect 2D11 by real-time PCR (lower panel). Data are representative of three independent experiments. \*, \(p < 0.05\) for day 0 vs day 1.

**FIGURE 5.** In vivo injection of the anti-TCR V\(\beta\)6 mAb results in protection from EAE. B10.PL mice were injected with an isotype control mAb (100 \(\mu\)g/mouse), an anti-TCR V\(\beta\)6 mAb (100 \(\mu\)g/mouse), or PBS on days 1 and 8 after EAE induction (day 0). Mice were monitored for paralytic disease as described in the Materials and Methods. \(p\) values between PBS/control and anti-TCR V\(\beta\)6 groups: 0.024 (day 15); 0.007 (day 16); 0.006 (day 17); 0.009 (day 18); 0.04 (day 19); 0.03 (day 20); 0.02 (day 21); 0.05 (day 22); and 0.04 (day 23). \(p\) values between IgG vs anti-TCR V\(\beta\)6 groups were as follows: 0.03 (day 20); 0.01 (day 21); 0.04 (day 22); and 0.04 (day 23).
Administration of anti-TCR Vβ6 mAb leads to protection from EAE

Activation rather than deletion of p42–50-reactive CD8αα Tregs allowed us to ask whether an injection of the anti-TCR Vβ6 mAb could protect mice from MBP-induced EAE. Groups of mice were immunized for EAE with MBP Ac1–9/CFA/PT followed by i.v. injection with a low dose of anti-TCR Vβ6 mAb (100 μg/mouse), an isotype control mAb, or PBS i.v. on days 1 and 8. The data in Fig. 5 show that an in vivo injection of the anti-TCR Vβ6 mAb results in protection from EAE in B10.PL mice (p < 0.05 between the PBS and the anti-TCR Vβ6 groups on days 15–23; p < 0.05 between the IgG and the anti-TCR Vβ6 groups on days 20–23). Table II shows a summary of the combined data from four independent experiments.

Injection of anti-TCR Vβ6 mAb prevents expansion of Th1-type MBP Ac1–9-reactive T cells in vivo

Although the role of Th1- and Th2-type cytokines in disease progression or remission is complex, it is well established that Th1-type cells contribute toward pathogenesis whereas Th2 cytokines prevent EAE in this system (33). To further study the mechanisms of protection from EAE following injection of the anti-TCR Vβ6 mAb, we examined the frequency of MBP-reactive Th1/Th2 cytokine-secreting cells in animals treated with anti-TCR Vβ6 mAb. Groups of mice were immunized for EAE with MBP Ac1–9/CFA/PT followed by i.v. injection with a low dose of anti-TCR Vβ6 mAb (100 μg/mouse) or an isotype control as described above. Splenocytes were analyzed for MBP Ac1–9–specific IFN-γ- or IL-4-secreting cells by ELISPOT. As shown in Fig. 6, IFN-γ-secreting cells are significantly decreased compared with IL-4-secreting cells in the anti-TCR Vβ6 mAb injected mice as detected by a significant decrease in the IFN-γ to IL-4 ratio (p < 0.05). Therefore, injection of the anti-TCR Vβ6 mAb results in the immune deviation of the response to MBP from the Th1 to the Th2 direction and in protection from the disease.

Discussion

The function and origin of CD8αα CD4+ TCRαβ (CD8αα T cells) in peripheral lymphoid organs other than the gut are not known. We have cloned and characterized a novel population of CD8αα T cells that are specific for a determinant derived from a conserved CDR2 region of the TCR Vβ8.2 chain (p42–50) and display regulatory function in an EAE model in H-2b mice (B10.PL or PL/J) (27). In this report, we further analyzed the TCR repertoire of the CD8αα CD4+ TCRαβ regulatory T cells (CD8αα Tregs). The data demonstrate that the TCR peptide-specific CD8αα Tregs predominantly use the TCR Vβ6 gene segment. Injection of anti-TCR Vβ6 mAb leads to activation of the majority of TCR Vβ6+ T cells followed by the down-modulation of their receptors or partial deletion. Ab treatment also results in apparent expansion of CD8αα T cells (TL-tetramer+ T cells) or the CD8αα Tregs in the peripheral lymphoid organs. In addition, in vivo injection of anti-TCR Vβ6 mAb leads to protection from EAE that correlates with the immune deviation of the immunodominant anti-MBP response.

