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Autoreactive T Cells in Healthy Individuals¹

Nancy A. Danke,* David M. Koelle,[†] Cassian Yee,[‡] Sucheta Beheray,* and William W. Kwok^{2*}

The presence of autoreactive CD4⁺ T cells in the peripheral blood of healthy human subjects was investigated after removal of CD4⁺CD25⁺ regulatory T cells (Treg). CD4⁺ T cells that were directed against the type 1 diabetes-associated autoantigen glutamic acid decarboxylase 65, the melanocyte differentiation Ag tyrosinase, and the cancer/testis tumor Ag NY-ESO-1 were readily derived from PBMC of healthy individuals. These autoreactive T cells could be visualized, using Ag-specific class II tetramer reagents, in the peripheral blood of most individuals examined. Addition of CD4⁺CD25⁺ Treg back to the CD4⁺CD25⁻ population suppressed the expansion of the autoreactive T cells. Autoreactive T cells were cloned based on tetramer binding, and expressed characteristic activation markers upon self-Ag stimulation. These results show that autoreactive T cells are present in most healthy individuals and that Treg likely play an important role of keeping these autoreactive T cells in check. *The Journal of Immunology*, 2004, 172: 5967–5972.

The deletion of autoreactive T cells through thymic selection is a principal, but not the only, mechanism for control of autoimmunity. Deletion is not 100% efficient, and even autoreactive T cells that have high affinity for autoantigens can escape the deletion process and migrate to the periphery. Peripheral tolerance mechanisms are capable of the suppression of those autoreactive T cells which escape thymic deletion. Recent evidence indicates that a population of regulatory cells, designated as CD4⁺CD25⁺ regulatory T cells (Treg),³ play a crucial role for the suppression of reactivity of autoreactive T cells. In the seminal experiments by Sakaguchi et al. (1), it was observed that the administration of CD4⁺CD25⁺ T cells to athymic nude mice would prevent the development of gastritis. Subsequent studies indicated that CD4⁺CD25⁺ T cells could also prevent the development of inflammatory bowel disease, diabetes, experimental autoimmune encephalomyelitis, and graft-versus-host disease in various rodent models (2–7), as well as demonstrate a suppressive effect in vitro (8, 9). CD4⁺CD25⁺ cells with in vitro suppressive activity can also be found in peripheral blood in humans. Most of the early human PBMC studies involved activation of T cells in an Ag-nonspecific fashion by anti-CD3 Ab (10–12). Recently, it was demonstrated that proliferative responses of PBMC toward the self-Ag human heat shock protein 60 and myelin oligodendrocyte glycoprotein as assayed by thymidine incorporation was significantly increased in the absence of CD4⁺CD25⁺ Treg (13, 14), indirectly implicating the presence of myelin oligodendrocyte glycoprotein or heat shock protein-specific T cells in PBMC.

We explored the extent and function of normal Treg by assessing activity of a spectrum of autoreactive human T cells. Direct demonstration of suppression of Ag-specific T cells in normal PBMC was accomplished by using class II tetramers. T cells directed against three different autoantigens were examined, including the diabetes-associated Ag glutamic acid decarboxylase (GAD) 65, the vitiligo and melanoma-associated Ag tyrosinase, and the cancer/testis Ag NY-ESO-1. The removal of the CD4⁺CD25⁺ population enabled a rapid expansion of these autoreactive T cells in vitro. Autoreactive T cells directed against GAD65, tyrosinase, and NY-ESO-1 can be detected after one round of in vitro stimulation in the absence of CD4⁺CD25⁺ T cells in most individuals examined. Addition of CD4⁺CD25⁺ T cells to the culture suppressed the expansion of the autoreactive T cells. These results indicate the presence of latent autoreactive T cells in healthy individuals and the role of CD4⁺CD25⁺ T cells suppressing their expansion.

Materials and Methods

Donor samples

All donors were healthy subjects with no history of autoimmune disease. Class II HLA typing was performed by using Dynal SSO typing kits (Dynal Biotech, Lake Success, NY). DR4 subtypings were determined by direct DNA sequencing. Only DRB1*0401 subjects were included in the study. A total of 12 DRB1*0401 subjects were recruited for the study.

