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Global expression profiling of peripheral Qa-1-restricted CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + regulatory T Cells reveals innate-like features: implications for immune regulatory repertoire

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Abstract

Among peripheral regulatory T cells, CD8⁺ T cells also play an important role in the maintenance of immune homeostasis. A subset of CD8⁺ Treg that express $\alpha\beta$ TCR and CD8 $\alpha\alpha$ homodimers can recognize TCR-derived peptides in the context of the class Ib MHC molecule Qa-1. To gain a better understanding of the nature and phenotype of CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + Treg, a global gene expression profiling using microarray, real-time quantitative PCR, and flowcytometry analysis was performed using functional Treg clones and lines. Our data show that CD8⁺ Treg shared gene profile expressed by innate-like lymphocytes, including murine intraepithelial lymphocytes and thymic CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + T cell populations. Additionally, this subset displays differential expression of several key regulatory molecules, including CD200. CD8 $\alpha\alpha$ + Treg expressed higher levels of a number of NK cell related receptors and molecules belonging to the TNF superfamily. Collectively, peripheral class Ib-reactive CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + T cells represent a unique regulatory population different from class Ia MHC-restricted conventional T cells. These studies have important implications for the regulatory mechanisms mediated by the CD8⁺ Treg population in general.

Keywords

CD8⁺ Treg; EAE; Microarray; Innate cells; Qa-1/HLA-E

Introduction

Among peripheral immune regulatory mechanisms, regulatory T cells (Treg) play a crucial role in the control of self-reactive, potentially pathogenic mature T cells (1, 2). Treg populations can be generally divided into several subgroups depending on the expression of

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different molecules, including co-receptors, CD4, or CD8, and can control immune responses in either an antigen-specific or -non-specific manner. In addition to naturally occurring Treg populations, including CD4⁺CD25⁺FoxP3⁺ T cells and NKT cells, CD8⁺ Tregs have also been described both in rodents and in humans (3, 4) and Human Immunology 2008, Vol. 69, issue 11). A detailed understanding of phenotypes and biology of different CD8⁺ Tregs is not well-understood.

MHC class Ib molecules, including Qa-1, Qa-2, H2-M3, and CD1d in rodents and HLA-E, HLA-G, and CD1 in humans, differ from MHC class Ia molecules in that they have limited polymorphism (5). MHC class Ib molecules have been shown to present both self-antigens as well as foreign antigens derived from a number of pathogens (6, 7). Class Ib-restricted CD8⁺ T cells have been shown to be involved in host defense, antitumor immunity (8-10) and in immune regulation mechanisms (11-14). MHC class Ib-restricted T cells appear to differ from MHC class Ia-restricted T cells in the following ways: 1) they generally have an activated phenotype and are CD44^{high}; 2) they are selected by hematopoietic cells (15), 3) they can be activated in the periphery by their thymic selecting ligand/agonist; 4) they rapidly produce cytokines upon activation (16); and 5) their gene expression phenotype has special features differing from that of adaptive T cells (17, 18).

Although, regulatory CD8⁺ T cells restricted by MHC class Ib molecules, Qa-1 in mouse and HLA-E in humans have recently been described (12, 19, 20), details of the cell surface phenotype and regulatory mechanisms have not been well characterized. We have identified a subset of Qa-1-restricted CD8 α ⁺TCR $\alpha\beta$ ⁺ Treg population (referred to as CD8 α ⁺ Treg) that recognizes an antigenic determinant in the conserved CDR2 region of the TCR V β 8.2 chain (4, 14, 21-23) and control experimental autoimmune encephalomyelitis (EAE), a prototype for multiple sclerosis (14, 23). We have demonstrated that CD8 α ⁺ Treg control disease by inducing apoptosis in activated, pathogenic CD4⁺ T cells following recognition of the TCR-peptide/Qa-1 complex on their cell surface (4, 14, 22, 23). Furthermore, mechanism of killing by CD8 α ⁺ Treg was dependent upon IFN- γ and perforin pathway (24).

To better understand the phenotype and regulatory mechanisms involved in the immune regulation mediated by CD8 α ⁺ Treg, we have recently generated and characterized several CD8 α ⁺TCR $\alpha\beta$ ⁺ T cell clones and lines that are physiologically primed by an antigenic determinant in the conserved CDR2 region of the TCR V β 8.2 chain and are involved in recovery and protection from EAE (4, 14, 21-23). Here we have compared global gene expression patterns in cloned populations of CD8 α ⁺TCR $\alpha\beta$ ⁺ Treg versus conventional CD8 $\alpha\beta$ ⁺TCR $\alpha\beta$ ⁺ T cells (Tconv). The results of microarray data analysis were further confirmed by real-time PCR and flow cytometric analysis. Our study reveals a unique gene expression signature in the CD8 α ⁺ Treg characterized by an enhanced expression of several inhibitory molecules associated with the maintenance of peripheral T cell regulation and homeostasis, as well as molecules typical of innate-like lymphocytes. These data have important implications for the identification as well as further characterization of the regulatory T cell repertoire with special emphasis on role of inhibitory pathways in immune regulation.