Earlier studies have suggested that thymus-derived lymphocytes migrate into the intestine and develop into CD8αα T cells and that CD8αα CD4+ TCRαβ thymocytes (CD8αα thymocytes) are generally absent in normal mice even at very early developmental stages, for example, embryonic day 14 (34, 35). However analysis of H-Y TCR transgenic male mice has shown that the H-Y transgenic thymus does contain detectable numbers of CD8αα thymocytes in the first 2 wk after birth, demonstrating that in the presence of agonistic Ags the thymus has the ability to positively select CD8αα T cells (1). One explanation for the absence of CD8αα thymocytes in the normal thymus is that most of the CD8αα thymocytes in normal mice, for example the TCR peptide-specific CD8αα thymocytes described here, develop in the thymus at very low numbers due to the relatively low concentration of a particular agonistic self-Ag available locally. An alternative possibility is that there may exist a particular population of precursors that are committed to the CD8αα lineage in the thymus but actually acquire the CD8αα homodimers in the intestine or under certain conditions in the peripheral lymphoid tissues. Indeed, a recent report indicates that triple-positive thymocytes (CD4+ CD8αα β− CD8αα+) represent the preselection stage: however, double-negative CD5+ TCRβ+ thymocytes are postselection precursors of the CD8αα iIELs. These precursors require further maturation into CD8αα iIELs after export from the thymus in an IL-15-rich environment, e.g., the gut (36).

Interestingly, nearly all CD8αα iIELs express FcɛRI γ-chains as part of the CD3 complex, whereas conventional TCRβ− iIELs express homodimers of CD3ε or heterodimers of CD3 ε and η (37). This characteristic phenotype appears to be akin to the expression of FcɛRI γ-chains in immature double-negative thymocytes and the fact that the immediate precursors of double-positive thymocytes are CD4+ CD8αα− (32). It is therefore possible that the CD8αα T cells may directly derive from DN thymocytes under the pressure of agonistic ligands (2). In addition, CD4+ CD8αα− thymocytes express high levels of anti-apoptotic factors of the Bcl-2 family (32), which is consistent with the finding from our and other laboratories that CD8αα T cells are resistant to apoptosis induced by TCR cross-linking (see below).

Table II. Activation of TCR Vβ6+ CD8αα+ T cells leads to protection from EAE

<table>
<thead>
<tr>
<th>Antibodiesa</th>
<th>Incidence of EAEb</th>
<th>Mean Days of Disease Onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>3/4 (5,3,3,0)</td>
<td>13.3 ± 1.2</td>
</tr>
<tr>
<td>IgG</td>
<td>10/12 (5,5,4,3,3,3,3,2,1,1,0,0)</td>
<td>14.8 ± 3.2</td>
</tr>
<tr>
<td>Anti-Vβ3</td>
<td>6/6 (5,5,4,4,2,1)</td>
<td>13.1 ± 2.2</td>
</tr>
<tr>
<td>Anti-Vβ6</td>
<td>5/12 (5,2,4,1,1,0,0,0,0,0,0,0)</td>
<td>26.7 ± 5.5</td>
</tr>
</tbody>
</table>

a Abs (100 μg) were injected i.v. on days 1 and 8 following immunization with MBP Ac1–9/CFA/PT for the induction of EAE.

b The number of animals with disease out of the total number of animals tested. Numbers in parentheses are the maximal individual disease scores.
We have further demonstrated that TL tetramer+ T cells (CD8α+ T cells) also exist in the peripheral lymphoid organs at a very low percentage in normal naive mice (<1%) (Figs 2A and Fig. 4C), which is consistent with the findings from other laboratories (38). It is important to note that the percentage of CD8α+ T cells is increased in both spleens (>10%) and among the iIELs (>20%) of MHC class Ia-deficient mice (5, 6, 38), suggesting that MHC class Ib molecules are important in the development of CD8α+ T cells in both the peripheral lymphoid organs and the intestine. However the relationship between these two groups of cells is not clear.

