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# Expansion of Functional Endogenous Antigen-Specific CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells from Nonobese Diabetic Mice<sup>1</sup>

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CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (T<sub>reg</sub>) are critical for controlling autoimmunity. Evidence suggests that T<sub>reg</sub> development, peripheral maintenance, and suppressive function are dependent on Ag specificity. However, there is little direct evidence that the T<sub>reg</sub> responsible for controlling autoimmunity in NOD mice or other natural settings are Ag specific. In fact, some investigators have argued that polyclonal Ag-nonspecific T<sub>reg</sub> are efficient regulators of immunity. Thus, the goal of this study was to identify, expand, and characterize islet Ag-specific T<sub>reg</sub> in NOD mice. Ag-specific T<sub>reg</sub> from NOD mice were efficiently expanded in vitro using IL-2 and beads coated with recombinant islet peptide mimic-MHC class II and anti-CD28 mAb. The expanded Ag-specific T<sub>reg</sub> expressed prototypic surface markers and cytokines. Although activated in an Ag-specific fashion, the expanded T<sub>reg</sub> were capable of bystander suppression both in vitro and in vivo. Importantly, the islet peptide mimic-specific T<sub>reg</sub> were more efficient than polyclonal T<sub>reg</sub> in suppressing autoimmune diabetes. These results provide a direct demonstration of the presence of autoantigen-specific T<sub>reg</sub> in the natural setting that can be applied as therapeutics for organ-specific autoimmunity. *The Journal of Immunology*, 2005, 175: 3053–3059.

The CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (T<sub>reg</sub>)<sup>4</sup> have been shown to be essential for controlling autoimmunity (1). This is apparent in NOD mice, a mouse model of type 1 diabetes in which depletion of T<sub>reg</sub> results in exacerbated T cell-mediated destruction of the insulin-producing islet cells of the pancreas. An unanswered question in understanding how CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> control autoimmunity is whether there is an Ag-specific T<sub>reg</sub> repertoire that is responsible for maintaining organ-specific tolerance (2). Early studies by Seddon and Mason (3) in rat models suggested that organ-specific tolerance was dependent on T<sub>reg</sub> that specifically recognize autoantigens expressed by the target organ. Subsequent studies showed that the transfer of polyclonal CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> was sufficient to prevent diabetes in NOD mice (4, 5). The process was inefficient, however, and required the infusion of high numbers of T<sub>reg</sub> possibly due to the low frequency of islet-specific cells. Recently, using a TCR transgenic (Tg) model, we and others showed that small numbers of islet

Ag-specific BDC2.5 TCR Tg<sup>+</sup> T<sub>reg</sub> were much more effective than polyclonal T<sub>reg</sub> in blocking and reversing diabetes in NOD mice (6, 7). Although an artificial system, these studies provide strong support for the hypothesis that Ag specificity is critical for optimal T<sub>reg</sub> function and imply that effective clinical therapy will benefit from the ability to identify and expand relevant Ag-specific T<sub>reg</sub> from polyclonal populations.

T<sub>reg</sub> have been shown to possess a diverse TCR repertoire that is biased toward self Ags; however, little is known about the Ag specificity of T<sub>reg</sub> arising under natural conditions (8). The low numbers of endogenous T<sub>reg</sub> have limited their study and therapeutic use. We recently described a method for the in vitro expansion of polyclonal T<sub>reg</sub> using a combination of IL-2 and beads coated with anti-CD3 and anti-CD28 mAb (6). This technique was used to expand Ag-specific T<sub>reg</sub> from BDC2.5 TCR Tg<sup>+</sup> mice that express a TCR specific for an islet Ag. These expanded T<sub>reg</sub> were shown to be efficacious in preventing and reversing diabetes in NOD mice (6). In the present study, we examined whether natural T<sub>reg</sub> that have the BDC2.5 specificity exist in conventional NOD mice using a novel protocol that selectively expands Ag-specific T<sub>reg</sub>. The expansion protocol was adapted from the one described above by substituting the anti-CD3 mAb with a rMHC class II I-A<sup>g7</sup> presenting the BDC2.5 TCR mimotope peptide 1040-31 (p31)<sup>4</sup> (9, 10). We show that not only do these cells exist in the unmanipulated T<sub>reg</sub> repertoire, but that these rare Ag-specific T<sub>reg</sub> can be expanded and used to prevent autoimmune diabetes in T<sub>reg</sub>-deficient NOD mice. These results have important implications not only for the basic understanding of T<sub>reg</sub> biology, but may also lead to an effective clinical therapy in autoimmune diseases.

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<sup>4</sup> Abbreviations used in this paper: T<sub>reg</sub>, regulatory T cell; Ct, threshold cycle; GAD, glutamic acid decarboxylase; GITR, glucocorticoid induced tumor necrosis factor receptor; HEL, hen egg lysozyme; mIgG, mouse IgG; T<sub>eff</sub>, effector T cell; Tg, transgenic.

## Materials and Methods

### Mice

NOD mice were purchased from Taconic Farms; BDC2.5 TCR Tg<sup>+</sup> mice were obtained from D. Mathis (Joslin Clinic, Harvard University, Boston, MA) (11); and glutamic acid decarboxylase (GAD)<sub>286</sub> TCR Tg<sup>+</sup> mice (12),

NOD.CD28<sup>-/-</sup> mice (13), and NOD.TCR $\alpha$ <sup>-/-</sup> mice (The Jackson Laboratory) were housed and bred under specific pathogen-free conditions at the University of California San Francisco Animal Barrier Facility in accordance with institutional guidelines.

