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Antigen-Specific T Cell Repertoire Modification of $CD4^+CD25^+$ Regulatory T Cells[1]

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T cell immune responses are regulated by the interplay between effector and suppressor T cells. Immunization with Ag leads to the selective expansion and survival of effector $CD4^+$ T cells with high affinity TCR against the Ag and MHC. However, it is not known if $CD4^+CD25^+$ regulatory T cells (T_{reg}) recognize the same Ag as effector T cells or whether Ag-specific TCR repertoire modification occurs in T_{reg}. In this study, we demonstrate that after a primary Ag challenge, T_{reg} proliferate and TCR repertoire modification is observed although both of these responses were lower than those in conventional T cells. The repertoire modification of Ag-specific T_{reg} after primary Ag challenge augmented the total suppressive function of T_{reg} against TCR repertoire modification but not against the proliferation of memory $CD4^+$ T cells. These results reveal that T cell repertoire modification against a non-self Ag occurs in T_{reg}, which would be crucial for limiting excess primary and memory $CD4^+$ T cell responses. In addition, these studies provide evidence that manipulation of Ag-specific T_{reg} is an ideal strategy for the clinical use of T_{reg}. ***The Journal of Immunology*****, 2004, 172: 5240–5248.**

The immune system has evolved several mechanisms to control self-reactive T cells that escape thymic negative selection (1–3). However, T cell nonresponsiveness to self-Ags does not exclusively result from clonal deletion, T cell anergy, or T cell ignorance (4, 5). Recent accumulating evidence has suggested that $CD4^+CD25^+$ regulatory T cells (T_{reg}),[3] which constitute 5–10% of the peripheral $CD4^+$ T cells in normal naive mice, play an important role in controlling self-reactive T cells and maintaining immunologic self-tolerance (3, 6, 7). T_{reg} in normal naive mice are nonresponsive to Ag-specific stimulation in vitro, but upon stimulation through the TCR, they potently suppress the activation of other $CD4^+$ T cells in an Ag-nonspecific manner (3, 6, 7). Indeed, T_{reg} can suppress autoreactive T cells that cause autoimmune gastritis in neonatal day 3 thymectomized mice (8) and also can suppress allo-MHC-responsive T cells in an allogeneic skin graft model (9). Also, depletion of total T_{reg} has been found to augment tumor-specific (10) or a parasite-specific T cell response (11). Therefore, the application of T_{reg} to clinical medicine (the reduction or increase in function of T_{reg}) such as treatment of tumors, autoimmune diseases, or allogeneic transplantation has been anticipated.

As for the lineage of T_{reg}, several recent papers have revealed that Foxp3 expression is crucial for their development (12–14). According to these results, Foxp3 is expressed in T_{reg} and to a lesser degree in $CD25^-$ T cells (14). Furthermore, the ectopic expression of Foxp3 in $CD4^+CD25^-$ T cells allowed such cells to become regulatory T cells that have the ability to suppress the T cell response (12, 14), suggesting that Foxp3 is at least one transcription factor critical for the development of T_{reg}.

Conventional $CD4^+CD25^-$ T cells (T_{conv}) proliferate in response to antigenic stimulation with the selective survival of cells with a high affinity TCR against the Ag (15–23). This T_{conv} TCR repertoire contraction plays an important role in augmenting primary and secondary immune responses against Ags, thus establishing Ag-specific T cell memory (17, 19). Although the total T cell immune response is thought to be regulated by the interplay between T_{conv}-derived effector/memory T cells and T_{reg}, it remains unclear if T_{reg} recognize the same Ag as effector T cells seen in vivo.

Thus, in this study we evaluated changes in TCR diversity in both T_{conv} and T_{reg} during the emergence of the primary and memory responses to pigeon cytochrome *c* (PCC) in nontransgenic animals to address if T_{reg} really recognize the same Ags as effector T cells do and contract their own repertoire. We found that T_{reg} do recognize the same Ag as T_{conv}, and expand while contracting their repertoire. Furthermore, Ag-specific T_{reg} suppress TCR repertoire modification but not cell proliferation of memory $CD4^+$ T cells.

Materials and Methods

Mice and immunization

The B10.A and B10.BR mice used were from The Jackson Laboratory (Bar Harbor, ME) and were crossed with each other to make F_1 mice. A primary immunization of 400 μg of PCC (Sigma-Aldrich, St. Louis, MO) in an emulsion with CFA was s.c. injected into the base of the mouse tail two days after cell transfer. PBS alone was used for the adjuvant only controls. Secondary challenge was a repeat of the primary regimen including adjuvant, also at the base of the tail, 8 wk after the initial priming.

