

# Definition of target antigens for naturally occurring CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells

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**The antigenic targets recognized by naturally occurring CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells (T reg cells) have been elusive. We have serologically defined a series of broadly expressed self-antigens derived from chemically induced mouse sarcomas by serological identification of antigens by recombinant expression cloning (SEREX). CD4<sup>+</sup> CD25<sup>+</sup> T cells from mice immunized with SEREX-defined self-antigens had strong suppressive activity on peptide-specific proliferation of CD4<sup>+</sup> CD25<sup>-</sup> T cells and CD8<sup>+</sup> T cells. The suppressive effect was observed without in vitro T cell stimulation. *Foxp3* expression in these CD4<sup>+</sup> CD25<sup>+</sup> T cells from immunized mice was 5–10 times greater than CD4<sup>+</sup> CD25<sup>+</sup> T cells derived from naive mice. The suppressive effect required cellular contact and was blocked by anti-glucocorticoid-induced tumor necrosis factor receptor family-related gene antibody. In vitro suppressive activity essentially disappeared 8 wk after the last immunization. However, it was regained by in vitro restimulation with cognate self-antigen protein but not with control protein. We propose that SEREX-defined self-antigens such as those used in this study represent self-antigens that elicit naturally occurring CD4<sup>+</sup> CD25<sup>+</sup> T reg cells.**

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Evidence for the essential role of regulatory T cells (T reg cells) in maintaining immunological homeostasis comes from a range of in vitro and in vivo experimental systems, and naturally occurring T reg cells coexpressing CD4 and CD25 are of central interest with regard to the regulation of autoreactive T cells (1–3). Although the expression of CD25 (IL-2R  $\alpha$  chain) is not restricted to naturally occurring T reg cells, IL-2 signaling appears to be essential for their generation in the thymus, their peripheral maintenance, and their suppressive function (4–6). Glucocorticoid-induced TNF receptor family-related gene (GITR) may possess more direct functional relevance with regard to naturally occurring T reg cells as indicated by evidence that anti-GITR antibody blocks the suppressive activity of T reg cells (7, 8). However, it has been shown that the reversal of suppression by anti-GITR antibody is attributable to the costimulatory activity of

anti-GITR antibody on the responder CD4<sup>+</sup> CD25<sup>-</sup> T cells (9), and therefore, the cellular target of anti-GITR antibody for blocking suppression needs to be reevaluated. Recently, the forkhead/winged helix transcription factor *Foxp3* has been shown to be essential in directing T cells toward a suppressor phenotype, and for this reason, *Foxp3* has become a key marker for naturally occurring T reg cells (10–12).

Naturally occurring T reg cells are thought to be maintained through interaction with self-antigens. However, despite the many indications that self-antigens play a critical role in the development of T reg cells (13–16), a limited number of self-antigen ligands of naturally occurring T reg cells have been identified (17). For this reason, molecular definition of these natural ligands is a top priority in the T reg cell field.

We have recently reported that immunization with serological identification of antigens by recombinant expression cloning (SEREX)-defined self-antigens results in enhancement of pulmonary metastasis of i.v.-challenged

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immunogenic transplantable tumor lines (18). These self-antigens were identified by SEREX, a methodology widely used to identify immunogenic molecules in mouse and human tumors (19–21).