### Reagents

Anti-CD3 $\epsilon$  (145-2C11), anti-CD28 (PV-1), anti-CD4 (GK1.5), and anti-glucocorticoid induced tumor necrosis factor receptor (GITR) (DTA-1; a gift from S. Sakaguchi, Kyoto University, Kyoto, Japan) were produced and purified in our laboratory. Anti-CD4 and anti-GITR were conjugated to FITC in our laboratory. R-PE-conjugated anti-CD25 (7D4) was purchased from Southern Biotechnology Associates. R-PE-conjugated anti-CTLA4 (UC10-4F10-11), biotinylated anti-V $\beta$ 2 TCR (B20.6), anti-V $\beta$ 4 TCR (KT4), anti-V $\beta$ 12 TCR (MR11-1), and anti-ICOS (7E 17G9) were purchased from BD Pharmingen. Allophycocyanin-conjugated anti-CD62L (MEL-14), anti-CD4 (GK1.5), and streptavidin were purchased from eBioscience. p31-I-A<sup>E7</sup>-mouse(m)IgG2a and hen egg lysozyme (HEL)-I-A<sup>E7</sup>-mIgG2a were produced in our laboratory, as previously described (10). Peptides were produced by the University of California Biomolecular Resource Center. The 1040-31 peptide, specific for BDC2.5 TCR Tg<sup>+</sup> T cells, consisted of amino acids YVRPLWVRME (9). The HEL<sub>11-25</sub> protein consisted of amino acids AMKRHGLDNYRGYSL. GAD<sub>286-300</sub> was a gift from E. Sercarz (Torrey Pines Institute for Molecular Studies, San Diego, CA).

Both latex and paramagnetic beads were used throughout the described studies with equal efficiency. For coating of latex beads, 1  $\times$  10<sup>8</sup> 5- $\mu$ m white sulfate latex beads (Interfacial Dynamics) were coated with 30  $\mu$ g of anti-CD28 and 3  $\mu$ g of either anti-CD3 or p31-I-A<sup>E7</sup>-mIgG2a. For coating of paramagnetic beads, 1  $\times$  10<sup>8</sup> Dynabeads M-450 Tosylactivated (DynaL Biotech) were coated with 50  $\mu$ g of anti-CD28 and 5  $\mu$ g of either anti-CD3 or p31-I-A<sup>E7</sup>-mIgG2a, following the manufacturer's instructions.

### Cell sorting and in vitro expansion of T cells

CD4<sup>+</sup> T cells were enriched from spleen and lymph nodes (inguinal, axillary, brachial, submandibular, pancreatic, and mesenteric) by negative selection using an AutoMACS (Miltenyi Biotec) or StemSep beads (Stem-Cell Technologies). Cells were stained and sorted, as previously described, on a Mo-Flo (DakoCytomation) into CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> T<sub>reg</sub> and CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>+</sup> T cell effector (T<sub>eff</sub>) populations to >97% purity (6). Purified T cells at 10<sup>6</sup>/ml were stimulated in flat-bottom plates at a 1:1 bead-cell ratio with anti-CD3 and anti-CD28- or p31-I-A<sup>E7</sup>-mIgG2a and anti-CD28-coated beads in medium supplemented with 2000 IU/ml human rIL-2 (Chiron). Complete DMEM was made, as previously described (6). Cultures were expanded with IL-2-supplemented medium when needed. Beads were removed at the end of the culture period before further experimentation. To remove coated latex beads, cultures were incubated with biotinylated anti-mouse IgG2a (Southern Biotechnology Associates) and biotinylated anti-hamster Ig (Vector Laboratories), washed, incubated with streptavidin microbeads (Miltenyi Biotec), washed, and run over an MS column (Miltenyi Biotec). Cells in the flow through fraction were collected. Dynabeads were removed using a Dynal MPC-L magnet (DynaL Biotech).

### Flow cytometry

For staining with peptide-I-A<sup>E7</sup> multimers, peptide-I-A<sup>E7</sup>-mIgG2a was complexed with Alexa 488-conjugated protein A (Molecular Probes), as previously described (10), or complexed with biotinylated protein A (Sigma-Aldrich) and allophycocyanin-conjugated streptavidin (eBioscience) at a 3:1:0.25 molar ratio. Cells (10<sup>6</sup>) were stained with 1  $\mu$ g of p31-I-A<sup>E7</sup>-mIgG2a for 2 h on ice. Abs against TCR V $\beta$  were added at the same time as the peptide-I-A<sup>E7</sup> multimers. Other Abs were added during the last 30 min of staining or when using peptide-I-A<sup>E7</sup>/biotinylated protein A multimers, during the secondary staining along with allophycocyanin-conjugated streptavidin. Isotype-matched Abs were used for control staining for CTLA-4 and ICOS expression on expanded T<sub>reg</sub>. Freshly isolated CD4<sup>+</sup>CD25<sup>-</sup> cells were used as negative control staining for GITR analysis. Flow cytometric analysis was performed on a FACSCalibur flow cytometer (BD Biosciences).

### In vitro suppression assays

The indicated numbers of expanded T<sub>reg</sub> cultures were added to 50,000 freshly isolated CD4<sup>+</sup> T cells with 50,000 NOD.TCR $\alpha$ -deficient spleen cells or NOD CD4<sup>+</sup>/CD8<sup>-</sup>-depleted spleen cells and either the indicated peptide at 0.1  $\mu$ M or anti-CD3 at 1  $\mu$ g/ml. Cultures were incubated for 72 h and pulsed with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine during the last 14 h.

### CFSE assays

The p31-I-A<sup>E7</sup>-expanded T<sub>reg</sub>, either unsorted or FACS sorted based on p31-I-A<sup>E7</sup> multimer binding, were labeled with 2.5  $\mu$ M CFSE and cultured with irradiated splenic APC and 1  $\mu$ M of either HEL or p31 peptide in the presence of 100 IU of IL-2/ml. Cells were collected at 72 h and analyzed by flow cytometry.