Cell transfer and purification

The protocol of cell transfer is shown (see Fig. 1*A*). The bone marrow (5×10^6) from 6-wk-old B10.A mice was transferred into 950 rad irradiated (B10.A $\times$ B10.BR)F_1 mice. Two months after bone marrow transplantation, lymph node cells from chimeric mice were stained with anti-B220, D^k, and CD8 mAbs and positive cells were removed by anti-rat mouse IgG-coated beads (Dynal Biotech, Oslo, Norway). The resultant cells were then stained with anti-CD25 mAb and $CD4^+CD25^-$ or $CD4^+CD25^+$ T cells were separated by negative or positive selection of $CD25^+$ cells by

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[3] Abbreviations used in this paper: T_{reg}, regulatory $CD4^+CD25^+$ T cells; T_{conv}, conventional $CD4^+CD25^-$ T cells; PCC, pigeon cytochrome *c*; CDR3, complementarity-determining region 3.

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anti-rat IgG-coated beads (Dynal Biotech) or anti-rat IgG-coated beads followed by LS+ magnetic column (Miltenyi Biotec, Cologne, Germany), respectively. The purity of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells in this preparation is >98% and D^k-positive cells are <1% and the $CD4^+CD25^-$ T cells contaminating the $CD4^+CD25^+$ T cell population is <1% (see Fig. 1*B*). Then, $CD4^+CD25^-$ or $CD4^+CD25^+$ purified T cells (1×10^6) were transferred into nonirradiated (B10.A × B10.BR)F_1 mice. The mice were immunized with PCC (400 μg) emulsified with CFA 2 days after cell transfer and activated donor-derived cells were purified from lymph nodes several days or weeks after immunization.

For the purification of activated cells for complementarity-determining region 3 (CDR3) analysis, lymph node cells from immunized mice were stained with anti-B220, D^k, and CD8 mAbs and positive cells were removed by anti-rat mouse IgG-coated beads (Dynal Biotech). Then resultant T cells were stained by anti-TCR-Vα11, TCR-Vβ3, CD44, and CD62 ligand (CD62L) mAbs, and $TCRV\alpha11^+TCRV\beta3^+CD44^{high}CD62L^{low}$ cells were sorted by a cell sorter as described (24). For the analysis of total Ag-specific cell number, after live cell count by trypan blue staining, spleen and lymph node cells were stained with PE-conjugated anti-TCR-Vα11, biotin-conjugated anti-TCR-Vβ3, allophycocyanin-conjugated anti-CD62L, FITC-conjugated anti-D^k, and FITC-conjugated anti-CD8 mAbs followed by streptavidin-CyChrome. Then percentage of $TCRV\alpha11^+TCRV\beta3^+CD62L^{low}$ cells gated in $CD8^-D^{k-}$ were evaluated by flow cytometer. The Ag-specific T cells were calculated by multiplying total live cell numbers and relative activated T cells. All Abs were purchased from BD Biosciences (San Francisco, CA). In some experiments, $CD25^+$ cells were depleted by injecting anti-CD25 mAb (7D4, 500 μg) 1 day before PCC immunization.

In some experiments, PCC-activated or naive $CD4^+CD25^+V\alpha11^+V\beta3^+$ T cells (1×10^6) were transferred into nonirradiated (B10.A × B10.BR)F_1 mice that had been immunized by PCC 8 wk earlier. For the purification of activated and naive $CD4^+CD25^+$ T cells, lymph node and spleen cells were first purified from PCC-immunized or unimmunized (B10.A × B10.BR)F_1 mice reconstituted with $CD4^+CD25^+$ T cells from 8-wk-old chimeric mice (B10.A bone marrow into irradiated B10.A × B10.BR mice). Then cells were stained with anti-B220, D^k, and CD8 mAbs and positive cells were removed by anti-rat mouse IgG-coated beads (Dynal Biotech). These resultant cells were then stained with anti-CD25 mAb, and $CD25^+$ T cells were separated by positive selection by anti-rat IgG-coated beads followed by LS+ magnetic column (Miltenyi Biotec). Then, cells were stained with anti-Vα11, Vβ3, CD62L, and CD44 mAbs and $V\alpha11^+V\beta3^+CD62L^{low}CD44^{high}$ T cells (activated $CD4^+CD25^+$ T cells) from PCC-immunized mice or $V\alpha11^+V\beta3^+CD62L^{high}CD44^{low}$ cells (naive $CD4^+CD25^+$ T cells) from PCC-unimmunized mice were sorted by a cell sorter. Each population was transferred into nonirradiated (B10.A × B10.BR)F_1 mice that had been immunized with PCC 8 wk earlier.