Tetramer synthesis

Biotinylated DR0401-soluble molecules were purified as described previously (15). Exogenous peptide loading onto biotinylated class II molecules were conducted with the following peptides, GAD_{555–567} (557I) NFIRMVIS NPAAT, GAD_{555–567} NFFRMVISNPAAT, NY-ESO-1_{120–131} GVLLKE FTVSGN, tyrosinase TYR_{450–462} (456V) SYLQDSVPSDFQD, TYR_{450–462} SYLQDSDPDSFQD, and type II collagen CII_{261–273} AGFKGEGQPKGEP. PE-streptavidin (BioSource International, Camarillo, CA) was used to cross-link the biotinylated molecules to generate DR0401 tetramers (15).

Generation of autoreactive T cells

PBMC were isolated from 150 ml of heparinized peripheral blood. CD4⁺ T cells were isolated from PBMC using auto-MACS with the “no touch” CD4⁺ T cell isolation kit (Miltenyi Biotec, Auburn, CA). CD4⁺ T cells were then stained with PE-conjugated anti-CD25 and CyChrome-conjugated anti-CD4 Abs and sorted by flow cytometry (FACS Vantage; BD Biosciences, San Diego, CA). Cells were gated on scattering and the top 1% of the CD4⁺CD25⁺ cells were considered as the CD4⁺CD25⁺ population, while the bottom 80% of the cells were considered as the CD4⁺CD25⁻ population. The yield of CD4⁺CD25⁺ T cells from 150 ml of blood was usually around 0.4×10^6 . Non-CD4⁺ cells that were retained in the MACS separation column were flushed out and were used to prepare

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³ Abbreviations used in this paper: Treg, regulatory T cell; GAD, glutamic acid decarboxylase; T1D, type 1 diabetes.

APC. These non-CD4⁺ cells were allowed to adhere to the tissue culture plates for 2 h, and nonadherent cells were removed by washing the wells repeatedly with RPMI 1640. The adherent cells were used as APC.

For *in vitro* peptide stimulation, 3×10^6 unfractionated CD4⁺ or CD4⁺CD25⁻ T cells were plated out per well onto a 24-well plate in the presence of 1.5×10^6 adherent APC. Cells were stimulated with one or two peptides per well (10 μ g/ml per peptide). Cells were fed with 20 U/ml IL-2 (Hemagen, Worcester MA) starting at day 7 and were refed every 2–3 days afterward. In some experiments, 5×10^6 unfractionated PBMC instead of CD4⁺ T cells were used as responders.

For suppression assays, 0.9×10^6 CD4⁺CD25⁻ T cells were cultured in the absence or presence of 0.3×10^6 autologous CD4⁺CD25⁺ cells/well in 48-well plates. A total of 0.7×10^6 adherent cells was used as APC. Cells were stimulated with 10 μ g/ml peptide.

Staining of T cells with tetramers

Fourteen days after *in vitro* stimulation, cells were stained with specific tetramers or the DR0401/CI₂₆₁₋₂₇₃ tetramers as control tetramers for 1 h at 37°C. PerCP-conjugated anti-CD4 and allophycocyanin-conjugated anti-CD3 Abs were then added for an additional 15 min at 4°C. In some experiments, cells were also stained with FITC-conjugated anti-CD25 Ab. All Abs were purchased from BD Biosciences.

For estimation of precursor frequency, CD4⁺CD25⁻ T cells were labeled with 0.3 μ M CFSE in PBS for 10 min at 37°C. Staining was stopped by adding 100% FBS and subsequent washing. Cells were stimulated with 10 μ g/ml peptide or with 2.5 μ g/ml PHA. Staining and flow cytometry of T cells were conducted at day 7 after *in vitro* peptide stimulation. Stimulation of T cells with PHA and IL-2 results in cell division with distinct fluorescence peaks, allowing determination of the number of cell divisions. Precursor frequency of Ag-specific T cells was estimated by dividing the fraction of tetramer-positive T cells by 2^x , where x is the average number of cell divisions as calculated by CFSE fluorescence.

Results

Detection of GAD-reactive T cells in DR0401 individuals without type 1 diabetes (T1D)

GAD65 is an autoantigen that is associated with the development of T1D. Using class II tetramer technology, Reijonen et al. demonstrated that recent onset DR0401 T1D subjects often have T cells directed against the GAD₅₅₇₁ epitope (GAD₅₅₅₋₅₆₇ with a F to I substitution at position 557) and the wild-type GAD₅₅₅₋₅₆₇ epitope, a naturally processed epitope (16). However, GAD-specific T cells were not detected by tetramers using this assay in subjects without T1D.