Ags recognized by CD8α+ T cells and role of these cells in the periphery have not been described. Recent data support the notion that T cells specific for any Ag have the ability to develop into CD8α+ T cells (1, 4). CD8α+ iIELs have been shown to be the only cell subset among iIELs that can suppress colitis induced by the adoptive transfer of TCR αβ+CD4+CD45RBhigh cells into SCID mice (9). However, due to the rarity of CD8α+ T cells in the secondary lymphoid organs, it had been extremely difficult to analyze their function in the periphery. Our data demonstrate that CD8α+ Tregs specific for TCR self-peptides constitute an important pool of regulatory T cells in the periphery, bearing similarity to the CD8α+ T cells among the iIELs that also display a regulatory function (9).

An effort to specifically deplete CD8α+ T cells to analyze their function has been made by the deletion of a CD8 locus enhancer (E8I), which leads to the selective reduction of CD8α homodimer expression on iIELs (39). Recent data generated from these mice suggest that conventional CD8α+CD8β+CD4+ TCRαβ+ T cells (CD8αβ T cells) also transiently express CD8α homodimers upon activation (CD8ααCD8αβ T cells) that were shown to contribute to the survival of CD8+ memory T cells (40). However, other laboratories have failed to confirm a general requirement for the CD8α homodimers in CD8+ T cell memory (39, 41, 42). Data from our and other laboratories have clearly shown that CD8α+ T cells from both peripheral lymphoid organs and iIELs have regulatory function. It is therefore tempting to speculate that CD8α homodimers actually endow a specific CD8+ T cell lineage with a regulatory function. The cloning of TCR peptide-specific CD8α+ Tregs provides an important tool for analyzing this unique group of cells.

Repertoire analysis of the p42–50-reactive CD8α+ Tregs described here reveals a predominant usage of the TCR Vβ6 gene segment that is reminiscent of two other populations in this system, the TCR Vβ8.2 peptide p76–101-specific CD4+ Tregs that predominantly use TCR Vβ14 (28), and the MBPAc1–9-specific CD4+ pathogenic T cells that predominantly use TCR Vβ8.2 (20, 21). The highly restricted usage of TCR Vβ gene segments in this system has made it easier to dissect different components in the immune system. It is important to note that the restricted usage of TCR variable gene segments to different degrees has been reported in immune responses to virus, allograft, tumor, β2-glycoprotein I, soluble hen egg white lysozyme, and others (26, 43–46). Therefore, the existence of dominant repertoires suggest that certain dominant T cell clones or even multiple clones can be targeted for therapy in T cell-mediated diseases.

Our data further suggest that although in vivo injection of anti-TCR Vβ6 mAb leads to activation of the CD8α+ Tregs, it results in down-modulation of the TCR or partial deletion of the bulk Vβ6+ T cell population (Figs. 3 and 4) as suggested by real-time PCR and CFSE-labeling experiments. Interestingly, the number and mRNA of CD8α+ T cells and the CD8α+ Treg clone, respectively, are actually increased upon in vivo injection of the anti-TCR Vβ6 mAb. Although, the underlying mechanism(s) remains unknown, it may be related to the relative resistance of CD8α+ T cells to apoptosis upon TCR cross-linking owing to unique signal transduction pathways (2, 32, 37). It is noteworthy that some anti-CD25 mAbs were recently found not to deplete the CD4+CD25+ Tregs, suggesting that regulatory T cells may have some common features that render them resistant to depletion by mAbs (47). Interestingly, polyclonal anti-lymphocyte serum has also been shown to spare the CD4+CD25+ Tregs from deletion although it deletes most of the other lymphocytes. At least 2 wk after anti-lymphocyte serum treatment it appeared that the residual CD4+CD25+ Tregs showed a much stronger in vitro suppressive activity (48). Although the mechanisms of the above polyclonal anti-lymphocyte sera appear complex, our data collectively suggest that anti-TCR Ab-mediated activation of the CD8α+ Tregs in vivo provides an important pathway for generating an effective regulatory response with potential clinical implications for the therapy of autoimmune diseases and transplant rejections.

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Disclosures
The authors have no financial conflict of interest.

References