PCR products

Several days after primary and secondary immunization, $CD8^+D^{k+}$-depleted single cells ($TCRV\alpha11^+V\beta3^+CD62L^{low}CD44^{high}$) from the lymph nodes were sorted into single cells and placed in a 5-μl cDNA reaction mixture (4 U/ml murine leukemia virus reverse transcriptase with recommended buffer, 0.5 nM spermidine, 100 μg/ml BSA, 10 ng/ml oligo(dT), 200 μM each dNTP, and 1% Triton X-100) and then immediately held at 37°C for 90 min. Aliquots (2 μl) of the cDNA reaction mixture were used for two separate 25 μl amplification reactions (2 U/ml *Taq* polymerase with the recommended reaction buffer, 0.1 mM each dNTP, 2 mM $MgCl_2$, and 1.2 μM primer), one for the TCR-Vα11 and one for the TCR-Vβ3, using primers specific for both the variable and constant regions of each chain. The following combinations of primers were used: Vα11, 5′-ATG CAGAGGAACCTGGGAGC-3′ and 5′-AATCTGCAGCGGCACATTG ATTTGGGA-3′; Vβ3, 5′-ATGGCTACAAGGCTCCTCTGGTA-3′ and 5′-CACGTGGTCAGGGAAGAA-3′. The total of 1 μl of the first PCR product was used for further 25 μl amplification reactions (2 U/ml *Taq* polymerase with the recommended reaction buffer, 0.1 mM each of dNTP, 2 mM $MgCl_2$, and 0.8 μM primer) for each chain of the TCR, using nested primers for: Vα11, 5′-AATCTGCAGTGGGTGCAGATTTGCTGG-3′ and 5′-GAGTCAAAGTCGGTGAACAGG-3′; Vβ3, 5′-AATCTGCAGA ATTCAAAAGTCATTCA-3′ and 5′-AATCTGCAGCACGAGGGTAGC CTTTTG-3′. Nested PCR product (7 μl) was run on a 1.5% agarose gel to screen for positives (single bands of the right size). The PCR product was then directly sequenced using an ABI 373 sequencing system.

T cell proliferation assay

Total lymph node cells (5×10^4/well) from (B10.A × B10.BR)F_1 mice 10 days after immunization with PCC were cultured with PCC (1 μM) and varying numbers of activated $CD4^+CD25^+D^{k-}V\alpha11^+V\beta3^+CD62L^{low}CD44^{high}$ T cells from PCC-immunized (B10.A × B10.BR)F_1 mice reconstituted with $CD4^+CD25^+$ T cells from bone marrow chimeric mice or naive $CD4^+CD25^+D^{k-}V\alpha11^+V\beta3^+CD62L^{high}CD44^{low}$ T cells from (B10.A × B10.BR)F_1 mice reconstituted with $CD4^+CD25^+$ T cells from bone marrow chimeric mice (B10.A bone marrow into irradiated B10.A × B10.BR mice). The 1 μCi/well [^{3}H]thymidine was pulsed during the final 8 of 72-h culture. [^{3}H]Thymidine incorporation was evaluated using an automated beta liquid scintillation counter.

Results

PCC immunization protocol

The PCC immunization protocol (24) was used to examine Ag-specific $CD4^+$ T cells as this system allowed us to analyze the TCR-Vα and TCR-Vβ CDR3 sequences critical for binding PCC presented by IE^k. The major PCC-responding $CD4^+$ T cell population expresses TCR-Vα11 and TCR-Vβ3 (24). We first examined the relative number of $TCRV\alpha11^+V\beta3^+$ cells in T_{conv} and