We compared the GAD tetramer reactivity of Treg-depleted CD4⁺ T cells from normal individuals with the Treg-undepleted fraction. In the first set of experiments, PBMC were isolated from a DR0401 individual and were either left unfractionated or fractionated into a CD4⁺CD25⁻ population. The cells were stimulated with the GAD₅₅₇₁ peptide. After 14 days of culture, tetramer-positive T cells could not be detected from the unfractionated PBMC. However, a population of DR0401-restricted GAD-reactive T cells was easily detected in the CD4⁺CD25⁻ samples with the DR0401/GAD5571 tetramers (Fig. 1A).

The GAD₅₅₇₁ peptide is a strong agonist with an isoleucine substitution at position 557 to enhance MHC binding and may be recognized as a “foreign” peptide. We investigated whether T cell responses toward wild-type GAD₅₅₅₋₅₆₇ peptide can also be detected in healthy individuals. T cell reactivities toward GAD₅₅₅₋₅₆₇ were assayed in both CD4⁺CD25⁻ and total CD4⁺ T cell populations. As shown in Fig. 1B, a strong response toward the GAD₅₅₅₋₅₆₇ peptide was observed in the CD4⁺CD25⁻ population and a weak response was observed in the unfractionated CD4⁺ population. A total of nine DR0401 individuals have been examined, and six of nine had responses toward the GAD₅₅₇₁/GAD₅₅₅₋₅₆₇ epitopes in the CD4⁺CD25⁻ population. In contrast, only one of nine shows positive tetramer staining in the total CD4⁺ population. The percentage of GAD tetramer-positive T cells at day 14 poststimulation among the responsive individuals ranged from 0.3 to 14%. The frequencies of GAD-reactive T cells in three samples were estimated by staining CFSE-labeling CD4⁺CD25⁻ T cells with GAD tetramers 7 days poststimulation. The frequencies were ~1 in 45,000 and 1 in 61,000 CD4⁺ T cells, respectively, in 2 healthy subjects (data not shown). The frequency of GAD-reactive T cells in the third subject could not be determined accurately at day 7 because the number of tetramer-positive events was very low. As we estimated that T cells at a frequency of 1 in 250,000 could be determined by the CFSE/tetramer approach, the frequency of GAD-reactive T cells for the third subject was lower than 1 in 250,000.

The activation status of the tetramer-positive cells was examined by staining with anti-CD25 Ab. Although the starting materials were CD4⁺CD25⁻ T cells, we observed that the Ag-specific