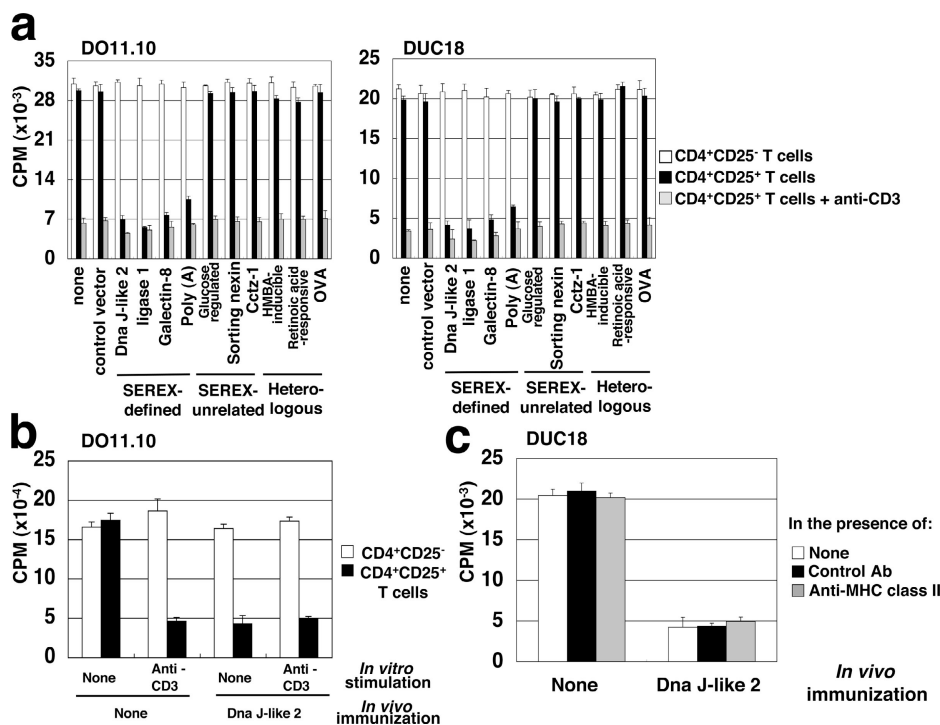
In this analysis, we have found that CD4<sup>+</sup> CD25<sup>+</sup> T cells obtained from mice immunized with SEREX-defined self-antigens display strong suppressive effect on IL-2 production and proliferation of CD4<sup>+</sup> CD25<sup>-</sup> T cells, and this effect depends on cellular contact and is independent of soluble mediators. Although these T reg cells closely resemble the widely studied naturally occurring CD4<sup>+</sup> CD25<sup>+</sup> T reg cells found in naive hosts, they have stronger immunosuppressive activity and do not require *in vitro* stimulation with anti-CD3 mAb. In addition, the CD4<sup>+</sup> CD25<sup>+</sup> T cells elicited by immunization with SEREX-defined self-antigens display significantly enhanced levels of *Foxp3* mRNA expression.

**RESULTS AND DISCUSSION**

**Immunization with SEREX-defined self-antigens elicits active CD4<sup>+</sup> CD25<sup>+</sup> T reg cells**

BALB/c mice were immunized twice at 2-wk intervals by gene gun with plasmids encoding the following SEREX-

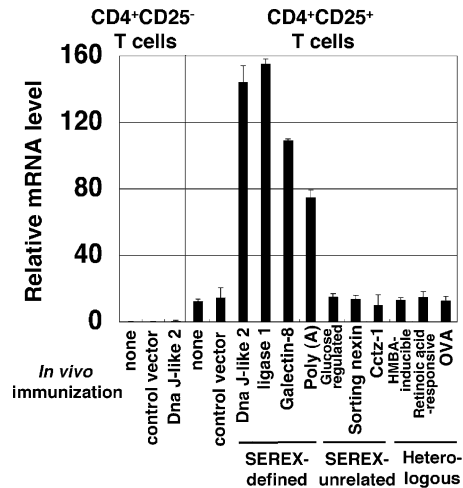
defined self-antigens: Mus heat shock protein Dna J-like 2 (Dna J-like 2; AF055664), Mus DNA ligase 1 (Ligase 1; U19604), Mus galectin-8 (Galectin-8; AF218069), or Mus poly (A) binding protein, cytoplasmic 1 (Poly (A); X65553). 1 wk after immunization, splenic CD4<sup>+</sup> CD25<sup>+</sup> T cells and CD4<sup>+</sup> CD25<sup>-</sup> T cells were isolated using microbeads (see Materials and methods) and added to cultures of naive CD4<sup>+</sup> CD25<sup>-</sup> T cells from DO11.10 mice or naive CD8<sup>+</sup> T cells from DUC18 mice stimulated with APC and cognate antigenic peptides. CD4<sup>+</sup> CD25<sup>+</sup> T cells from mice immunized with SEREX-defined self-antigens showed significant suppression of the peptide-specific proliferation of CD4<sup>+</sup> CD25<sup>-</sup> T cells and CD8<sup>+</sup> T cells, whereas CD4<sup>+</sup> CD25<sup>+</sup> T cells from naive BALB/c mice or mice immunized with plasmids encoding the following SEREX-unrelated molecules (nonimmunogenic molecules randomly picked from the SEREX screening library) showed essentially no suppression: Mus glucose-regulated protein (Glucose-regulated; D78645), Mus sorting nexin 1 (Sorting nexin; AB019214), or Mus *Cctz-1* gene for chaperon-containing TCP-1-ζ-1 subunit (*Cctz-1*; AB022159), or with control vector (Fig. 1 a). Furthermore, no proliferative suppression of CD4<sup>+</sup> CD25<sup>-</sup>