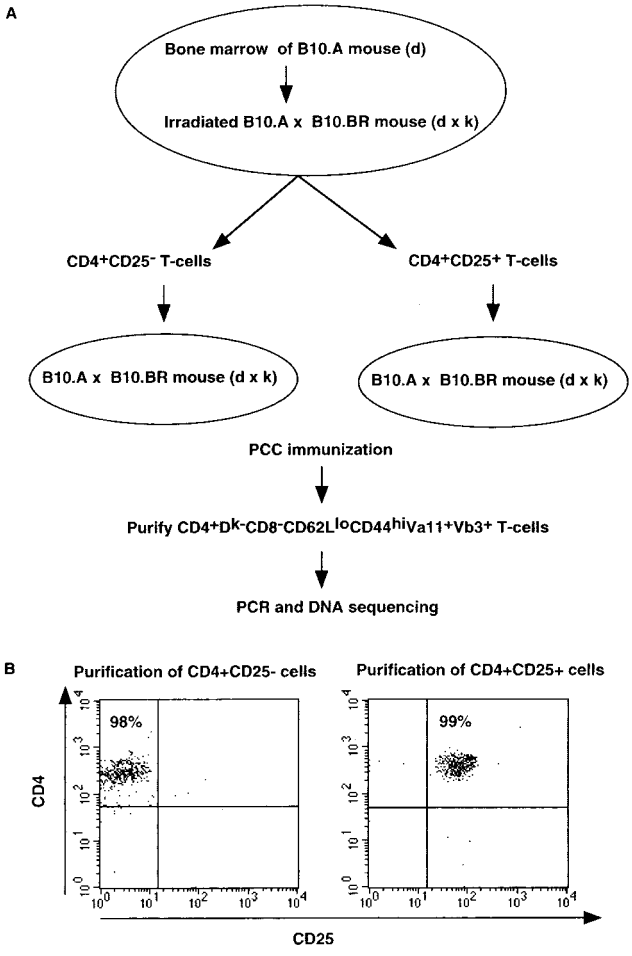

FIGURE 1. Scheme of experimental system. *A*, Irradiated (B10.A × B10.BR)F_1 mice were reconstituted with bone marrow from B10.A mice, and donor-derived T_{conv} or T_{reg} collected and purified 2 mo after reconstitution as described in *Materials and Methods*. T_{reg} from chimeric mice had T cell suppressive activity comparable to T_{reg} from B10.A mice (data not shown). Each cell population was then transferred into nonirradiated (B10.A × B10.BR)F_1 mice, and 2 days later mice immunized with PCC emulsified in CFA. Several days or weeks after PCC immunization, single donor-derived activated ($CD4^+CD8^-B220^-CD11b^-V\alpha11^+V\beta3^+D^{k-}CD62L^{low}CD44^{high}$) cells were sorted from lymph nodes by flow cytometry and CDR3 sequences evaluated by PCR followed by DNA sequencing. *B*, After purification of $CD4^+CD25^-$ (*left panel*) or $CD4^+CD25^+$ (*right panel*) T cells as described in *Materials and Methods*, cells were stained by PE-conjugated anti-CD4 and FITC-conjugated anti-CD25 mAbs (7D4) and evaluated by flow cytometry.

CD4+CD25- T-cells

TCRVα11 position 93

	primary							memory			
E	32	31	41	41	40	42		42	43	43	43
%	52	60	87	95	95	98		98	100	100	100
L	6	5	1								
V	1	1									
A											
D	3	2									
G	5	2	4	2							
S	7	8	1		2	1		1			
T											
H	2										
Q	1										
N	1	1									
R											
Y	1										
P	3	2									
Day	0	4	8	10	14	18		0	4	8	10
n	62	52	47	43	42	43		43	43	43	43

CD4+CD25+ T-cells

TCRVα11 position 93

	primary							memory			
E	24	23	21	26	30	31		25	25	25	22
%	57	56	50	62	71	74		60	60	60	52
L	8	6	7	1							
V	3	3	2								
A											
D	4	3	2								
G	3	4	4	6	2	1		2	2	2	5
S			6	9	7	7		12	12	12	14
T											
H		1									
N					3	3		2	2	2	1
Day	0	4	8	10	14	18		0	4	8	10
n	42	40	42	42	42	42		42	42	42	42

TCRVα11 position 95

	primary							memory			
S	22	23	36	37	38	39		39	42	42	43
%	35	44	77	86	90	91		91	98	98	100
A	11	9	2	1		2		2			
G	14	10	6	2	2	2		1	1		
N	13	10	3	2	2						
Y	1										
E	1										
F				1							
D								1	1	1	
Day	0	4	8	10	14	18		0	4	8	10
n	62	52	47	43	42	43		43	43	43	43