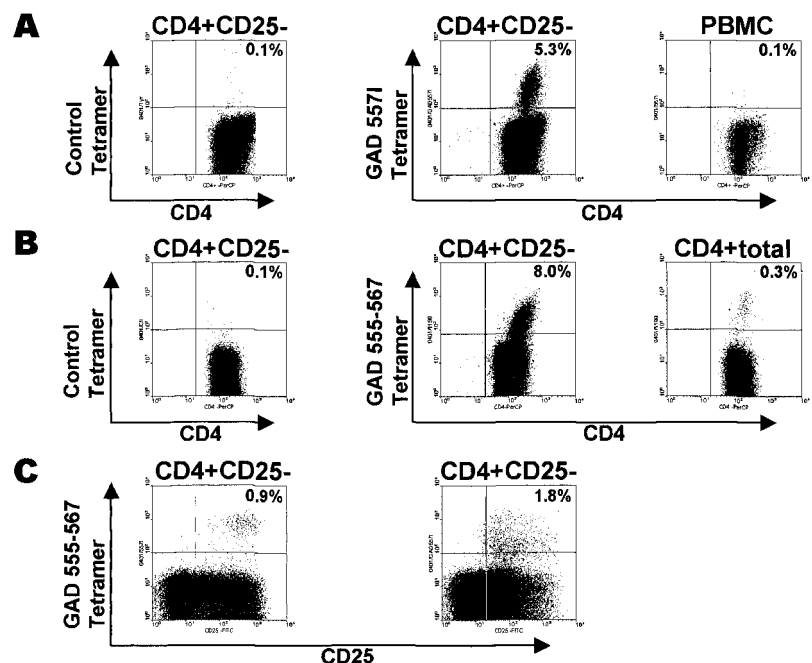


FIGURE 1. Detection of GAD65-autoreactive T cells in CD4⁺CD25⁻ T cell populations. **A**, Detection of GAD₅₅₇₁-specific T cells. CD4⁺CD25⁻ T cells or unfractionated PBMC from a DR0401 subject were stimulated with 10 μ g/ml GAD₅₅₇₁ peptide. Cells were assayed by flow cytometry 14 days poststimulation with DR0401/GAD₅₅₇₁ tetramers or DR0401/TYR₄₅₀₋₄₆₂ tetramers as control tetramers. For flow analysis, cells were gated on scattering and CD3⁺ cells. **B**, Detection of CD4⁺ GAD₅₅₅₋₅₆₇-autoreactive T cells in CD4⁺CD25⁻ T cell populations. CD4⁺CD25⁻ T cells or unfractionated CD4⁺ T cells from a DR0401 subject were stimulated with 10 μ g/ml GAD₅₅₅₋₅₆₇ peptide. Cells were assayed with DR0401/GAD₅₅₅₋₅₆₇ tetramers or DR0401/CI₂₆₁₋₂₇₃ as control tetramers. **C**, GAD65-reactive T cells are CD25⁺. CD4⁺CD25⁻ T cells from two different DR0401 subjects were stimulated with GAD₅₅₅₋₅₆₇ peptide. Cells were assayed by flow 14 days poststimulation.

T cells were activated during the 14-day culturing period in the presence of IL-2. Thus, all of the tetramer-positive T cells were also CD25 positive, as shown in Fig. 1C.

Detection of other autoreactive T cell epitopes in healthy individuals

In addition to the presence of GAD-autoreactive T cells, the presence of autoreactive T cells that are specific for two additional autoantigens, NY-ESO-1 and tyrosinase, were also evaluated. NY-ESO-1 is a cancer/testis Ag that is only expressed in tumors or germ cells. T cells directed against the NY-ESO-1 can be detected in healthy and cancer patients by functional assays (17–19). Tyrosinase is a melanocyte differentiation Ag. Both class I- and class II-restricted T cells that are directed against tyrosinase have been found in patients with melanoma (20, 21). Tyrosinase is also a target autoantigen in vitiligo and Vogt-Koyanagi-Harada disease (22). It has been recognized that generation of these tumor Ag-specific lines from normal subjects is a difficult and tedious process. Repeated antigenic stimulation in the presence of dendritic cells as APC is required (17). We examined whether NY-ESO-1- and tyrosinase-specific T cells can be more easily expanded from normal individuals after depletion of CD4⁺CD25⁺ T cells. The results of these experiments are shown in Fig. 2. For the expansion of NY-ESO-1-specific T cells, CD4⁺CD25⁻ T cells were stimulated with the NY-ESO-1_{120–131} peptide. NY-ESO-1-specific T cells can be observed by staining with DR4/NY-ESO-1_{120–131} tetramers 14 days later. The NY-ESO-1 T cells were detected in five of seven DR0401 individuals examined. The percentage of NY-ESO-1 tetramer-positive T cells at day 14 poststimulation among the responsive individuals ranged from 0.3 to 10%.

For the expansion of tyrosinase-specific T cells, CD4⁺CD25⁻ T cells were stimulated with the wild-type tyrosinase peptide TYR_{450–462}. Cells were stained with DR0401/TYR_{450–462} tetramer 14 days later. A small population of tyrosinase-specific T

cells can be easily recognized in the tyrosinase-stimulated sample. The TYR_{450–462} peptide with a Val substitution at position 456 has been identified as a better agonist compared with the wild-type peptide (21). T cell response to the modified tyrosinase peptide, i.e., the TYR_{456V} peptide can also be observed in the current study (data not shown). We were able to detect tyrosinase-specific CD4⁺ T cells in the CD4⁺CD25⁻ population in seven of eight DR0401 individuals examined. Tyrosinase-specific T cells could also be detected by tetramer analysis of cells stimulated from the total CD4⁺ population in one of eight individuals tested. The percentage of TYR tetramer-positive T cells at day 14 poststimulation among the responsive individuals ranged from 0.4 to 14%. The frequency of tyrosinase-specific T cells was found to be 1 in 55,000 and 1 in 240,000 CD4⁺ T cells, respectively, in 2 individuals examined (Fig. 3).