**Figure 1. Immunization with SEREX-defined self-antigens elicits active CD4<sup>+</sup> CD25<sup>+</sup> T reg cells.** (a) BALB/c mice were immunized with plasmids encoding SEREX-defined self-antigens, SEREX-unrelated molecules, heterologous molecules, or control vector. 1 wk after the second immunization, 5 × 10<sup>4</sup> CD4<sup>+</sup> CD25<sup>+</sup> T cells or 5 × 10<sup>4</sup> CD4<sup>+</sup> CD25<sup>-</sup> T cells from spleens were added to cultures of 5 × 10<sup>4</sup> CD4<sup>+</sup> CD25<sup>-</sup> T cells from DO11.10 mice or 5 × 10<sup>4</sup> CD8<sup>+</sup> T cells from DUC18 mice with 5 × 10<sup>4</sup> MMC-treated BALB/c splenic Thy-1<sup>-</sup> APC and cognate peptides. (b) 5 × 10<sup>4</sup> CD4<sup>+</sup> CD25<sup>-</sup> T cells from DO11.10 mice were cultured with 5 × 10<sup>4</sup> MMC-

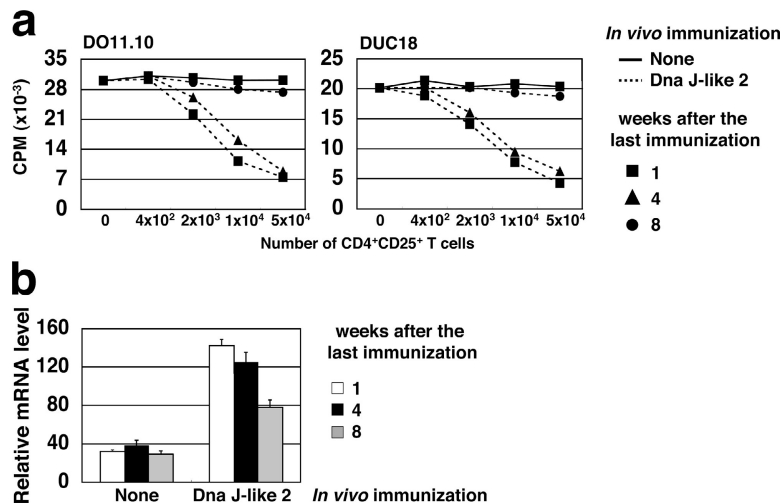
treated BALB/c splenic Thy-1<sup>-</sup> APC and cognate peptides in the presence of FACSvantage-sorted 5 × 10<sup>4</sup> CD4<sup>+</sup> CD25<sup>+</sup> or CD4<sup>+</sup> CD25<sup>-</sup> T cells from naive or Dna J-like 2-immunized BALB/c mice. (c) 5 × 10<sup>4</sup> CD8<sup>+</sup> T cells from DUC18 mice were cultured with 5 × 10<sup>4</sup> CD4<sup>+</sup> CD25<sup>+</sup> T cells from naive or Dna J-like 2-immunized BALB/c mice with 5 × 10<sup>4</sup> MMC-treated BALB/c splenic Thy-1<sup>-</sup> APC and cognate peptides with or without anti-MHC class II mAb. Proliferation was assessed as described in Materials and methods. These experiments were repeated three (a) or two (c) times with similar results. Data are expressed as mean ± SD.