### Real-time PCR analysis

Total RNA was isolated from expanded T cells using QIAshredder and RNeasy Mini Kit spin columns (Qiagen). cDNA was synthesized from 50–600 ng of sample RNA using SuperScript III reverse transcriptase and oligo(dT)12-18 primer (Invitrogen Life Technologies). Real-time PCR contained 1.25–28.5 ng of cDNA. Primers and probes for Foxp3 and 18S rRNA were purchased as premixed reagents from Applied Biosystems. Real-time PCR was performed on an ABI PRISM 7900 HT Sequence Detection System using TaqMan Universal PCR Master Mix (Applied Biosystems). All samples and controls were run in duplicate, and average threshold cycle (Ct) values were used to normalize the signal levels in samples relative to an endogenous control (18S rRNA). The normalized sample values were then used to calculate the fold difference in expression of Foxp3 between expanded T<sub>reg</sub> and T<sub>eff</sub>. The expression ratios of T<sub>reg</sub> to T<sub>eff</sub> were calculated by the following formula:  $(T_{reg}/T_{eff})_{Foxp3} = 2^n$ ,  $n = (T_{eff}Ct_{Foxp3} - T_{eff}Ct_{18S}) - (T_{reg}Ct_{Foxp3} - T_{reg}Ct_{18S})$ .

### Cytokine ELISA

To measure cytokine production, 1  $\times$  10<sup>6</sup> p31-I-A<sup>E7</sup>-expanded T<sub>reg</sub> were cultured with 5  $\times$  10<sup>6</sup> irradiated APC, 100 nM 1040-31 peptide, and 1  $\mu$ g/ml anti-CD28. Culture supernatants were harvested at 48 h. Cytokine concentrations were determined by ELISA using Ab pairs purchased from BD Pharmingen.

### Adoptive transfer

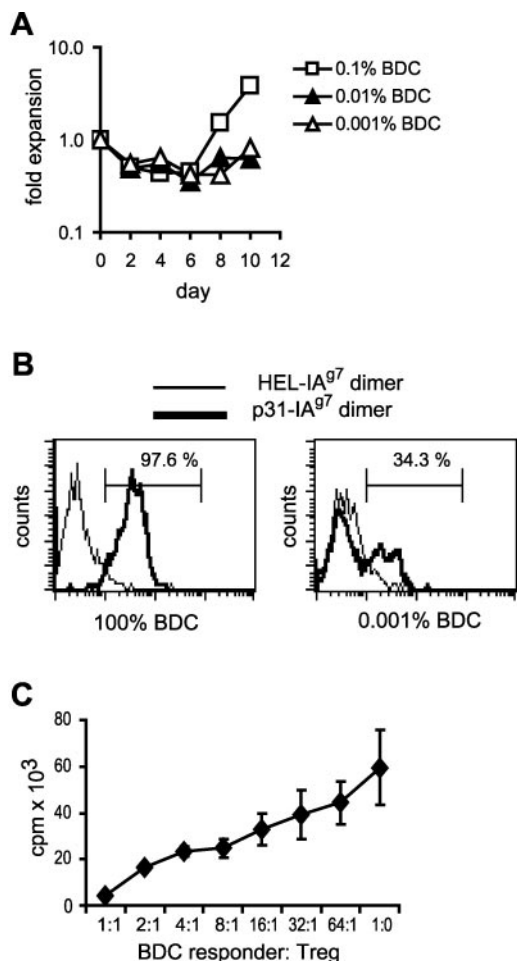
The indicated numbers of p31-I-A<sup>E7</sup>-expanded T<sub>reg</sub> cultures or expanded polyclonal T<sub>reg</sub> cultures were transferred to NOD.CD28<sup>-/-</sup> mice via retro-orbital injection. Nonfasting blood glucose levels in recipient mice were monitored using an Accu-Check glucometer (Roche Diagnostic Systems). Mice were considered diabetic after two readings above 250 mg/dL.

## Results

### Recombinant peptide-MHC can specifically expand rare Ag-specific T<sub>reg</sub>

To selectively expand Ag-specific T<sub>reg</sub>, we adapted the technique used to expand polyclonal T<sub>reg</sub> by substituting the anti-CD3 mAb with a rMHC class II I-A<sup>E7</sup> presenting the BDC2.5 TCR mimotope peptide 1040-31 (p31) (10). The mimotope peptide was used because the endogenous BDC2.5 Ag is not yet identified (9). Initial experiments were conducted with BDC2.5 Tg<sup>+</sup> T<sub>reg</sub> to determine whether p31-I-A<sup>E7</sup>- and anti-CD28-coated beads could expand low frequency Ag-specific cells from a polyclonal population and whether T<sub>reg</sub> expanded in this manner retained suppression activity. Polyclonal CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> from NOD mice were seeded with low frequencies of sorted BDC2.5 TCR Tg<sup>+</sup> T<sub>reg</sub> at 0.1–0.001% of the total population and cultured with p31-I-A<sup>E7</sup>- and anti-CD28-coated beads in the presence of IL-2. The p31-I-A<sup>E7</sup>-coated beads were extremely efficient in expanding CD4<sup>+</sup>CD25<sup>+</sup> BDC2.5 TCR Tg<sup>+</sup> T<sub>reg</sub>. Although cultures initially seeded at 0.01 and 0.001% BDC2.5 TCR Tg<sup>+</sup> T<sub>reg</sub> did not expand appreciably based on total cell number (Fig. 1A), flow cytometric analysis using p31-I-A<sup>E7</sup> multimers revealed that BDC2.5 TCR Tg<sup>+</sup> T<sub>reg</sub> had expanded in all cultures. At the lowest seeding, BDC2.5 TCR Tg<sup>+</sup> T<sub>reg</sub> grew from 0.001 to 34.3% of the population (Fig. 1B), reflecting greater than 12 cell divisions during the 10-day culture period and resulting in nearly a 5000-fold expansion of the Ag-specific cells.