Materials and Methods

Animals

B10.PL and PL/J mice (H-2^u) 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). Ova-reactive OT-1 TCR transgenic and MBP-reactive V β 8.2 TCR transgenic mice were kindly provided by Drs. S.P. Schoenberger (LIAI, San Diego) and

Juan Lafaille (NYU, New York), respectively. 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.

Generation of regulatory CD8 α +TCR α β + T cell lines and clones

Regulatory CD8 α +TCR α β + T cell lines (XT-14, PL-1) and clones (2D11, 1C9) have been generated and characterized as described earlier (4, 14, 21-23). Briefly, groups of mice were immunized subcutaneously with the peptide p42–50 (20 μ g/mouse) of the V β 8 TCR chain, and draining lymph node cells were cultured with p42-50-pulsed irradiated splenocytes in complete DMEM medium containing 20% FBS. The resulting cultures were then cloned at 100 or 1000 cells/well in the presence of 10 IU of IL-2 and p42–50 pulsed irradiated syngenic splenocytes. To generate CD8 α + Treg lines, the re-stimulated draining lymph node cells were expanded using and 2% conditioned-medium in 24-well plates. Conditioned medium contained IL-2 (50 IU/ml), IL-15 (2 IU/ml) and Con A/MLR supernatant (4%). The lines were supplemented with fresh medium every 3 to 4 days and re-stimulated with p42–50-pulsed irradiated splenocytes every two weeks. To generate SIINFEKL (OVAp257-264)-specific CTL clones, splenocytes from OT-1 transgenic mice (25) were stimulated with an engineered fibroblast cell line, MEC.B7.SigOVA. Clonality was confirmed by staining with specific antibodies and by flow cytometry using anti-CD8 α , anti-CD8 β , anti-TCR- β and anti-V β 6 (for Treg) and anti-V β 5/V α 2 (for OVA) mAbs. MBPAc1-9-reactive T cells (3C10) were generated following peptide immunization from wild-type B10.PL (H-2u) animals or following in vitro challenge of splenocytes derived from a V β 8.2-TCR-transgenic B10.PL mice, essentially as described before (14, 26, 27). Both OVA- and MBP-reactive cloned T cells were cultured under identical conditions.

Microarray Gene Expression Analysis

Total RNA was isolated from T cell lines and clones generated from different mice using RNAqueous reagents (Ambion). Briefly, following lysis of frozen cell pellets with lysis reagent, equal volume of 64% ETOH buffer was added, mixed by pipetting and transferred to columns and centrifuged @ 14,000 RPM for 30 seconds. After washing, RNA was eluted twice with warm elution buffer, EB, (75°C) by centrifugation and concentration estimated in UV-spectrophotometer and the quality was evaluated using formaldehyde/agarose RNA gels. RNA was used to generate biotinylated cRNA for hybridization to mouse GeneChip Genome 430 2.0 Array (Affymetrix, Santa Clara, CA) using GeneChip one-cycle target labeling and control reagents (Affymetrix, Santa Clara, CA) in accordance with the manufacturer's protocol.

GCRMA was used for background correction, quantile normalization and summarization [1]. This method uses probe sequence information to estimate probe affinity to non-specific binding. One way ANOVA statistical analysis of the microarray data was performed. Benjamini-Hochberg false discovery rate (FDR) method with a specified p-value cutoff (or error rate) of 0.05 and a fold change >2 was used to obtain differentially expressed probe sets, as described earlier (28).

Real-time PCR Analysis

Total mRNA was extracted from cells using RNeasy mini kit (Qiagen), and subjected to cDNA synthesis using oligo(dT)12-18 primer. Real-time PCR was performed to detect enrichment of genes using the Brilliant SYBR Green quantitative PCR kit (Stratagene) on a Stratagene Mx3000p machine. Each PCR contained 2 μ l of cDNA, 25 μ l of 2X Brilliant SYBR Green QPCR Master Mix (Stratagene), 1 μ l of forward and reverse primers (10 μ M),

0.75 μ l of ROX Reference Dye (Stratagene), and 20.25 μ l of sterile water. A typical cycle was as follows: denatured at 95°C for 11 min, 40 $\times$ 2.25-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 calculation of comparative mRNA expression was designated as relative quantity after normalization against an internal control gene (L32) and after consideration of the amplification efficiency of individual genes.

All gene-specific primers were designed using Primer3 software of Rozen and Skaletsky. Each primer pair is picked using standard Primer3 parameters to be 18bp-22bp in length and have melting temperature between 59°C and 61°C. Major primers used in the above studies include: L32 sense (5'-GAAACTGGCGGAAACCCA-3') and L32 antisense (5'-GGATCTGGCCCTTGAACCTT-3'); TCR-V β 6 sense (5'-CTCTCACTGTGACATCTGCCC-3'); and C β 145 antisense (5'-ACTGATGTTCTGTGTGACA-3')

Flow cytometry analysis

Briefly, 1–4 $\times$ 10⁵ cells per 100 μ l 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 FACSCalibur. Abs were purchased from BD Pharmingen include 145-2C11 (anti-CD3), 53-6-7 (anti-CD8 α), 53-5.8 (anti-CD8 β .2), H1.2F3 (anti-CD69), and RR4-7 (anti-TCR V β 6). Rat anti-mouse CD200:FITC. Clone OX-90 Ab (anti-CD200) was purchased from Serotec. Other antibodies were acquired from e-Biosciences. Staining and flowcytometry analysis was essentially carried out as described earlier (14, 23).