T cells or CD8<sup>+</sup> T cells was observed with CD4<sup>+</sup> CD25<sup>+</sup> T cells from mice immunized with the following plasmids encoding heterologous human antigens (21): *Homo sapiens* HMBA-inducible (HMBA-inducible; XM\_008348), human retinoic acid-responsive protein (Retinoic acid-responsive; U50383), or OVA. However, CD4<sup>+</sup> CD25<sup>+</sup> T cells from all groups suppressed the proliferation of CD4<sup>+</sup> CD25<sup>-</sup> T cells and CD8<sup>+</sup> T cells when anti-CD3 mAb was added to the cultures, in accordance with previous reports (2, 3, 22). To exclude the possibility that multivalent cross-linking by antibodies conjugated to microbeads during CD4<sup>+</sup> CD25<sup>+</sup> T cell purification alters their function, the cells were purified on a FACSVantage from negatively selected CD4<sup>+</sup> T cells. Consistent with previous experiments, CD4<sup>+</sup> CD25<sup>+</sup> T cells purified on a FACSVantage from mice immunized with Dna J-like 2 (one of the SEREX-defined self-antigens) showed similar suppressive capacity (Fig. 1 b). Suppression of CD4<sup>+</sup> CD25<sup>-</sup> T cell and CD8<sup>+</sup> T cell proliferation by CD4<sup>+</sup> CD25<sup>+</sup> T cells derived from mice immunized with Dna J-like 2 was contact dependent, cytokine independent, blocked by anti-GITR, and associated with suppression of IL-2 production by responder T cells (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20041959/DC1>). Finally, CD4<sup>+</sup> CD25<sup>+</sup> T cells derived from mice immunized with Dna J-like 2 had a suppressive effect on CD8<sup>+</sup> T cell proliferation even in the presence of anti-MHC class II mAb, indicating “no necessity” for in vitro stimulation of CD4<sup>+</sup> CD25<sup>+</sup> T cells by self-antigens in the context of MHC class II (Fig. 1 c). Thus, this set of experiments shows that immunization with SEREX-defined self-antigens elicits CD4<sup>+</sup> CD25<sup>+</sup> T cells with extremely potent regulatory activity.



**Figure 2.** Expression of *Foxp3* mRNA is enhanced in CD4<sup>+</sup> CD25<sup>+</sup> T cells from mice immunized with SEREX-defined self-antigens. BALB/c mice were immunized with plasmids encoding SEREX-defined self-antigens, SEREX-unrelated molecules, heterologous molecules, or control vector. 1 wk after the second immunization, CD4<sup>+</sup> CD25<sup>+</sup> T cells and CD4<sup>+</sup> CD25<sup>-</sup> T cells were purified from spleens and their levels of *Foxp3* mRNA were assessed by real-time quantitative RT-PCR. Normalized *Foxp3* mRNA expression values were calculated as the ratio of *Foxp3* mRNA expression to HPRT mRNA expression. These experiments were repeated three times with similar results. Data are expressed as mean  $\pm$  SD.