Isolation of autoreactive T cells through tetramer sorting

To demonstrate that the tetramer-positive T cells are indeed Ag specific, tetramer-positive T cells were single cell sorted and cloned by culture with PHA, allogenic feeder cells, and IL-2. GAD-specific, tyrosinase-specific, and NY-ESO-1-specific T cell clones were obtained by single-cell sorting with their respective tetramers. After in vitro expansion with PHA, these T cells show Ag specificity by both proliferation assay and tetramer staining. These results are shown in Fig. 4.

Suppression of autoreactive T cells by Treg

Studies with CD4⁺CD25⁺ Treg indicate that Treg can suppress T cell activation in vitro. We examined whether CD4⁺CD25⁺ Treg can suppress the generation of autoreactive T cells from the CD4⁺CD25⁻ population. CD4⁺CD25⁻ T cell cultures were stimulated with autoantigenic peptides in the absence or presence of autologous CD4⁺CD25⁺ Treg (at a ratio of 1 CD4⁺CD25⁺ Treg to 3 CD4⁺CD25⁻ T cells). Tetramers were used to assay for the

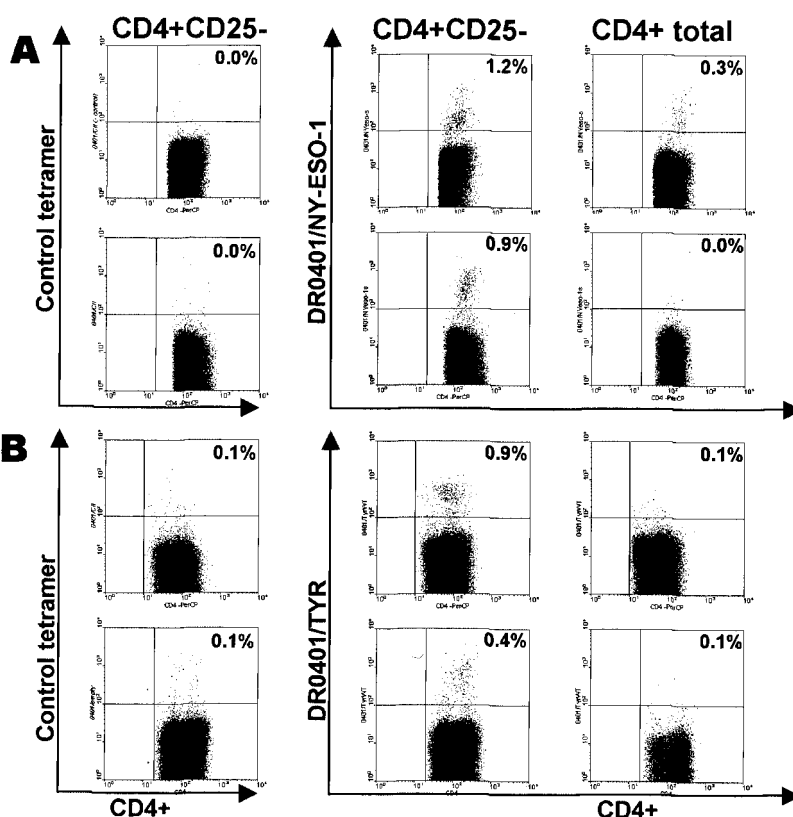
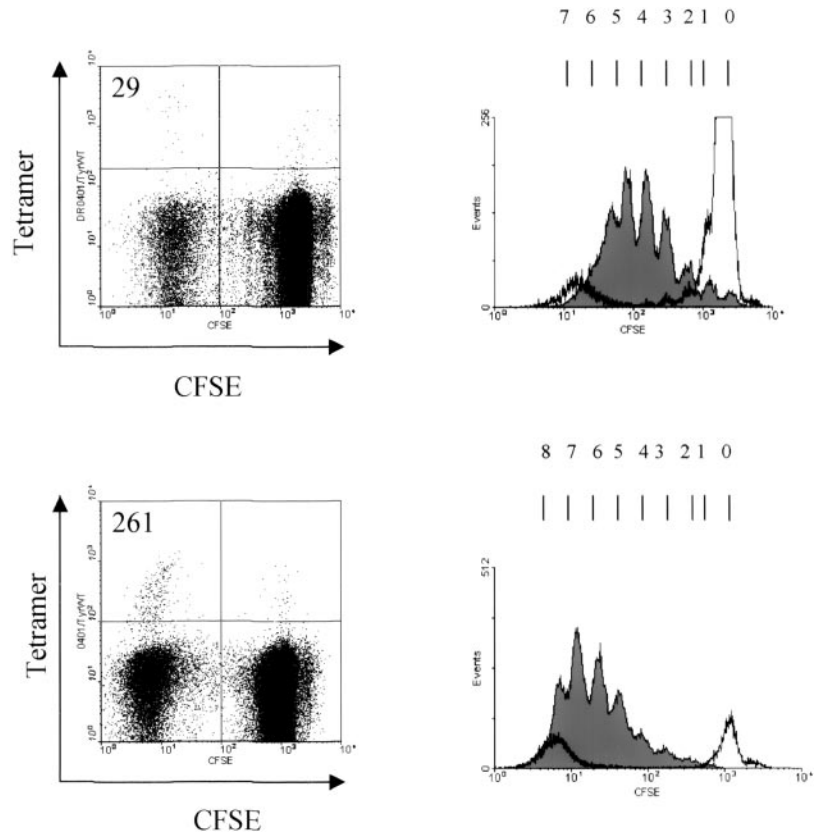