Results

Microarray gene analysis of the regulatory CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + vs. non-regulatory CD $\alpha\beta$ +TCR $\alpha\beta$ +T cells

A detailed phenotype and gene expression profile of CD8+ Treg is not known. To determine specific cell surface markers on Treg, microarray gene analysis of long-term functional CD8 $\alpha\alpha$ + Treg clones was undertaken and compared to non-regulatory “conventional” T cells. Suppressor activity of CD8+ Treg clones and lines used in the present study has already been reported earlier and showed their ability to kill only activated target T cells in vitro as well as in vivo studies. Furthermore, upon adoptive transfer, CD8 $\alpha\alpha$ + Treg lines and clones significantly suppress EAE (14, 23, 29). Usage of clonal populations enabled us to avoid potential contamination from non-regulatory cells. Additionally, to ensure that the phenotype was not unique to these functional clones, we analyzed additional short-term CD8 $\alpha\alpha$ + Treg cell lines for the expression of selected RNA transcripts using realtime quantitative PCR. Differential gene expression at the clonal level was determined between a functional CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + Treg (2D11) (14, 22) and OVA-reactive CD8 $\alpha\beta$ +TCR $\alpha\beta$ + T cells (OT-1), cultured under identical conditions in the presence of their respective antigen and IL-2. The total RNA isolated and then hybridized independently to an Affymetrix MOE 430 2.0 Array that has 45,101 probe-sets roughly corresponding to 35,000 genes. The data were normalized using GCRMA, followed by one-way ANOVA and FDRCI analysis, resulting in 562 differentially expressed probe sets corresponding to 461 genes. Interestingly, 188 genes showed increased (>2 fold) and 273 genes showed decreased (>2 fold) expression in 2D11 in comparison to OT-1. These genes can be classified into functional classes according to their involvement in immune/defense responses, metabolism, signal transduction, transport, transcription, cell-death/growth and cell-adhesion. Hierarchical cluster analysis of these genes (Fig. 1) revealed a unique set of up- or down-regulated genes in CD8 $\alpha\alpha$ + Treg, suggesting a specific molecular signature compared to

CD8 $\alpha\beta$ + T cells. Notably, several genes that are relatively over-expressed in the CD4+CD25+FoxP3+ Treg population are downregulated in CD8 $\alpha\alpha$ + Treg, including IL-2R α (CD25) and Lag3. Scatter plots displaying values of normalized signal intensities for these genes are shown as a distance from the diagonal (Fig. 1). The center diagonal indicates no change and the outer lines indicate a 3-fold expression difference. The points in the scatter plot are colored by the normalized signal values of the control cell line.

We then selected a number of differentially expressed genes on the CD8 $\alpha\alpha$ + Treg for further validation by quantitative real-time PCR (qPCR) analysis (Table 1) as well as by flowcytometry analysis (Figure 3). Furthermore, to eliminate possible artifacts resulting from long term in vitro culture of clonal CD8 $\alpha\alpha$ + Tregs, in parallel real-time PCR analysis was performed on freshly generated short-term antigen-reactive CD8 $\alpha\alpha$ + Treg cell lines (XT-14, and PL1) (Figure 2). For most of the enriched genes, profiles among both Treg lines as well as another clone (1C9) were found to be similar to Treg clone 2D11. Furthermore, expression profiles of the differentially expressed genes among Ova-reactive T cells derived from H-2b mice vs. MBP-reactive T cells derived from the wild type or V β 8.2-TCR transgenic B10.PL (H-2u) mice were quite similar (data not shown).

Enrichment of ligands and receptors of the tumor necrosis factor superfamily in CD8+ Tregs

To further determine genes involved in immunoregulatory pathways preferentially expressed in CD8 $\alpha\alpha$ + Treg, real-time PCR analysis of genes belonging to the tumor necrosis factor superfamily (Tnfsf) was carried out. Among the 10 Tnfsf genes identified we have found preferential expression of 8 of these genes (see Table 3). Transcripts for receptors Tnfrsf14 (HVEM) and Tnfrsf11b showed 4.9-fold and 4.5-fold enrichment, respectively, while receptor Tnfrsf4 (OX-40) showed a smaller 2.2-fold increase. Five ligands from the Tnf superfamily, including Tnf, Tnfaip1, Tnfsf8 (CD30-L), Tnfsf11 (Rank-L), and Tnfsf14 (Light), were also upregulated (>2.4 fold) in the CD8 $\alpha\alpha$ + Treg population. As shown in Figure 3, Tnf receptor HVEM is expressed at higher levels on the surface of CD8 $\alpha\alpha$ + Treg and has been shown to associated with immune regulatory pathways (30, 31). Interestingly, another potential regulatory molecule the TNF-related apoptosis-inducing ligand (TRAIL), produced by CD8+ T cells to mediate suppression of inflammatory responses (32), is not enriched in CD8 $\alpha\alpha$ + Treg population.