TCRVα11 position 95

	primary							memory			
S	12	23	23	32	31	30		28	26	26	23
%	28	58	55	76	74	71		67	62	62	55
A	12	3		2		7		6	8	8	6
G	9	6	9	5	5	4		7	7	7	11
N	9	5	7	1	2						
Y											
E	1	3	3	1	2						
F				1	1						
D											1
H						1		1	1	1	1
Day	0	4	8	10	14	18		0	4	8	10
n	43	40	42	42	42	42		42	42	42	42

TCRVα11 CDR3 length

	primary							memory			
8	45	41	40	40	41	41		42	42	42	43
%	73	79	85	93	98	95		98	98	98	100
9	9	5	4	1	1	2					
7	6	5	3	2				1	1	1	
6	2	1									
Day	0	4	8	10	14	18		0	4	8	10
n	62	52	47	43	42	43		43	43	43	43

TCRVα11 CDR3 length

	primary							memory			
8	30	31	35	37	34	37		39	39	37	37
%	70	78	83	88	81	88		93	93	88	88
9	7	3	4	2	4	2		2	2	2	3
7	6	5	3	2	4	3		1	1	3	2
6		1		1							
Day	0	4	8	10	14	18		0	4	8	10
n	43	40	42	42	42	42		42	42	42	42

Jα usage

	primary							memory			
16	3	4	5	6	9	8		6	7	7	7
22	4	4	7	4	7	7		6	4	10	6
34	2	5	8	9	4	6		7	8	8	11
17	2	2	2	1	1	1		4	4	3	4
%	18	25	49	47	50	51		53	53	65	65
21	2	2	3	1	1	1		3	2	2	1
11	1	4	3	2	2	1		2	2	2	2
50	8	3	2	3	3	3		2	2	2	2
56	10	4	2	2	3	3		2	2	2	2
48	6	4	3	3	3	3		1	1	0	1
13	5	3	2	2	1	1		1	2	1	2
12	6	4	2	2	2	2		1	1	1	0
33	4	2	2	1	1	1		1	1	1	2
9	3	1	2	1	0	1		2	1	1	0
27	2	2	1	1	1	1		1	1	1	1
40	1	2	2	1	1	1		1	2	0	0
23	1	2	1	2	1	1		1	1	1	1
4	1	2	0	1	1	1		1	1	1	1
49	1	2	0	1	1	1		1	0	0	0
Day	0	4	8	10	14	18		0	4	8	10
n	62	52	47	43	42	43		43	43	43	43

Jα usage

	primary							memory			
16	2	3	4	4	3	4		4	3	4	4
22	3	4	3	4	5	2		4	4	3	3
34	2	3	3	2	4	4		4	4	2	2
17	1	2	2	2	1	2		0	1	3	3
%	19	30	29	29	31	29		29	29	29	29
21	2	2	2	1	2	1		3	2	3	3
11	1	2	1	2	2	1		2	2	4	4
50	7	3	2	4	5	6		6	5	6	7
56	8	1	7	6	6	6		5	6	3	6
48	3	4	3	3	3	3		1	2	2	1
13	2	3	2	2	1	1		1	2	1	2
12	4	3	2	2	2	2		3	1	1	1
33	1	2	3	1	1	2		1	1	1	2
9	1	1	2	3	2	1		2	1	1	0
27	1	1	3	1	1	1		2	1	2	1
40	1	1	2	1	2	2		1	2	0	0
23	1	1	1	2	1	1		1	2	1	1
4	1	2	0	1	2	2		1	1	1	1
49	1	2	0	1	1	1		1	2	0	0
Day	0	4	8	10	14	18		0	4	8	10
n	42	40	42	42	42	42		42	42	42	42

FIGURE 2. Ag-driven modification of preferred TCR-Vα11 CDR3 sequence parameters from PCC-specific TCR. The three most-preferred CDR3 sequences for TCR-Vα11 (position α93, position α95, CDR3 length, and Jα usage) observed in PCC-specific TCR are shown. Each number represents the number of cells with the indicated CDR3 parameter from a single cell analysis (after day 0), with the preferred CDR3 sequence parameters (E) presented at the *top* of each panel and the percentage of single cells expressing this characteristic listed in the next row. Data are shown as a progression over time (*left to right*) before injection (day 0), after primary immunization (days 4, 8, 10, 14, and 18), and after memory immunization (days 0, 4, 8, and 10). The second immunization was done 8 wk after the primary immunization. The number of sequences used in the analysis (*n*) is displayed below the days shown on the *x*-axis. The day 0 group for primary immunization includes Vα11Vβ3-expressing T cells before injection with resting $CD44^{low}CD62L^{high}$ phenotypes.