Expanded CD4<sup>+</sup>CD25<sup>+</sup> BDC2.5 T<sub>reg</sub> were then tested for suppressive activity in in vitro cultures using freshly isolated CD4<sup>+</sup>CD25<sup>-</sup> BDC2.5 Tg<sup>+</sup> cells as responders and the p31 peptide as an Ag-specific stimulus. Expanded CD4<sup>+</sup>CD25<sup>+</sup> BDC2.5 T<sub>reg</sub> efficiently suppressed the response the BDC2.5 Tg<sup>+</sup> responder



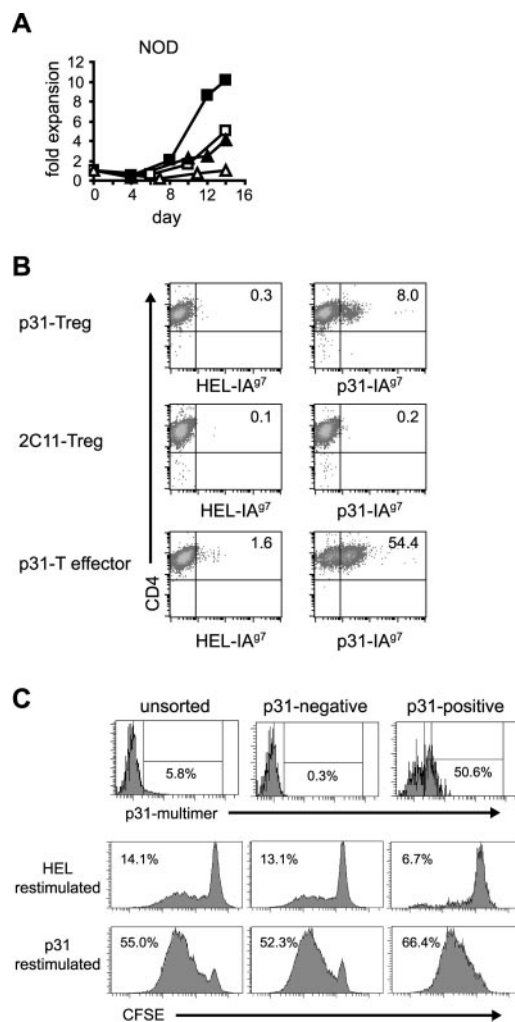
**FIGURE 1.** Peptide-I-A<sup>g7</sup> beads can expand low frequency Ag-specific BDC2.5 TCR Tg<sup>+</sup> T<sub>reg</sub> in vitro. **A**, CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> BDC2.5 TCR Tg<sup>+</sup> T cells were seeded at the indicated frequencies into CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> NOD cells and cultured with p31-I-A<sup>g7</sup>- and anti-CD28-coated beads along with IL-2. The fold expansion of total cell numbers relative to the initial cell input of BDC2.5 and NOD cells is shown. Cultures were quantitated by viable cell counting. **B**, Cultures initially seeded at 100 or 0.001% BDC2.5 Tg<sup>+</sup> T<sub>reg</sub> were expanded for 10 days with p31-I-A<sup>g7</sup> beads, stained with either p31-I-A<sup>g7</sup> multimers or control HEL-I-A<sup>g7</sup> multimers, and analyzed by flow cytometry. **C**, Graded doses of BDC2.5 Tg<sup>+</sup> T<sub>reg</sub> expanded with p31-I-A<sup>g7</sup> and anti-CD28 beads were cultured with 5 × 10<sup>4</sup> freshly isolated CD4<sup>+</sup>CD25<sup>-</sup> BDC2.5 Tg<sup>+</sup> responder cells and 5 × 10<sup>4</sup> irradiated APC. Responder cells alone and T<sub>reg</sub> alone are also shown. Cultures were stimulated with 100 nM BDC2.5 mitotope peptide p31 for 72 h. Proliferation was measured by [<sup>3</sup>H]thymidine incorporation.

cells in a dose-dependent manner (Fig. 1C). Thus, the expansion procedure using recombinant peptide-MHC class II as an Ag-specific stimulus resulted in a large expansion of rare Ag-specific T<sub>reg</sub> that retained suppressive function.

#### Rare Ag-specific T<sub>reg</sub> can be expanded from wild-type NOD mice

This approach was applied next to the expansion of Ag-specific CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> from conventional NOD mice. Initial staining of freshly isolated T<sub>reg</sub> from NOD mice with the p31-I-A<sup>g7</sup> multimer failed to detect positively staining cells above background levels, indicating that, if present, these Ag-specific cells were present at low frequency (data not shown). Sorted CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> cells from 4- to 8-wk-old NOD mice were

cultured with p31-I-A<sup>g7</sup> beads, as described in *Materials and Methods*. Over a 2-wk period, T<sub>reg</sub> cultured in this manner generally expanded from 1- to 10-fold based on total cell number, with 4- to 5-fold expansion being the most typical. Expansion profiles of four representative cultures are shown in Fig. 2A. The lower fold expansion was usually seen when lower numbers of purified T<sub>reg</sub> (<0.5 × 10<sup>6</sup>) were seeded into the wells. The reason for the greater expansion observed in some cultures remains unknown. There was no correlation with the age of mice, as starting populations of CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> cells taken from 4- or 8-wk-old mice demonstrated the same range of expansion of 1- to 10-fold. Flow cytometry analysis demonstrated that after expansion with the p31-I-A<sup>g7</sup> beads, up to 10% of CD4<sup>+</sup>CD25<sup>+</sup> cells stained positive for the p31-I-A<sup>g7</sup> multimer (Fig. 2B). Control cultures of CD4<sup>+</sup>CD25<sup>+</sup> cells expanded with anti-CD3-coated beads did not



**FIGURE 2.** Ag-specific T<sub>reg</sub> can be expanded from wild-type NOD mice. **A**, Sorted CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> from wild-type NOD mice were cultured with p31-I-A<sup>g7</sup> and anti-CD28 beads in the presence of IL-2. The expansion of four representative cultures over a 2-wk period is shown. **B**, CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> cells cultured with either p31-I-A<sup>g7</sup> beads or anti-CD3 (2C11) beads were stained with anti-CD4 and either p31- or HEL-I-A<sup>g7</sup> multimers to identify Ag-specific cells. Staining of CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>+</sup> T<sub>eff</sub> cultured with p31-I-A<sup>g7</sup> beads is also shown. **C**, Cultures of p31-I-A<sup>g7</sup>-expanded T<sub>reg</sub> were left unsorted or sorted into p31-I-A<sup>g7</sup> multimer-positive and -negative populations using FACS. The resulting populations were CFSE labeled and stimulated with HEL or p31 peptide in the presence of irradiated APC and IL-2. Cultures were analyzed at 72 h by flow cytometry for CFSE intensity.