CD8+ Tregs express several NK cell related genes

The microarray gene analysis revealed over expression of several NK cell receptors on CD8 $\alpha\alpha$ + Tregs in comparison to that among conventional CD8 $\alpha\beta$ + T cells. We further examined the expression profile of NK cell receptors and molecules acting downstream of these receptors using real-time PCR and flowcytometry. A high enrichment of killer cell activating receptor associated protein DAP12 (Tyrobp) is observed in CD8 $\alpha\alpha$ + Treg when compared to CD8 $\alpha\beta$ + Tconv (Figure 4B). CD8 $\alpha\alpha$ + Treg were also enriched with Ly49 family NK receptors as well as CD94. Real-time PCR data revealed a 1.7 to 19-fold increase in Ly49A, Ly49C, Ly49E, Ly49G, Ly49I, Ly49D, CD94, and Ly49F transcripts. Among the preferentially expressed NK receptors, Ly49E showed the greatest degree of enrichment as also shown by flow cytometry analysis (Figure 3B). In contrast with the enrichment of Ly49 family receptors, we have observed diminished mRNA levels for NK1.1 (Klrb1c), Klrb1a, Ly49H, and Nkg2d. The majority of the NK receptors enriched in CD8 $\alpha\alpha$ + Treg are inhibitory receptors, including most of the tested Ly49 family receptors and CD94. In the Ly49 family, all of the enriched receptors are inhibitory, with the exception of Ly49D, which is activating. DAP12 may be either activating or inhibiting depending on the target of regulation (33).

CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + Tregs are enriched in several immunoregulatory molecules/pathways

Both the microarray and real-time PCR data indicate significant enrichment of several regulatory molecules, including CD200 in the CD8 $\alpha\alpha$ + Treg. As shown in figures 2 and 3, real-time PCR and flow cytometry data respectively revealed a considerable increase of up to 198-fold in the transcript levels of the immunosuppressive CD200 molecule (34). In agreement with our earlier observations (14), CD8 $\alpha\alpha$ + Treg were enriched in CD28 transcripts, as shown by real-time PCR. We have also observed an increased transcript level of the CTLA-4 ligand, CD80. The CD8 $\alpha\alpha$ + Treg population also displayed a unique expression pattern of granzymes, with a preferential expression of granzymes D (CTLA-5), E (CTLA-6), and G (CTLA-7). As shown in table 2, granzyme transcripts are increased at least 3.1-fold, with granzyme G showing the greatest degree of enrichment (>10 fold) in CD8 $\alpha\alpha$ + Treg. Interestingly, the three granzymes that showed significant down-regulation in CD8 $\alpha\alpha$ + Treg (up to 447-fold), granzyme A, B and K are cytotoxic effector molecules (35, 36), in agreement with our previous finding of perforin being the principle pathway in CD8 $\alpha\alpha$ + Treg-mediated cytotoxicity (24).

Differentially expressed transcription factors involved in key pathways

To better understand CD8 $\alpha\alpha$ + Treg usage of cellular and molecular pathways involved in their development and function, we were able to identify 15 transcription factors from microarray gene analysis that are differentially expressed (>5-fold difference) in CD8 $\alpha\alpha$ + Treg. Out of the 15 identified transcription factors, 6 were preferentially upregulated in CD8 $\alpha\alpha$ + Treg, and the rest were down-regulated (Table 4). The transcription factor Rora is a positive regulator of transcription and showed the greatest enrichment (17.7-fold) in CD8 $\alpha\alpha$ + Treg. As opposed to Rora, Mycn is a negative regulator of transcription and showed the most down-regulation in CD8 $\alpha\alpha$ + Treg (57.5-fold). Notably, a protein tyrosine phosphatase encoding gene PTPN22 gene is strikingly downregulated in CD8 $\alpha\alpha$ + Treg in comparison to conventional T cells. The PTPN22 gene encodes the lymphoid tyrosine phosphatase (LYP), an important negative modulator of TCR-signaling (37, 38). One important regulatory element that was not up-regulated in CD8 $\alpha\alpha$ + Treg was the forkhead transcription factor Fox P3, considered to be the master regulator of the development and function of naturally occurring CD4+CD25+ Treg. This is consistent with our earlier flow cytometry data indicating basal level of FoxP3 expression in TCR-peptide-reactive CD8 $\alpha\alpha$ + and CD4+ Treg cells (4, 14, 21-23, 39).

Discussion

The phenotypic markers as well as potential immunoregulatory molecules expressed by CD8+ Treg populations are poorly understood. Here we have attempted to characterize the phenotype and other potential regulatory features of a recently identified novel population of peripheral, Qa-1-restricted CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + regulatory T cells that are involved in controlling autoimmune disease. In this study for the first time we have performed a global gene expression analysis of functional Treg clones and polyclonal lines to analyze differentially expressed markers on CD8 $\alpha\alpha$ + Treg compared to the conventional CD8 $\alpha\beta$ + T cells. Our data suggest a unique phenotype of CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + Tregs in that they are enriched in a number of molecules expressed by NK cells, members of TNF-super family as well as in negative signaling molecules, including CD200.