FIGURE 2. Detection of tumor Ag-specific T cells. *A*, Detection of CD4⁺ NY-ESO-1 T cells. CD4⁺ or CD4⁺CD25⁻ T cells from two DR0401 subjects were stimulated with 10 μg/ml NY-ESO-1_{120–131} peptide. Cells were assayed with DR0401/NY-ESO-1_{120–131} tetramers or DR0401/CII_{261–273} as control tetramers. *B*, Detection of CD4⁺ tyrosinase T cells. CD4⁺ or CD4⁺CD25⁻ T cells from two different DR0401 subjects were stimulated with 10 μg/ml tyrosinase TYR_{450–462} peptide. Cells were assayed with DR0401/TYR_{450–462} tetramers or DR0401/CII_{261–273} as control tetramers. Each row represents samples from single subjects.

FIGURE 3. Estimation of frequency of TYR-specific CD4⁺ T cells in peripheral blood by CFSE dye dilution. CD4⁺ T cells from two DR0401 subjects (*top and bottom panels*) were labeled with CFSE and stimulated with 10 μg/ml TYR₄₅₀₋₄₆₂ peptide. Samples were analyzed for DR0401/TYR₄₅₀₋₄₆₂ tetramer binding 7 days later. The number of cell divisions was estimated by measuring the CFSE staining intensity of a PHA-stimulated sample (*right panels*). Overlaying of the histogram of the TYR-stimulated sample (lined) onto the histogram of the PHA sample (filled) indicate the cells have undergone a total of seven divisions (*top panel*) and eight divisions (*bottom panel*) in the 7-day period. A total of 55,000 CD4⁺CD3⁺ events was analyzed and the number within each panel indicates the number of tetramer-positive events. Staining with irrelevant tetramers DR0401/CI₂₆₁₋₂₇₃ was negative.



presence of autoreactive T cells 14 days later. GAD65, tyrosinase, and NY-ESO-1 T cell expansions were all inhibited in the presence of CD4⁺CD25⁺ T cells (Fig. 5).

Discussion

These experiments directly confirm the presence of autoreactive T cells in normal circulating peripheral blood and show that CD4⁺CD25⁺ Treg can suppress the expansion of these autoreactive T cells. CD4⁺ T cells directed against three different autoan-

tigens were visualized by class II tetramers in the current study. Notably, these autoreactive T cells were detected in the majority of healthy subjects studied after removal of Treg cells. Table I summarizes the prevalence of the response. The epitopes recognized by the autoreactive T cells have also been previously identified as naturally processed epitopes (16, 19, 21). These results demonstrate directly that autoreactivity is a “normal” T cell phenotype.