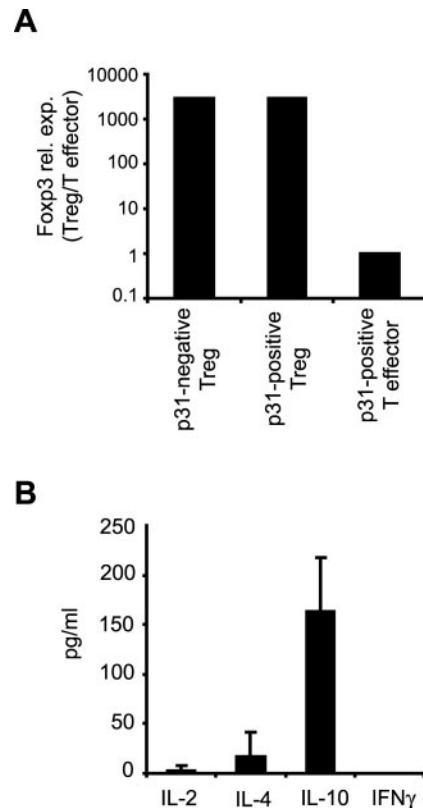
stain positive for p31-I-A<sup>g7</sup> above background levels. In cultures of CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>+</sup> T<sub>eff</sub> expanded with p31-I-A<sup>g7</sup>-coated beads under the same conditions, 40–50% of the population stained positive for the p31-I-A<sup>g7</sup> multimer (Fig. 2B). The p31-I-A<sup>g7</sup>-expanded T<sub>reg</sub> were also tested for specificity against peptides in proliferation assays. The p31-I-A<sup>g7</sup>-expanded T<sub>reg</sub> responded to p31 peptide, but not control HEL peptide when cultured with APC and IL-2 (data not shown).

As shown in Fig. 2B, in general, 90% of the p31-I-A<sup>g7</sup>-expanded T<sub>reg</sub> population failed to stain for the p31-I-A<sup>g7</sup> multimer when analyzed by flow cytometry. It was possible that the unstained population was comprised of p31-I-A<sup>g7</sup>-reactive cells with TCRs with too low of an affinity to be detected by flow cytometry (14). Alternatively, this population could be comprised of cells that were expanding in an Ag-nonspecific manner. To distinguish between these two alternatives, p31-I-A<sup>g7</sup>-expanded T<sub>reg</sub> populations were sorted into p31-I-A<sup>g7</sup> multimer-positive and -negative populations using FACS. The resulting populations were then labeled with CFSE and stimulated with the polyclonal activator anti-CD3 mAb or with the p31 peptide or a control HEL peptide in the presence of APC and IL-2. All three populations, unsorted, p31-I-A<sup>g7</sup> multimer positive, and p31-I-A<sup>g7</sup> multimer negative, expanded equally well to anti-CD3 mAb when analyzed for CFSE dilution (data not shown). As shown in Fig. 2C, >50% of the T<sub>reg</sub> from the unsorted population entered into cell cycle when stimulated with the p31 peptide compared with only ~14% when stimulated with the HEL peptide. The background proliferation with the HEL peptide probably reflected the residual presence of beads in the culture before cell sorting into p31-I-A<sup>g7</sup> multimer-positive and -negative populations. In the p31-I-A<sup>g7</sup> multimer-positive population, >65% of the cells entered into the cell cycle when stimulated with the p31 peptide. Importantly, >50% of the cells from the p31-I-A<sup>g7</sup> multimer-negative population entered into cell cycle when stimulated with the p31 peptide, demonstrating the presence of p31-reactive cells in this population. The expansion protocol using recombinant peptide-MHC class II was therefore very efficient in expanding Ag-specific T<sub>reg</sub> with TCRs with a broad range of affinities for Ag.

#### Characterization of expanded Ag-specific T<sub>reg</sub>

Expanded Ag-specific T<sub>reg</sub> were examined for the expression of the T<sub>reg</sub> lineage marker Foxp3 using quantitative real-time PCR. To ensure that p31-I-A<sup>g7</sup>-reactive cells were analyzed, p31-I-A<sup>g7</sup>-expanded T<sub>reg</sub> and T<sub>eff</sub> were sorted into p31-I-A<sup>g7</sup> multimer-positive and -negative populations by FACS before analysis. In a representative experiment shown in Fig. 3A, expanded p31-I-A<sup>g7</sup> multimer<sup>+</sup> T<sub>reg</sub> expressed ~3000-fold more Foxp3 relative to expanded p31-I-A<sup>g7</sup> multimer-positive T<sub>eff</sub>. Upon challenge with Ag and APC, p31-I-A<sup>g7</sup>-expanded T<sub>reg</sub> expressed low levels of the proinflammatory cytokines IL-2, IL-4, and IFN- $\gamma$ , and expressed high levels of the anti-inflammatory cytokine IL-10 (Fig. 3B). Peptide-I-A<sup>g7</sup>-expanded T<sub>reg</sub> retained high levels of expression of CD62L and CD25 throughout the culture period (Fig. 4A). In contrast, peptide-I-A<sup>g7</sup>-expanded T<sub>eff</sub> down-regulated CD62L and expressed relatively lower levels of CD25. Peptide-I-A<sup>g7</sup>-expanded T<sub>reg</sub> also expressed high levels of CTLA-4, GITR, and ICOS (Fig. 4B). Peptide-I-A<sup>g7</sup>-expanded T<sub>eff</sub> expressed levels of CTLA-4 comparable to peptide-I-A<sup>g7</sup>-expanded T<sub>reg</sub> (data not shown). GITR was also up-regulated on the expanded T<sub>eff</sub> as compared with naive T<sub>eff</sub>, but remained lower than levels observed on expanded T<sub>reg</sub>. ICOS expression was higher on expanded T<sub>eff</sub> as compared with expanded T<sub>reg</sub> (data not shown).