CD4+CD25- T-cells

TCRVβ3 position 100

	primary							memory			
N	**23**	**22**	**39**	**38**	**37**	**38**		**38**	**39**	**41**	**41**
%	37	42	83	88	87	88		88	90	95	95
G	24	14	3	3	2	2			4	2	2
S	3	8	3	2							
Q	1	1									
T	5	1									
D	1				3	3		5			
Y	1										
R	1	1									
H	1	1									
P	1										
F	1										
E		2									
M	1										
Day	0	4	8	10	14	18		0	4	8	10
n	62	52	47	43	42	43		43	43	43	43

TCRVβ3 position 102

	primary							memory			
A **G**	**15** **18**	**27** **12**	**40** **7**	**39** **4**	**37** **3**	**41** **2**		**40** **2**	**42** **1**	**43**	**43**
%	53	75	100	100	95	100		98	100	100	100
N	10	5									
S	5	8		2				1			
Q	4										
R	4										
V	1										
T	1										
Y	2										
D	1										
L	1										
P											
W											
H											
Day	0	4	8	10	14	18		0	4	8	10
n	62	52	47	43	42	43		43	43	43	43

TCRVβ3 CDR3 length

	primary							memory			
9	**51**	**47**	**44**	**41**	**40**	**42**		**42**	**43**	**41**	**42**
%	82	90	94	95	95	98		98	100	95	98
8	9	3	1	1		1		1		1	1
7	2	2									
10			2	1	2					1	
Day	0	4	8	10	14	18		0	4	8	10
n	62	52	47	43	42	43		43	43	43	43

Jβ usage

	primary							memory			
1.2 **2.5**	**11** **2**	**15** **7**	**27** **7**	**25** **8**	**24** **8**	**24** **8**		**21** **11**	**25** **12**	**26** **14**	**26** **15**
%	30	55	80	78	76	76		76	88	95	97
1.3	2	1	1	1	2	2		2	1		
2.3	4	2	1	1	1	1		1			
1.4	4	2	1	2	2	1		1	2		
2.1	3	3	1								
2.7	8	3	2	2	3	3		2	1	1	1
2.4	6	4	2	2	2	3		3	1	1	
1.1	2	2		1							
2.2	1	1									
Day	0	4	8	10	14	18		0	4	8	10
n	43	40	42	42	42	42		42	42	42	42

CD4+CD25+ T-cells

TCRVβ3 position 100

	primary							memory			
N	**19**	**16**	**24**	**36**	**29**	**33**		**31**	**32**	**30**	**31**
%	44	40	57	86	69	79		74	76	71	74
G	15	18	11	3	5	1					
S	1	1	2	3	8	8		11	10	12	11
Q	1										
T	3	1									
D			1								
Y		1									
R	1	3									
H	3										
P											
F			4								
E											
M											
Day	0	4	8	10	14	18		0	4	8	10
n	43	40	42	42	42	42		42	42	42	42

TCRVβ3 position 102

	primary							memory			
A **G**	**10** **14**	**7** **18**	**20** **13**	**33** **3**	**33** **6**	**32** **9**		**29** **8**	**24** **15**	**30** **10**	**32** **10**
%	56	63	79	86	93	98		88	93	95	100
N	4	6	3						1	1	
S	3	1	4	2							
Q	4		1								
R	3	5	1								
V											
T	1										
Y	2										
D	1										
L	1										
P		1									
W		1									
H				4	3	1		5	2	1	
Day	0	4	8	10	14	18		0	4	8	10
n	43	40	42	42	42	42		42	42	42	42

TCRVβ3 CDR3 length

	primary							memory			
9	**35**	**28**	**35**	**38**	**33**	**36**		**38**	**37**	**35**	**36**
%	81	70	83	90	79	86		90	88	83	86
8	7	4	3	2	5	3		2	3	3	2
7		3	1								
6	1										
10		5	3	2	4	3		2	2	4	4
Day	0	4	8	10	14	18		0	4	8	10
n	43	40	42	42	42	42		42	42	42	42