**High *Foxp3* mRNA expression is found in CD4<sup>+</sup> CD25<sup>+</sup> T cells from mice immunized with SEREX-defined self-antigens**  
*Foxp3* mRNA expression in CD4<sup>+</sup> T cell subsets was examined 1 wk after immunization with SEREX-defined self-antigens (Fig. 2). Enhanced expression of *Foxp3*



**Figure 3.** Changes in CD4<sup>+</sup> CD25<sup>+</sup> T cell suppressive activity after immunization are correlated with *Foxp3* mRNA expression levels. (a) 1, 4, or 8 wk after the second immunization with plasmids encoding Dna J-like 2, CD4<sup>+</sup> CD25<sup>+</sup> T cells were obtained from spleens. Graded doses of these cells were added to cultures of  $5 \times 10^4$  CD4<sup>+</sup> CD25<sup>-</sup> T cells from DO11.10 mice or  $5 \times 10^4$  CD8<sup>+</sup> T cells from DUC18 mice with  $5 \times 10^4$

MMC-treated BALB/c splenic Thy-1<sup>-</sup> APC and cognate peptides. Proliferation was assessed as described in Materials and methods. (b) cDNA was prepared from CD4<sup>+</sup> CD25<sup>+</sup> T cells at various time intervals after immunization with Dna J-like 2. The expression of *Foxp3* mRNA was analyzed as described in the legend of Fig. 2. These experiments were repeated three times with similar results. Data are expressed as mean  $\pm$  SD.

mRNA was observed in CD4<sup>+</sup> CD25<sup>+</sup> T cells from mice immunized with each of the four different SEREX-defined self-antigens. Expression levels were 5–10 times higher than those found in CD4<sup>+</sup> CD25<sup>+</sup> T cells from naive mice, mice immunized with the control vector, mice immunized with SEREX-unrelated molecules, or heterologous molecules. Levels of *Foxp3* mRNA expression in CD4<sup>+</sup> CD25<sup>+</sup> T cells from all groups of mice remained unchanged after immunization.

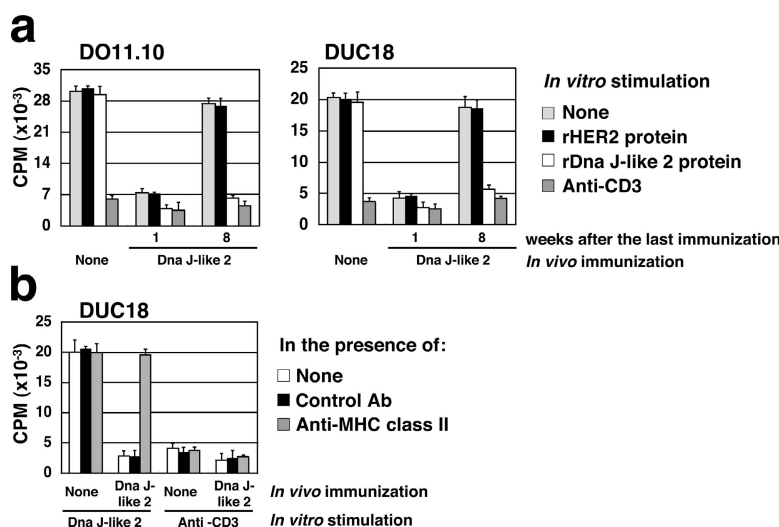
We then examined the suppressive capacity and levels of *Foxp3* mRNA expression in CD4<sup>+</sup> CD25<sup>+</sup> T cells from mice immunized with SEREX-defined self-antigens at various time intervals after immunization. Suppressive activity was strongest when CD4<sup>+</sup> CD25<sup>+</sup> T cells were obtained 1 wk after immunization, becoming marginal when the cells were obtained 8 wk after immunization (Fig. 3 a). In parallel to the decrease in suppressive activity of CD4<sup>+</sup> CD25<sup>+</sup> T cells, *Foxp3* mRNA expression was also highest when CD4<sup>+</sup> CD25<sup>+</sup> T cells were obtained 1 wk after immunization and decreased thereafter (Fig. 3 b).