As mentioned earlier, owing to their low abundance in periphery and to avoid contamination from other non-regulatory T cells, we have used antigen-specific, functional T cell lines and clones of CD8 $\alpha\alpha$ + Tregs and analyzed the microarray and real-time PCR data with a special emphasis on the differentially expressed genes of immunological interest. Treg preferentially express several surface receptors and ligands from the tumor necrosis factor

(Tnf) super family. The Tnf receptor HVEM has been shown to exert regulatory effects by interacting with BTLA, which leads to the activation of inhibitory signaling cascade in effector T cells (30). Consistent with its regulatory role, HVEM^{-/-} mice are more susceptible to MOG-peptide induced EAE (31). Our preliminary data showing a significant inhibition of the suppression in the presence of anti-HVEM antibodies suggest that HVEM may be involved in immune regulation mediated by CD8 $\alpha\alpha$ + Treg. These *in vitro* findings, along with our previous data showing the ability of CD8 $\alpha\alpha$ + Treg to inhibit pathogenic T cells *in vivo* during EAE (14, 23) further suggest regulatory role of HVEM+ CD8 $\alpha\alpha$ + Treg. We are currently backcrossing gene-deficient mice to H-2u background to further investigate the role of the HVEM/BTLA pathway in CD8 $\alpha\alpha$ + Treg-mediated immune regulation. It is noteworthy that though several TNFsf members are enriched in the CD8 $\alpha\alpha$ + Treg, Fas (CD95)-Fas-ligand (CD95L) or TRAIL do not appear to be involved in the apoptotic killing mediated by CD8 $\alpha\alpha$ + Treg (40).

Consistent with their role in controlling EAE, CD8 $\alpha\alpha$ + Tregs showed significant enrichment of another important inhibitory cell surface molecule, CD200 (Figure 1 and 4). The CD200/CD200R pathway is known to be capable of delivering a crucial intracellular signal leading to immunosuppression (41). Earlier study showed that antibody-mediated blockade of CD200R leads to an aggravated clinical course of EAE (42). Another study also found significant down-regulation of CD200 in human multiple sclerosis (MS) lesions (43). Collectively these data suggest that CD200 expression on CD8 $\alpha\alpha$ + Treg may play an important role in downregulation of the anti-MBP response in the CNS: for example CD200R-expressing activated microglia can be down-regulated (44). Therefore CD8+ Treg not only will be able to suppress antigen-presenting cells in lymphoid organs but will also be able to suppress microglia activation locally in the CNS for an effective regulation. We are breeding CD200^{-/-} mice onto H-2u background to examine this hypothesis. Interestingly, CD28 and CD80 are also elevated in CD8 $\alpha\alpha$ + Treg population. CD28 and its co-stimulatory molecule CD80 have been shown to be important in maintaining peripheral tolerance in CD8 T cells. In the periphery, a CD28 signal enhances T cell activation and expansion when paired with alloantigens of intermediate avidity. However when TCR ligation exceeds a threshold, a CD28 signal can down-regulate peripheral T cell response by reducing T cell expansion and increasing apoptosis (45). Moreover a CD28 signal was shown to be critical for tolerance induction in CD8+ T cells in the periphery (46).

Another notable feature we have identified here is the enrichment of a unique repertoire of NK receptors by CD8 $\alpha\alpha$ + Treg populations. Interestingly, the most highly enriched NK receptor is an orphan receptor from the Ly49 family, Ly49E. It is known that Ly49E is incapable of reacting with polymorphic MHC class I molecules (47). However, CD8 $\alpha\alpha$ + Treg are restricted by the non-classical MHC class Ib molecule Qa-1, so it remains possible that a non-classical class I molecule could be a potential ligand for Ly49E. Also consistent with immunoregulatory properties of CD8 $\alpha\alpha$ + Treg the preferentially expressed NK receptors are predominantly inhibitory, Ly49A, Ly49C, Ly49F and Ly49G (Fig. 4b). However, it is interesting that activating receptor Ly49D is also expressed on Treg population. Since Ly49D is also expressed on T cells in general following activation. Collectively our data suggest that CD8 $\alpha\alpha$ + Treg preferentially express a unique repertoire of inhibitory NK receptors. Notably, a CD8+ regulatory T cell subset expressing the heterodimeric CD8 $\alpha\beta$ chain has also recently been shown to express inhibitory Ly49 receptors (48, 49). Consistent with their regulatory role, a defect in CD8+ Treg activity in B6-Yaa mutant mice that develops SLE-like disease has been reported (49).

It is notable that similar to the CD8 $\alpha\alpha$ + Treg population described here, a polyclonal population of CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + IEL (17) and *in vitro* generated Thymic CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + T cell populations (18) also preferentially express a set of innate-like genes. For example,

several members of the inhibitory NK receptors were detected to be preferentially over expressed among a polyclonal CD8 $\alpha\alpha$ + IEL population (17). These genes included Ly49A, Ly49C, Ly49D, Ly49E, Ly49G, Ly49I, 2B4 and CD94. Notably, several genes enriched in thymic cells are also preferentially expressed in CD8 $\alpha\alpha$ + Treg populations, for example, FcEr1g, Kegl2, and Kit (18). It has been reported that the TCR repertoire of CD8 $\alpha\alpha$ + IELs is nonrandom and that there is a predominant expression of the TCR V β 6 and V β 11 gene segments. Notably, we have found a preferential use of the V β 6 TCR in our CD8 $\alpha\alpha$ + Treg populations in H-2^u mice (14, 23). Furthermore it appears that a subset of IEL may recognize antigens in the context of non-classical MHC molecules and may even possess immune-regulatory ability similar to the peripheral Qa-1-restricted CD8 $\alpha\alpha$ + Treg population (50, 51).