Jβ usage

	primary							memory			
1.2 **2.5**	**12** **1**	**12** **2**	**14** **3**	**16** **5**	**16** **5**	**14** **6**		**15** **4**	**16** **6**	**16** **5**	**14** **5**
%	30	35	40	50	50	48		45	52	50	45
1.3	2	2	2	2	2	3		2	2	2	2
2.3	3	2	3	2	1	2		3	2	3	2
1.4	4	2	1	1	2	2		3	2	2	2
2.1	3	3	3	4	3	3		3	3	2	3
2.7	7	6	7	5	6	5		6	4	5	6
2.4	8	6	6	5	5	4		4	5	5	5
1.1	1	2	2	2	2	3		2	2	2	2
2.2	2	1	1					1			1
Day	0	4	8	10	14	18		0	4	8	10
n	43	40	42	42	42	42		42	42	42	42

FIGURE 3. Ag-driven modification of preferred TCR-Vβ3 CDR3 sequences of PCC-specific TCR. The TCR-Vβ3 CDR3 regions of PCC-specific TCR were sequenced as in Fig. 2. The three most-preferred TCR-Vβ3 CDR3 (position β100, position β102, CDR3 length, and Vβ usage) sequences are shown. Each number represents the number of cells having the indicated CDR3 characteristic after single cell analysis (from day 0), with data organized as in Fig. 1. The day 0 primary immunization group includes Vα11Vβ3-expressing T cells before injection with a resting CD44^{low}CD62L^{high} phenotype.

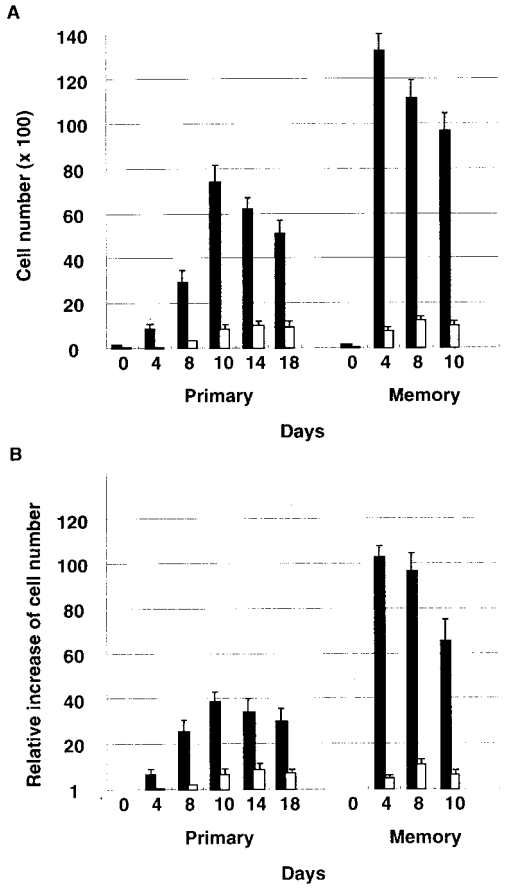

FIGURE 4. Frequencies of TCR Vα11Vβ3-expressing PCC-specific T cells. *A*, The total number of Ag-specific cells ($CD8^-V\alpha11^+V\beta3^+CD62L^{low}D^{k-}$) from the lymph nodes and spleen of each animal was calculated. After live cell counts by trypan blue staining, spleen and lymph node cells were stained with PE-conjugated anti-TCRVα11, biotin-conjugated anti-TCR-Vβ3, allophycocyanin-conjugated anti-CD62L, FITC-conjugated anti-D^k, or FITC-conjugated anti-CD8 mAbs followed by streptavidin-CyChrome. The percentage of $TCRV\alpha11^+TCRV\beta3^+CD62L^{low}$ cells gated in $CD8^-D^{k-}$ were evaluated by flow cytometry. Ag-specific T cell numbers were calculated by multiplying total live cell numbers by the relative proportion of activated T cells. Total cell counts on the *y*-axis represents the mean of six animals ± SEM. Total cell counts of T_{conv} (■) and T_{reg} (□) are shown. There was no significant difference in the adjuvant-only response across different days (data not shown). *B*, Relative increases in Ag-specific cell counts against day 0 for primary or secondary Ag challenge were calculated. The *y*-axis represents the mean of six relative cell counts per animal ± SEM. Total cell counts of T_{reg} (□) and T_{conv} (■) are shown.