#### Antigen-specific stimulation restores suppressive activity of CD4<sup>+</sup> CD25<sup>+</sup> T cells from Dna J-like 2 immunized mice

As the suppressive activity of CD4<sup>+</sup> CD25<sup>+</sup> T cells declines after immunization, we asked whether self-antigens used for *in vivo* immunization could restimulate CD4<sup>+</sup> CD25<sup>+</sup> T cells *in vitro*. CD4<sup>+</sup> CD25<sup>+</sup> T cells obtained 8 wk after immunization with Dna J-like 2 lost suppressive activity (Fig. 4 a). However, they became strongly suppressive when rDna J-like 2 protein, but not control rHER2 protein, was added to the cultures. The regained suppressive capacity of *in vitro*–

stimulated CD4<sup>+</sup> CD25<sup>+</sup> T cells was also contact dependent and cytokine independent (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20041959/DC1>). The presence of anti-MHC class II mAb abrogated the reactivation of the suppressive effect of these CD4<sup>+</sup> CD25<sup>+</sup> T cells by rDna J-like 2 protein (Fig. 4 b). Anti-MHC class II mAb did not interfere with the suppressive activity of CD4<sup>+</sup> CD25<sup>+</sup> T cells from naive or Dna J-like 2-immunized mice induced by anti-CD3 mAb.

Our studies have shown that immunization with SEREX-defined self-antigens generates CD4<sup>+</sup> CD25<sup>+</sup> T cells that exert potent suppressive activity and express elevated levels of *Foxp3* mRNA expression. The characteristic of these CD4<sup>+</sup> CD25<sup>+</sup> T cells clearly resembles naturally occurring T reg cells. It is unclear at present whether increased suppressive activity and elevated *Foxp3* mRNA expression are due to the clonal expansion of SEREX-defined self-antigen-specific CD4<sup>+</sup> CD25<sup>+</sup> T cells or to the increase of activity per cell. Although recent reports have demonstrated that CD4<sup>+</sup> CD25<sup>+</sup> T cells proliferate *in vivo* in an antigen-specific fashion like CD4<sup>+</sup> CD25<sup>+</sup> T cells (23–25), we could not detect any increase in the percentage of CD4<sup>+</sup> CD25<sup>+</sup> T cells in spleen, lymph node, and pulmonary cells from mice immunized with Dna J-like 2 (18). These data are in line with the report that tolerization to dnaJp1 by mucosal immunization increases *Foxp3* expression in CD4<sup>+</sup> CD25<sup>bright</sup> T cells without an obvious increase in the total number of dnaJp1-specific T cells (26). Nevertheless, it is still possible that SEREX-defined self-antigen-specific CD4<sup>+</sup> CD25<sup>+</sup> T cells clonally expand, but this expansion is under the detection level of flow cytometric analysis. It is also possible that expression of



**Figure 4. Antigen-specific stimulation restores suppressive activity of CD4<sup>+</sup> CD25<sup>+</sup> T cells from Dna J-like 2-immunized mice.** (a) 1 or 8 wk after the second immunization with plasmids encoding Dna J-like 2, 5 × 10<sup>4</sup> CD4<sup>+</sup> CD25<sup>+</sup> T cells were obtained from spleens. The suppressive activity was analyzed as described in the legend of Fig. 1 a. (b) 5 × 10<sup>4</sup> CD8<sup>+</sup> T cells from DUC18 mice were cultured with 5 × 10<sup>4</sup> CD4<sup>+</sup> CD25<sup>+</sup>

T cells from mice 8 wk after the second immunization with 5 × 10<sup>4</sup> MMC-treated BALB/c splenic Thy-1<sup>+</sup> APC and cognate peptides, with or without anti-MHC class II mAb, and with or without *in vitro* stimulation as indicated. Proliferation was assessed as described in Materials and methods. These experiments were repeated three (a) or two (b) times with similar results. Data are expressed as mean ± SD.

*Foxp3* mRNA could be increased on a per cell basis after stimulation with SEREX-defined self-antigens in vivo.