We then examined the V $\beta$  repertoire of the p31-I-A<sup>g7</sup>-expanded

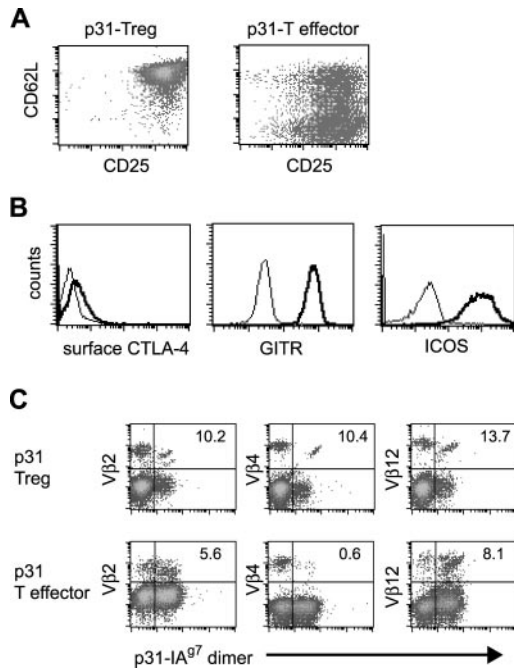


**FIGURE 3.** Expanded Ag-specific T<sub>reg</sub> express Foxp3 and IL-10. A, p31-I-A<sup>g7</sup>-expanded cells were sorted into p31-I-A<sup>g7</sup> multimer-positive and -negative populations by FACS. Levels of Foxp3 mRNA expression were determined for the indicated populations by real-time PCR analysis. The expression levels of Foxp3 in p31-I-A<sup>g7</sup>-expanded CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> relative to p31-I-A<sup>g7</sup>-expanded CD4<sup>+</sup>CD25<sup>-</sup> T<sub>eff</sub> are shown. B, p31-I-A<sup>g7</sup>-expanded T<sub>reg</sub> were stimulated with p31 peptide in the presence of irradiated APC. Secreted cytokine levels were determined 48 h after stimulation by ELISA.

T cells. The BDC2.5 TCR expresses a TCR $\beta$  derived from the V $\beta$ 4 family (11). However, when p31-I-A<sup>g7</sup>-expanded T<sub>reg</sub> and T<sub>eff</sub> were costained with p31-I-A<sup>g7</sup> multimers and different TCR V $\beta$  reagents, neither population was monoclonal. Although there was a significant number of V $\beta$ 4<sup>+</sup> p31-I-A<sup>g7</sup> multimer<sup>+</sup> T cells in the T<sub>reg</sub> culture, other TCR V $\beta$  were also present in significant numbers (Fig. 4C). For instance, V $\beta$ 2 and V $\beta$ 12 accounted for 10.2 and 13.7% of the p31-I-A<sup>g7</sup> multimer<sup>+</sup> T<sub>reg</sub>, respectively, in this representative culture. TCR V $\beta$ 4<sup>+</sup> T cells were generally present in the p31-I-A<sup>g7</sup> multimer<sup>+</sup> T<sub>eff</sub> population, but at a lower percentage. Together, these results suggest that broad repertoires of T<sub>reg</sub> and T<sub>eff</sub> reactive against the islet peptide mimic are resident in conventional NOD mice, and that the islet peptide mimic-reactive T<sub>reg</sub> repertoire is not identical with the islet peptide mimic-reactive T<sub>eff</sub> repertoire.

#### Suppression by Ag-specific T<sub>reg</sub> in vitro

Previous studies have shown that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> can suppress proliferation of CD4<sup>+</sup> effectors in vitro and that the suppressive effect is dependent on stimulation of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> through their TCR. Therefore, expanded p31-I-A<sup>g7</sup> T<sub>reg</sub> were examined for suppressive activity and specificity in vitro. Expanded p31-I-A<sup>g7</sup> T<sub>reg</sub> effectively suppressed the proliferation of Ag-specific CD4<sup>+</sup> BDC2.5 TCR Tg<sup>+</sup> cells in a dose-specific manner when cultures were stimulated with either anti-CD3 or 1040-31 peptide (Fig. 5A).