It is also noteworthy that several key differences in gene expression profile exist between CD8 $\alpha\alpha$ + Treg and IEL counterparts: for example genes upregulated in IEL CD8 $\alpha\alpha$ + populations but did not show enrichment in CD8 $\alpha\alpha$ + Treg, include LAG-3, NK1.1, and Nkg2d. Similarly expression of Ccr5, Cxcr3, Ccl5, Lat2, Fgl2, Klrb1a, Xdh, and 2B4 also differed among CD8 $\alpha\alpha$ + Treg vs. in vitro thymic culture-derived CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + T cells. Additionally, CD8 $\alpha\alpha$ + Treg showed a unique expression profile for members of the tumor necrosis factor super family, which has yet to be described in CD8 $\alpha\alpha$ IEL counterparts. Also several of the granzymes associated with cytotoxic lymphocytes showed significant differential expression in CD8 $\alpha\alpha$ + Treg, which were not illustrated in CD8 $\alpha\alpha$ IEL. CD8 $\alpha\alpha$ + Treg use different immune regulatory mechanisms for controlling autoimmunity. CD8 $\alpha\alpha$ + IEL regulate immune responses using cytokines such as IL-10 and TGF- β (17, 51). We have failed to detect significant levels of IL-10 or TGF- β secretion among CD8 $\alpha\alpha$ Tregs, but confirmed that IFN- γ was required for their immune control (14, 22, 24). It will be interesting to investigate whether these differences are due to polyclonal nature of the IEL or thymic populations that comprise of both regulatory as well as non-regulatory T cells or whether CD8 $\alpha\alpha$ + Treg represent a unique population of peripheral regulatory lymphocytes.

Protein tyrosine phosphatases have been shown to be able to either upregulate or suppress signaling via the T cell receptor and accordingly have been shown to be involved in autoimmune diseases (37, 38). It is noteworthy that transcripts for one of the important lymphoid tyrosine phosphatase, PTPN22 a potent negative modulator of TCR signaling, are drastically reduced in CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + Tregs in comparison to the conventional CD8 $\alpha\beta$ +TCR $\alpha\beta$ + T cells. In fact a missense single nucleotide polymorphism in PTPN22 has been shown to be associated with a number of autoimmune diseases in humans, including type 1 diabetes and lupus (37, 38). Although reduced levels of PTPN22 in CD8 $\alpha\alpha$ + Tregs could either influence their thymic development or their ability to proliferate, it will be interesting to investigate whether there exists any relation to autoimmunity. It is also possible that while negative TCR signaling associated with PTPN22 is required in pathogenic CD8 $\alpha\beta$ +TCR $\alpha\beta$ + T cells and not in CD8 $\alpha\alpha$ + Tregs whose role is to control autoreactivity.

Notably, the signature transcription factor, FoxP3, a forkhead transcriptional repressor, important for the development and function of CD4+CD25+ Treg cells, is not overexpressed in CD8 $\alpha\alpha$ + Treg populations. These data are consistent with our earlier data showing only low level of expression for FoxP3 among functional CD4+ as well as CD8 $\alpha\alpha$ + Treg cells (4, 14, 21-23). However an important retinoid-related orphan nuclear receptor ROR alpha or NR1F1 is significantly over expressed in CD8 $\alpha\alpha$ + Treg. Interestingly, RORa has been shown to be a negative regulator of the inflammatory response (52). It is interesting that FoxP3 also interacts with Rora leading to inhibition of transcriptional activation mediated by Rora (53).

It is clear from several studies that CD8⁺ Treg plays immunoregulatory role in EAE and in multiple sclerosis. In addition to the potential negative signaling via CD200/CD200R pathway, it has been shown that CD8⁺ Treg are able to kill or induce apoptosis in activated target pathogenic T cells (11, 12, 14, 29, 54, 55). We and others have recently shown that the mechanism of killing is dependent upon IFN- γ and perforin molecules (24, 56-61). Collectively, our data suggest that the regulation mediated by CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + Treg is efficient as it may not only kill the activated encephalitogenic T cells but also down-regulate antigen-presentation by negatively signaling dendritic cells or microglia populations. These studies are not only important for getting crucial insights into the phenotype of these cells but will form the basis of future investigations into the development and details of molecular mechanism of immune regulation mediated by the CD8⁺ Treg population.

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FIGURE 1.