A unique finding in our analysis is that although SEREX-defined self-antigens, but not heterologous antigens, elicit CD4<sup>+</sup> CD25<sup>+</sup> T reg cells with potent suppressive activity, both antigens can induce equivalent CD4<sup>+</sup> T cell-dependent helper T cell activity for enhancing tumor-specific CTLs (21). The reason for this difference is currently unknown. Although several recent reports have described the presence of CD4<sup>+</sup> T cells for heterologous antigens with suppressive activity (27, 28), constant stimulation with self-antigens, represented by SEREX-defined self-antigens, may create a favorable environment for activation of self-antigen-specific CD4<sup>+</sup> CD25<sup>+</sup> T reg cells that maintain immunological homeostasis. Immunization with SEREX-defined self-antigens under the condition of our study appears to favor prompt elicitation or recall of these CD4<sup>+</sup> CD25<sup>+</sup> T reg cells.

Although SEREX was introduced to identify tumor antigens that elicited a humoral immune response in the tumor-bearing host, the vast majority of these antigens were not restricted to tumors but were broadly expressed, nonmutated self-antigens (19–21). Thus, SEREX has the potential of defining the repertoire of immunogenic self-antigens. As these immunogenic molecules are detected by IgG class antibodies, SEREX also represents a powerful indirect way to study the CD4<sup>+</sup> T cell repertoire. Our analysis indicates that a number of these immunogenic wild-type molecules detected by SEREX are recognized by CD4<sup>+</sup> CD25<sup>+</sup> T reg cells and elicit CD4<sup>+</sup> CD25<sup>+</sup> T reg cells with suppressive activity. As coimmunization with SEREX-defined self-antigens and a CTL epitope enhances CD8<sup>+</sup> CTL induction in a CD4<sup>+</sup> T cell-dependent manner (21), these self-antigens are also likely to be recognized by CD4<sup>+</sup> helper T cells. Two possibilities to explain these findings regarding the relation between SEREX-defined self-antigens and helper and T reg cells are that CD4<sup>+</sup> T cells with different functions yet recognizing the same self-antigens exist in the periphery, or that single CD4<sup>+</sup> precursors gain distinct functions under different circumstances of antigenic stimulation. We have recently found that IFN- $\gamma$ , but not several other cytokines, produced by CD8<sup>+</sup> CTLs recognizing CTL epitopes used for coimmunization with SEREX-defined self-antigens suppresses generation of CD4<sup>+</sup> CD25<sup>+</sup> T reg cells recognizing SEREX-defined self-antigens (unpublished data). This ability of IFN- $\gamma$  to nullify suppression provides an explanation for the paradoxical effect of immunization with self-antigens and CTL epitopes, where strong helper activity is generated in contrast to the suppression seen when mice are immunized with self-antigens alone. Our interpretation of these findings is that IFN- $\gamma$  produced by CD8<sup>+</sup> T cells responding to a CTL epitope inhibits generation of CD4<sup>+</sup> CD25<sup>+</sup> T reg cells, permitting CD4<sup>+</sup> CD25<sup>-</sup> helper T cells to become dominant. The role of SEREX-defined self-antigens in generating and maintaining CD4<sup>+</sup> CD25<sup>+</sup> T reg cells and CD4<sup>+</sup> helper T cells is clearly a critical area for future exploration. An important

next step will be to define the peptide sequences in these self-antigens that elicit diverse CD4<sup>+</sup> T cell responses.

## MATERIALS AND METHODS

**Mice.** Female BALB/c mice and C.B-17 SCID mice were purchased from CLEA Japan and used at 7–10 wk of age. DUC18 mice, transgenic for  $\alpha\beta$  TCR reactive with the K<sup>d</sup>-restricted mERK2<sub>136–144</sub>, were established as described previously (29). DO11.10 mice, transgenic for  $\alpha\beta$  TCR reactive with the I-A<sup>d</sup>-restricted OVA<sub>323–339</sub> (30), were provided by K.M. Murphy (Washington University, St. Louis, MO). Mice were maintained at the Animal Center of Mie University School of Medicine. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Mie University School of Medicine.

**Immunization by gene gun.** Gold particles coated with plasmid DNA (1  $\mu$ g/injection) were prepared and delivered into shaved skin of the abdominal wall of BALB/c mice by a Helios Gene Gun System (Bio-Rad Laboratories) at a helium discharge pressure of 350–400 psi, as described previously (21).