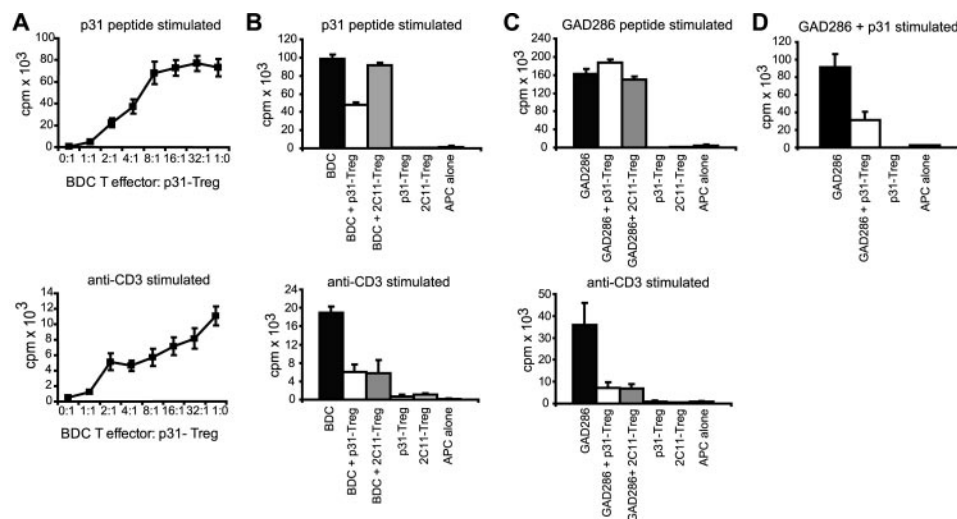
**FIGURE 4.** Surface phenotype of expanded Ag-specific  $T_{reg}$ . *A*, Cultures of p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  or  $T_{eff}$  were stained for expression of CD25 and CD62L. *B*, Surface staining of p31-I-A<sup>g7</sup>-expanded  $T_{reg}$ . Light lines indicate control staining. Heavy lines indicate staining of p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  populations. *C*, Cultures of p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  and  $T_{eff}$  were costained with p31-I-A<sup>g7</sup> multimers and the indicated TCR V $\beta$  Abs. Numbers shown represent the percentage of p31-I-A<sup>g7</sup> multimer-positive cells staining positive for the indicated TCR V $\beta$ .

In contrast, p31-I-A<sup>g7</sup>-expanded CD4<sup>+</sup>CD25<sup>+</sup>  $T_{eff}$  failed to suppress freshly isolated BDC2.5 CD4<sup>+</sup> T cells and resulted in augmentation of proliferation (data not shown). The p31-I-A<sup>g7</sup>-expanded  $T_{reg}$ , but not the  $T_{eff}$ , were anergic to stimulation (both p31 peptide and anti-CD3) in the absence of CD28 costimulation or addition of IL-2 consistent with reports for freshly isolated  $T_{reg}$  (Fig. 5*B* and data not shown). To further characterize the Ag spec-

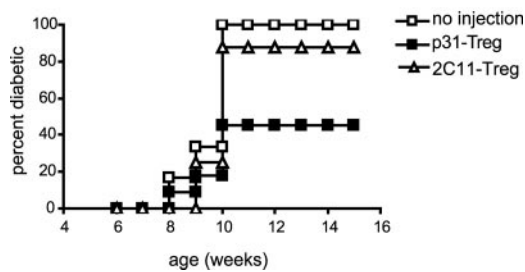
ificity of the expanded  $T_{reg}$ , expanded polyclonal  $T_{reg}$  and p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  were assessed for the ability to suppress BDC2.5 TCR Tg<sup>+</sup> or GAD<sub>286</sub> TCR Tg<sup>+</sup> CD4<sup>+</sup> cells (12) through either polyclonal T cell activation via anti-CD3 or through Ag-specific T cell activation. Both polyclonal  $T_{reg}$  and p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  suppressed BDC2.5 TCR Tg<sup>+</sup> responders when stimulated with anti-CD3, whereas only the p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  suppressed cultures stimulated with the BDC2.5 1040-31 peptide (Fig. 5*B*). Similarly, both polyclonal  $T_{reg}$  and p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  suppressed the response of GAD<sub>286</sub> TCR Tg<sup>+</sup> CD4<sup>+</sup> T cells when stimulated with anti-CD3 (Fig. 5*C*, bottom). However, neither the polyclonal  $T_{reg}$  population nor the p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  population suppressed GAD<sub>286</sub> TCR Tg<sup>+</sup> responders when stimulated with the GAD<sub>286-300</sub> peptide (Fig. 5*C*, top). Most significantly, p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  were capable of suppressing GAD<sub>286</sub> TCR Tg<sup>+</sup> responders when the culture was stimulated with both the GAD<sub>286-300</sub> and the 1040-31 peptide (Fig. 5*D*). Collectively, these data demonstrate that the suppressive activity of the p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  is dependent on Ag-specific stimulation through the TCR, although, once stimulated with cognate Ag, p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  are capable of exerting bystander suppression.

#### Expanded Ag-specific $T_{reg}$ prevent autoimmune diabetes

We then tested the ability of small numbers of p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  to suppress diabetes in CD28<sup>-/-</sup> NOD mice. CD28<sup>-/-</sup> NOD mice have normal numbers of  $T_{eff}$  and Th1 responses and undergo an accelerated form of autoimmune diabetes due to a deficiency in  $T_{reg}$  (4, 15). Previous studies have shown that delay or prevention of diabetes in this model required the transfer of high numbers of polyclonal  $T_{reg}$  ( $8-20 \times 10^6$ ) (4). In sharp contrast, the transfer of as few as  $1.8 \times 10^6$  p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  into 5- to 7-wk-old mice prevented the development of diabetes in the majority of mice for at least 15 wk of age (Fig. 6). The in vivo administered p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  cultures were an admixture of ~10% high avidity cells, as determined by the ability to bind p31-I-A<sup>g7</sup> multimers, and lower avidity cells that were not detected by the p31-I-A<sup>g7</sup> multimers when analyzed by flow cytometry, but were capable of responding to p31 peptide presented by APC (see Fig.



**FIGURE 5.** p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  suppress in an Ag-specific manner in vitro. *A*,  $5 \times 10^4$  freshly isolated BDC2.5 CD4<sup>+</sup>CD25<sup>-</sup> T cells were cultured with varying amounts of p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  and  $5 \times 10^4$  irradiated APC. Cultures were stimulated with either 100 nM 1040-31 peptide or 1  $\mu$ g/ml anti-CD3. Proliferation was determined, as described in Fig. 1*C*. *B* and *C*,  $5 \times 10^4$  p31-I-A<sup>g7</sup> or 2C11-expanded  $T_{reg}$  were cultured with freshly isolated BDC2.5 TCR Tg<sup>+</sup> CD4<sup>+</sup>CD25<sup>-</sup> responders (*B*) or GAD<sub>286</sub> TCR Tg<sup>+</sup> CD4<sup>+</sup>CD25<sup>-</sup> responders (*C*),  $5 \times 10^4$  irradiated APC and the 1040-31 peptide (*B*) or GAD<sub>286-300</sub> peptide (*C*), or anti-CD3. Proliferation was determined, as described above. *D*, p31-I-A<sup>g7</sup>-expanded  $T_{reg}$  were cultured with GAD<sub>286</sub> TCR Tg<sup>+</sup>CD4<sup>+</sup>CD25<sup>-</sup> responder cells, as described in *C*, and stimulated with a combination of the 1040-31 and GAD<sub>286-300</sub> peptides.