Scatter plot displays the log of raw intensity values for the genes of interest between CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + Treg vs. conventional CD8 $\alpha\beta$ +TCR $\alpha\beta$ + T cell populations. Genes with a fold change expression greater than 2 are shown on the left and right hand side of the outer diagonals whereas the center diagonal indicates less than 2 fold change. Immunoregulatory genes are shown in red whereas the remaining ones are shown in blue. The complete microarray data has been submitted to the public database GEO: submission number GSE22985.

FIGURE 2.

Relative expression of some characteristic genes enriched among clonal as well as polyclonal populations of CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + Treg cells. mRNAs were isolated from respective CD8 $\alpha\alpha$ + Treg clone (2D11) and lines (XT14 and PL1), and expression of > 50 genes was analyzed by real-time PCR and only expression of some of the most relevant genes is shown. Gene expression from the OVA-reactive CD8 $\alpha\beta$ +TCR $\alpha\beta$ + OT-1 T cells was used as control. The data were representative of one of two independent experiments and are presented as relative fold change after normalization against internal control gene L32.

FIGURE 3.

CD8αα+TCRαβ+ Treg showed elevated cell surface expression of immunoregulatory molecules CD200, HVEM (A) and Ly49 (B) cell surface molecules. CD8αα+TCRαβ+ Treg (2D11) and OVA-reactive CD8αβ+TCRαβ+ T cells are stained with the indicated antibodies or relevant isotype controls and analyzed by flow cytometry. The thick line represents staining of CD8αα+TCRαβ+ Treg and the thin line represents staining of CD8αβ+TCRαβ+ T cells. An isotype control is also included and is represented by the dotted line. While y-axis denotes the % max fluorescence, x-axis shows fluorescence intensity relative to staining with anti-HVEM or anti-CD200 antibodies (3A) and several anti-Ly-49 markers as indicated on the top (3B). Data are representative of three independent experiments.

TABLE 1
Genes of immunological interest differentially expressed by CD8 $\alpha\alpha$ +TCR $\alpha\beta$ + Treg

CD8 $\alpha\alpha$ + vs. CD8 $\alpha\beta$ +	Gene Name	Gene Alias	Annotation	Entrez Gene ID
574.94	H2-D1	H-2D	histocompatibility 2, D region locus 1	14964
114.25	Xist		inactive X specific transcripts	213742
62.75	H2-Q1	Q1b; Q1d; Q1k; Qa-1	histocompatibility 2, Q region locus 1	15006
56.42	Gpr15		G protein-coupled receptor 15	71223
33.55	Tgfr3	TBRIII	transforming growth factor, beta receptor III	21814
27.8	Ctse	CE, CatE	cathepsin E	13034
19.5	St6galnac2	Siat7, Siat7b	ST6 (alpha-N-acetyl-neuraminy1-2,3- beta-galactosyl-1,3)-N- acetylglactosaminide alpha-2,6-sialyltransferase 2	20446
16.5	B3gnt1	iGAT, iGNT	UDP-GlcNAc:betaGal beta-1,3-N- acetyl-glucosaminyltransferase 1	108902
8.39	Arhgap1	Cdc42GAP	Rho GTPase activating protein 1	228359
8.06	Sgne1	7B2	secretogranin V	20394
4.44	Zfp537	Tsh3, teashirt3	teashirt zinc finger family member 3	243931
4.33	Ptger2	EP2	prostaglandin E receptor 2	19217
4.28	Nr1d2	RVR	nuclear receptor subfamily 1, group D, member 2	353187
4.24	Itga6	Cd49f	integrin alpha 6	16403
3.44	Igf1r	CD221,	insulin-like growth factor I receptor	16001
2.22	CD80	B7.1, Cd28l, Ly53, MIC17	CD80 antigen	12519
2.10	IL7r	CD127	interleukin 7 receptor	16197
0.43	CD69	AIM, VEA	CD69 antigen	12515
0.41	CD44	HERMES, Ly-24, Pgp-1	CD44 antigen	12505
0.40	Ccr5	CD195, Cmkbr5	chemokine (C-C motif) receptor 5	12774
0.35	Ahnak	DY6	AHNAK nucleoprotein (desmoyokin)	66395
0.30	IL-10ra	mIL-10R	interleukin 10 receptor, alpha	16154
0.19	Fcgrt	FcRn	Fc receptor, IgG, alpha chain transporter	14132
0.19	Ly6c	Ly-6C, Ly6c	lymphocyte antigen 6 complex, locus C1	17067
0.19	Lat2	LAB, NTAL, Wbscr5	linker for activation of T cells family, member 2	56743
0.17	Fut4	CD15, FAL, LeX,	fucosyltransferase 4	14345
0.13	CD25	Il2ra, Ly43,	interleukin 2 receptor, alpha chain	16184
0.09	Ifi30	GILT, IP30	interferon gamma inducible protein 30	65972
0.04	Xdh	XO, Xor	xanthine dehydrogenase	22436
0.04	CD72	Ly19, Ly32	CD72 antigen	12517

CD8 $\alpha\alpha$ + vs. CD8 $\alpha\beta$ +	Gene Name	Gene Alias	Annotation	Entrez Gene ID
0.021	Tm6sf1		transmembrane 6 superfamily member 1	107769
0.015	Ifitm3	Cd225, Fgls,	interferon induced transmembrane protein 3	66141
0.012	Anxa2	Cal1h	annexin A2	12306
0.004	Plac8	C15	placenta-specific 8	231507
0.003	Camk2b		calcium/calmodulin-dependent protein kinase II, beta	12323

Values are expressed as normalized mRNA fold expression between CD8 $\alpha\alpha$ + Treg clone 2D11 and CD8 $\alpha\beta$ +TCR $\alpha\beta$ + T cell clone OT-1 (CD8 α CD8 $\alpha\alpha$ vs. CD8 $\alpha\beta$). Data are sorted based on gene expression preference in CD8 $\alpha\alpha$ + Treg populations.