There is speculation that autoreactive T cells which escape negative selection are of low avidity toward autoantigens and cannot

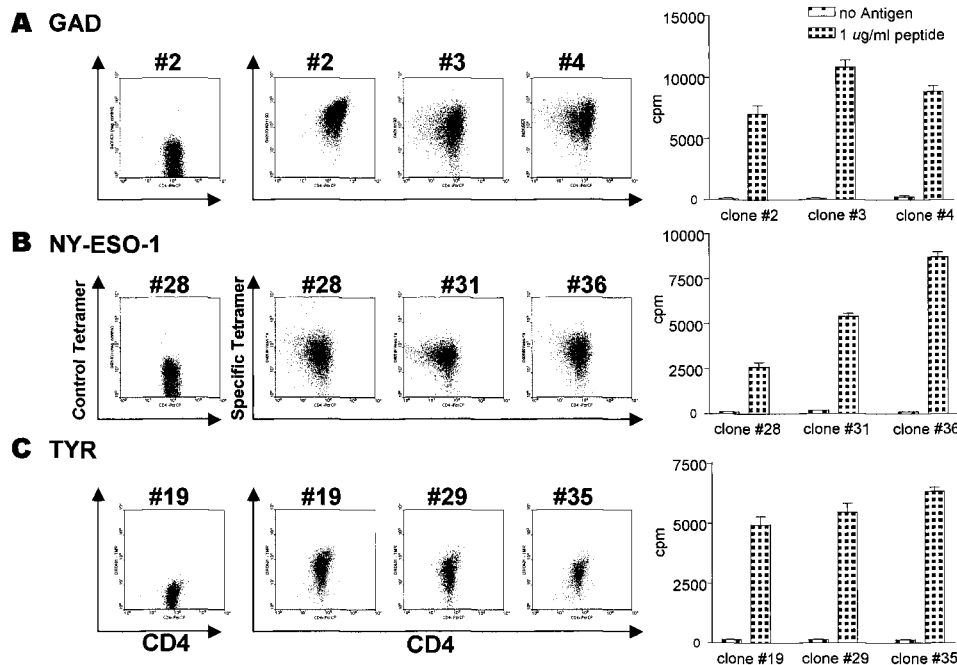


FIGURE 4. Specificity of DR0401-restricted GAD65, NY-ESO-1, and TYR T cell clones. Ag specificities of T cell clones obtained by cell sorting were confirmed by both tetramer staining and peptide-dependent proliferation assays. Three clones each specific for GAD65 (A), NY-ESO-1 (B), and TYR (C) are shown. The control tetramer used was DR0401/CI₂₆₁₋₂₇₃. Proliferation assays were conducted with PBMC from a DR0401 subject as APC and with 1 μg/ml GAD₅₅₅₋₅₆₇, NY-ESO-1₁₂₀₋₁₃₁, or TYR₄₅₀₋₄₆₂ peptide.

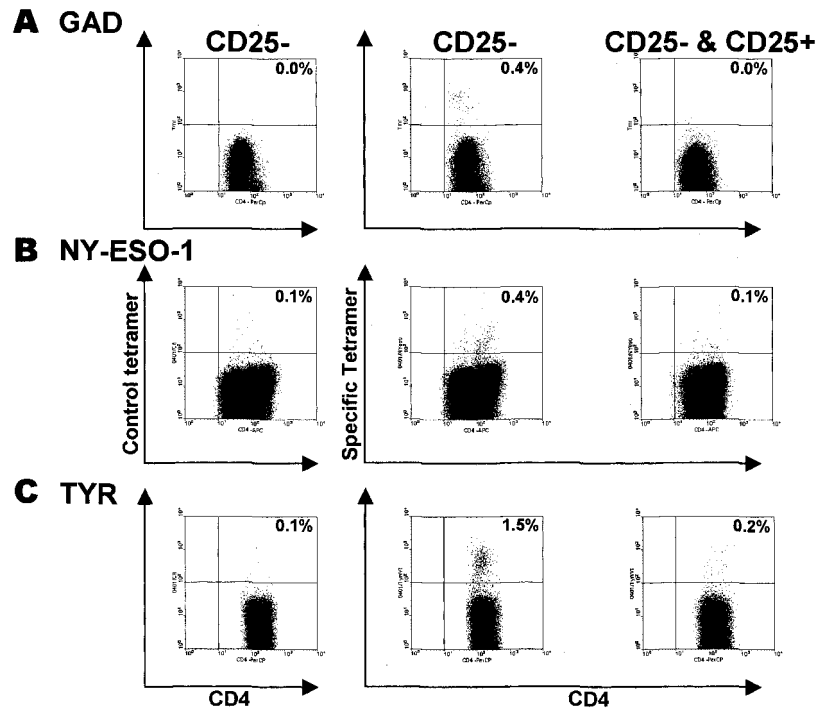


FIGURE 5. $CD4^+CD25^+$ Treg can suppress the expansion of autoreactive T cells. $CD4^+CD25^-$ T cells were stimulated with 10 $\mu\text{g/ml}$ GAD_{555–567} (A), NY-ESO-1_{120–131} (B), or TYR_{450–462} (C) in the absence or presence of $CD4^+CD25^+$ suppressors at a ratio of three responders to one suppressor. Cells were stained with either the control DR0401/CII_{261–273} tetramers or specific tetramers 14 days poststimulation.