**Antibodies and reagents.** Anti-CD3 mAb (145-2C11; hamster IgG) and anti-I-A<sup>d</sup> mAb (M5/114; rat IgG2b) were purified from ascites of each hybridoma in C.B-17 SCID mice on a protein A column. FITC-conjugated anti-CD25 antibody (PC61; rat IgG1) was purchased from eBioscience. PE-conjugated anti-CD4 (H129.19; rat IgG2a) was purchased from BD Biosciences. Synthetic mERK2<sub>136–144</sub>-9m peptide QYIHSANVL (21) and OVA<sub>323–339</sub> peptide ISQAVHAHAHAINEAGR (30) were obtained from TAKARA SHUZO. cDNA encoding SEREX-defined molecules, control SEREX-unrelated molecules, and heterologous molecules were cloned into pBK-CMV (Stratagene) and purified using the EndoFree Plasmid Mega Kit (QIAGEN).

**Purification of CD4<sup>+</sup> CD25<sup>+</sup> T cells and CD4<sup>+</sup> CD25<sup>-</sup> T cells.** Spleen cells were fractionated into CD25<sup>+</sup> and CD25<sup>-</sup> using MACS Anti-FITC MultiSort Kit (Miltenyi Biotec). Anti-FITC microbeads bound to CD25<sup>+</sup> cells were detached using MACS MultiSort Release Reagent and MACS MultiSort Stop Reagent according to the manufacturer's instructions. The resultant CD25<sup>+</sup> and CD25<sup>-</sup> cells were further enriched for CD4<sup>+</sup> cells by positive selection on a MACS column after reacting with anti-CD4 microbeads. CD4<sup>+</sup> CD25<sup>+</sup> and CD4<sup>+</sup> CD25<sup>-</sup> T cell preparations were confirmed to contain >96 and >93%, respectively. CD4<sup>+</sup> T cells were enriched by negative selection using a CD4<sup>+</sup> isolation kit (Miltenyi Biotec) followed by purification of CD4<sup>+</sup> CD25<sup>+</sup> and CD4<sup>+</sup> CD25<sup>-</sup> T cells on a FACSVantage (BD Biosciences) after staining with FITC-anti-CD25 and PE-anti-CD4. The purity of these CD4<sup>+</sup> CD25<sup>+</sup> and CD4<sup>+</sup> CD25<sup>-</sup> T cells was >99%.

**Proliferation assay.**  $5 \times 10^4$  CD4<sup>+</sup> CD25<sup>-</sup> T cells from DO11.10 mice or  $5 \times 10^4$  CD8<sup>+</sup> T cells from DUC18 mice were cultured with  $5 \times 10^4$  mitomycin C (MMC)-treated splenic Thy-1<sup>-</sup> APCs from wild-type BALB/c mice in the presence of 10  $\mu$ g/ml OVA<sub>323–339</sub> peptide or mERK2<sub>136–144</sub>-9m peptide in the wells of 96-well flat-bottomed plates. To these cultures, CD4<sup>+</sup> CD25<sup>+</sup> or CD4<sup>+</sup> CD25<sup>-</sup> T cells were added. Proliferation was evaluated by pulsing with 0.5  $\mu$ Ci/well [<sup>3</sup>H]thymidine for the last 6 h of the 72-h culture.

**Real-time quantitative RT-PCR.** Oligo-dT-primed first strand cDNA of *Foxp3* or HPRT was synthesized for real-time RT-PCR. Real-time RT-PCR was performed as described previously (10).

**Online supplemental material.** Fig. S1 shows that the suppressive effect of active CD4<sup>+</sup> CD25<sup>+</sup> T reg cells was dependent on cellular contact and blocked by GITR antibody. Fig. S2 shows that the regained suppressive capacity of in vitro-stimulated CD4<sup>+</sup> CD25<sup>+</sup> T cells was also contact dependent and cytokine independent. Figs. S1 and S2 are available at <http://www.jem.org/cgi/content/full/jem.20041959/DC1>.

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