**FIGURE 6.** Expanded Ag-specific T<sub>reg</sub> prevent diabetes in vivo. Five- to 7-wk-old prediabetic CD28-deficient NOD mice were injected with  $1.8-2 \times 10^6$  p31-I-A<sup>g7</sup>-expanded T<sub>reg</sub> ( $n = 11$ ),  $2 \times 10^6$  ( $n = 6$ ), or  $8 \times 10^6$  ( $n = 2$ ) 2C11-expanded T<sub>reg</sub> or left untreated ( $n = 6$ ). Diabetes was determined by monitoring blood glucose levels.

2, B and C). Thus, the Ag-specific p31-I-A<sup>g7</sup>-expanded cells were highly efficient in protecting the onset of diabetes induced by a fully functional polyclonal T cell response. Suppression of autoimmunity by the p31-I-A<sup>g7</sup>-expanded T<sub>reg</sub> appeared to be organ specific. Although animals that received the p31-I-A<sup>g7</sup>-expanded T<sub>reg</sub> were protected from diabetes, the mice continued to exhibit other autoimmune syndromes based on continued lymphocytic infiltration in the salivary and thyroid glands (data not shown).

## Discussion

The identification of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells as important regulators of tolerance has opened a major area of investigation in autoimmunity. The field has been limited, however, by the small numbers of circulating T<sub>reg</sub> and the inability to define their antigenic specificities. In this study, we demonstrated that Ag-specific CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> reactive to an islet peptide mimic reside in the periphery of diabetes-susceptible NOD mice. Furthermore, we demonstrated that these rare Ag-specific cells can be selectively expanded in vitro from a polyclonal population, and that these expanded T<sub>reg</sub> retain phenotypic and functional characteristics of freshly isolated CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub>. The in vitro expanded T<sub>reg</sub> retained high expression of CD62L important for allowing trafficking to lymph nodes upon administration of these cells in vivo, and also retained expression of molecules such as Foxp3, GITR, and CTLA-4, implicated as important for T<sub>reg</sub> function. Activation of suppressive activity of the in vitro expanded T<sub>reg</sub> was Ag specific, but once activated, the T<sub>reg</sub> were capable of suppressing T<sub>eff</sub> with other specificities. This aspect is important for controlling diseases such as type 1 diabetes in which numerous self Ags are targeted by pathogenic effector cells. Most importantly, we showed that in vivo, expanded Ag-specific T<sub>reg</sub> are highly efficient at controlling organ-specific autoimmunity. These results support previous studies demonstrating that immune regulation by CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> is dependent on the Ag specificity of the T<sub>reg</sub> (6, 7) and are not consistent with reports suggesting that T<sub>reg</sub> function in an Ag-nonspecific fashion by competing for T cell niches (16). Thus, efficient T<sub>reg</sub> function is likely to require Ag-specific activation either for maintenance in the periphery or for function at the site of inflammation or the draining lymph node.

The BDC2.5 mimotope-reactive T<sub>reg</sub> expanded from wild-type NOD mice described in these studies were not as efficient at controlling autoimmune diabetes as previously described T<sub>reg</sub> expressing a Tg BDC2.5 TCR and expanded with anti-CD3-coated beads (6). Several possibilities could explain this difference. One possi-

bility is that the BDC2.5 TCR Tg<sup>+</sup> T<sub>reg</sub> possess a TCR with much higher affinity for the expanding p31 peptide Ag as well as the endogenous islet Ag than the in vitro expanded T<sub>reg</sub> from wild-type NOD mice. As shown in Figs. 2C and 4C, the in vitro expansion method with recombinant peptide-MHC produces a population with a broad repertoire with varying affinity for the expanding p31 peptide Ag. Thus, it is possible that the p31-I-A<sup>g7</sup>-expanded T<sub>reg</sub> culture as a population possesses a lower affinity for self Ag or that only a subset of the expanded T<sub>reg</sub> responds to the endogenous self Ag. Differences in the methods of stimulation between the two studies (anti-CD3 vs peptide-MHC) are unlikely to be the cause of the variation in function because BDC2.5 TCR Tg<sup>+</sup> T<sub>reg</sub> expanded with p31-I-A<sup>g7</sup> beads are comparable in function in vivo to BDC2.5 TCR Tg<sup>+</sup> T<sub>reg</sub> expanded with anti-CD3 beads (E. Masteller and J. Bluestone, unpublished data).

The ability to expand functional Ag-specific T<sub>reg</sub> has important implications for the development of T<sub>reg</sub>-based approaches for clinical therapy. The expansion of small numbers of autoantigen-specific T<sub>reg</sub> with restricted repertoires is likely to be clinically efficacious because of the ability to suppress polyclonal pathogenic T cell responses either by bystander cytokine production and/or recruitment of endogenous regulatory cells while avoiding pan-immune suppression. Finally, it should be noted that many organ-specific Ags have been identified that contribute to autoimmune diseases such as type 1 diabetes and multiple sclerosis. It is possible that currently available human MHC multimer reagents could be used to expand human organ-specific T<sub>reg</sub> from blood for effective treatment of autoimmune diseases (17).

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## Disclosures

The authors have no financial conflict of interest.

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