bind to Ag-specific class II tetramers. Autoreactive T cells described in this work could be detected by tetramer staining, indicating that these T cells were of medium to high avidity for the Ag. Significant proliferation of the tetramer-sorted cells was observed in the presence of 1 $\mu\text{g/ml}$ specific peptide, and a low level of proliferation was detected when the cells were activated with 0.1 $\mu\text{g/ml}$ peptide (data not shown). Upon specific activation, IFN- γ secretion could also be detected (data not shown). These observations support the concept that central deletion of autoreactive T cells is incomplete, even when the TCR shows moderate or high affinity for self-Ags.

In our experimental protocol, there was no prior activation of $CD4^+CD25^+$ T cells with anti-CD3 in the suppressive assay. It is possible that Ag-specific Treg can be activated in a specific manner by their respective exogenous peptide which is present in the culture. However, this is an unlikely scenario since cell contact appears to be required for suppression (9), and the frequencies of both Ag-specific Treg and Ag-specific responder cells are both very low. Two alternative scenarios may explain the suppressive effect of Treg in our assays. First, a population of activated Treg that is responsible for the suppression of immune responses is always present in peripheral blood and this population of “preactivated” $CD4^+CD25^+$ T cells is active in the suppression assay. Alternatively, the Treg are being activated by endogenous peptides and pooled human serum proteins are presented by autologous APC during the in vitro culture. The latter scenario is also in agreement with the observation that $CD4^+$ T cells become autoreactive in the absence of class II and that the disappearance of $CD4^+CD25^+$ Treg in a MHC class II null environment occurs (23).

It is of interest that $CD4^+$ tumor-specific T cells in peripheral blood could be detected by tetramer staining. Although class I tumor-reactive T cells have been detected by tetramers (24), a convincing demonstration of staining $CD4^+$ tumor-specific T cells with class II tetramers is lacking (25). The interaction between TCRs and pMHC class I is on the average almost 10-fold stronger compared with that of pMHC class II (26). The high-affinity interaction between pMHC class I complexes and TCR probably contributes to the better staining of class I tetramer toward $CD8^+$

tumor-specific T cells. Published results in the use of class II tetramers for staining autoreactive T cells are very limited (16). Staining of collagen-reactive PBMC with class II tetramers in both relapsing polychondritis and rheumatoid arthritis patients have failed to visualize the class II-autoreactive T cells (27, 28). Experiments with HLA class II-transgenic mice also indicate that self-reactive $CD4^+$ T cells fail to bind class II tetramers (29).

The current data do not imply that T cells specific for all autoantigenic epitopes can be easily expanded. There were subjects in whom we could not detect TYR_{450–462}, GAD_{555–567}, and NY-ESO-1_{120–131}-specific T cells. There were also subjects that show responses to TYR_{450–462} and not to GAD_{555–567}. These differences in responses among different subjects may be a reflection of both genetic and environmental factors. We have also attempted to identify collagen-specific T cells directed against CII_{261–273} in three healthy subjects using the same protocol and the results were negative. The frequency of high-affinity T cells for the CII epitopes may be so low that repeated peptide stimulations or other manipulations are needed for the observation of these Ag-specific T cells.

In summary, this study directly demonstrates the presence of autoreactive T cells in peripheral blood of healthy individuals and finds that Treg can suppress the expansion of these autoreactive T cells. Ag-specific autoreactivity is a normal component of the $CD4^+$ T cell population, and the appropriate manipulation of Treg may be beneficial for subjects with autoimmune disease or cancer.

Table I. Autoreactive T cell responses in healthy DR4 subjects^a

	GAD	NY-ESO-1	TYR	CII
No. of individuals examined	9	7	8	3
No. of individuals with positive response	6	5	7	0

^a Autoreactive T cell responses were measured by Ag-specific tetramer stainings as described in the text.

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