TABLE 2
Differential expression of granzymes in the CTLA (cytotoxic T-lymphocyte-associated protein) family in CD8 $\alpha\alpha$ + Treg

CD8 $\alpha\alpha$ + vs. CD8 $\alpha\beta$ +	Gene Name	Gene Alias	Annotation	Entrez Gene ID
9.95	Gzmg	Ctla7	granzyme G	14944
5.54	Gzmd	CCP5; Ctla5	granzyme D	14941
3.12	Gzme	CCP3; Ctla6	granzyme E	14942
0.47	Gzmb	CCP1, Ctla1	granzyme B	14939
0.007	Gzmk		granzyme K	14945
0.002	Gzma	Ctla3, Hf, SE1, TSP1	granzyme A	14938

Values are expressed as normalized mRNA fold expression between CD8 $\alpha\alpha$ + Treg clone 2D11 and CD8 $\alpha\beta$ + T cell clone OT-1 (CD8 $\alpha\alpha$ + vs. CD8 $\alpha\beta$ +). Data are sorted based on gene expression preference in CD8 $\alpha\alpha$ + Treg populations.

TABLE 3
Differential expression of genes in the Tnf superfamily in CD8 $\alpha\alpha$ + Treg

CD8 $\alpha\alpha$ vs. CD8 $\alpha\beta$	Gene Name	Gene Alias	Annotation	Entrez Gene ID
4.89	Tnfrsf14	Atar, Hvem,	tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator)	230979
4.47	Tnfrsf11b	Opg, TR1	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	18383
3.28	Tnfsf14	HVEML, LIGHT,	tumor necrosis factor (ligand) superfamily, member 14	50930
3.1	Tnfsf11	ODF, OPGL, RANKL,	tumor necrosis factor (ligand) superfamily, member 11	21943
2.87	Tnfsf8	CD153, d30l	tumor necrosis factor (ligand) superfamily, member 8	21949
2.46	Tnf	DIF, Tnfa,	tumor necrosis factor	21926
2.36	Tnfaip1	Edp1, Tnfip1	tumor necrosis factor, alpha-induced protein 1	21927
2.21	Tnfrsf4	CD134, Ox40	tumor necrosis factor receptor superfamily, member 4	22163
0.38	Tnfsf10	Trail	tumor necrosis factor (ligand) superfamily, member 10	22035
0.21	Tnfrsf9	4-1BB, Cd137	tumor necrosis factor receptor superfamily, member 9	21942

Values are expressed as normalized mRNA fold expression between CD8 $\alpha\alpha$ + Treg clone 2D11 and CD8 $\alpha\beta$ + T cell clone OT-1 (CD8 $\alpha\alpha$ + vs. CD8 $\alpha\beta$ +). Data are sorted based on gene expression preference in CD8 $\alpha\alpha$ + Treg populations.

TABLE 4
Transcription factors differentially expressed in CD8 $\alpha\alpha$ + Treg

CD8 $\alpha\alpha$ + vs. CD8 $\alpha\beta$ +	Gene Name	Gene Alias	Annotation	Entrez Gene ID
17.7	Rora	Nr1f1	RAR-related orphan receptor alpha	19883
8.4	Pgr	NR3C3, PR	progesterone receptor	18667
8.4	EST		RIKEN cDNA 2610305D13 gene	112422
6.6	EST		RIKEN cDNA 4930422I07 gene	71640
5.9	Onecut2	Oc2	one cut domain, family member 2	225631
5.7	Hmgn1	Hmg14	high mobility group nucleosomal binding domain 1	15312
0.12	Zfp422	Krox25, Krox26	zinc finger protein 422	67255
0.12	Zfp429	Rsl2	zinc finger protein 429	72807
0.09	Rai14	Ankycorbin, Norpeg	retinoic acid induced 14	75646
0.06	EST	Zfp874	zinc finger protein 874	238692
0.04	Tanc2		tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2	77097
0.02	Ctnnd2	GT24, NPRAP	catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein)	1501
0.12	Basp1	CAP23, AP22	brain abundant, membrane attached signal protein 1	10409
0.12	EST		cDNA sequence BC094916	545384
0.09	Mycn	N-myc	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	18109

Values are expressed as normalized mRNA fold expression between CD8 $\alpha\alpha$ + Treg clone 2D11 and CD8 $\alpha\beta$ + T cell clone OT-1 (CD8 $\alpha\alpha$ + vs. CD8 $\alpha\beta$ +). Data are sorted based on gene expression preference in CD8 $\alpha\alpha$ + Treg populations.