T_{reg}. Both T_{conv} and T_{reg} contained ~1% $TCRV\alpha11^+V\beta3^+$ cells (data not shown). Because CD25, the IL-2R α-chain, can be upregulated following T_{conv} stimulation (6), we established the following experimental system to clearly discriminate between T_{reg} and activated T_{conv} (Fig. 1*A*). Although both B10.A and B10.BR mice express the MHC class II k haplotype, B10.A and B10.BR express the MHC class I^d and I^k haplotypes, respectively. Irradiated (B10.A × B10.BR)F_1 mice were reconstituted with bone marrow from B10.A mice to remove donor-derived $CD4^+$ T cells reactive against B10.BR-derived Ags (Fig. 1*A*). Donor-derived $CD4^+CD25^+D^{k-}$ (T_{reg}) or $CD4^+CD25^-D^{k-}$ (T_{conv}) cells were then purified as described in *Materials and Methods* (Fig. 1*B*) and transferred into nonirradiated (B10.A × B10.BR)F_1 mice (designated $CD25^+$ chimeric mice or $CD25^-$ chimeric mice, respectively) (Fig. 1*A*). Using this protocol, donor- and host-derived cells could be discriminated according to MHC class I haplotype expression.

After immunization of the chimeric mice with PCC emulsified in CFA, single donor-derived activated cells ($CD4^+CD8^-B220^-CD11b^-V\alpha11^+V\beta3^+D^{k-}CD62L^{low}CD44^{high}$) from lymph nodes were sorted by flow cytometry. The CDR3 regions of each cell were then amplified by PCR and sequenced as previously described (24). In parallel, total donor-derived activated cell ($CD8^-D^{k-}V\alpha11^+V\beta3^+D^{k-}CD62L^{low}$) numbers from the spleen and lymph nodes were counted as described in *Materials and Methods*. Both the primary and memory responses were examined, we determined eight CDR3 sequence parameters from TCR-Vα11 and TCR-Vβ3 of PCC-specific T cells (Figs. 2 and 3) (24), and measured total activated cell numbers (Fig. 4). The eight CDR3 sequence parameters from the PCC-specific T cells were CDR3 length, DNA sequence at positions 93 and 95, and Jα usage for TCR-Vα11 (Fig. 2), and CDR3 length, DNA sequence at positions 100 and 102 and Jβ usage for TCR-Vβ3 (Fig. 3). Figs. 2 and 3 summarize the results of one representative experiment and show CDR3 sequence information for TCR-Vα11 and TCR-Vβ3 chains from T cells obtained after PCC immunization. A summary of the combined data obtained from individual animals is shown in Fig. 5.

T_{reg} proliferation after primary and secondary PCC immunization

T_{reg} and T_{conv} had similar frequencies of TCR-Vα11 and TCR-Vβ3 CDR3 sequence parameters before PCC immunization (Figs. 2 and 3), which suggested that both populations had similar TCR repertoires in terms of recognition of PCC presented by IE^k. PCC immunization led to cell proliferation of both T_{conv} and T_{reg} after both primary and memory responses (Fig. 4*A*). However, the degree of proliferation of activated cells was lower and the amount of time to reach the peak day was longer for T_{reg} than for T_{conv} after primary immunization (Fig. 4*B*). For the memory response, peak proliferation occurred earlier for both T_{reg} and T_{conv} (Fig. 4*B*). These findings indicated that polyclonal T_{reg} proliferated in response to Ag immunization with adjuvant in vivo, although these findings are in contrast with a previous report of T_{reg} anergy after stimulation via TCR ligation in vitro and in vivo (25, 26).

TCR repertoire modification after primary PCC immunization

We next analyzed the changes in TCR-Vα11 and TCR-Vβ3 CDR3 sequence parameters using lymph node T cells obtained after primary and memory responses. All preferred TCR-Vα11 or TCR-Vβ3 CDR3 sequence parameters (TCR-Vα11 positions 93 and 95, CDR length, and Jα; TCR-Vβ3 positions 100 and 102, CDR length, and Jβ) from PCC-specific T_{conv} changed during the primary immune response (Figs. 2 and 3). The percentage of PCC-specific T_{conv} with preferred TCR-Vα11 or TCR-Vβ3 CDR3 sequence parameters of TCR-Vα11 positions 93 and 95, TCR-Vα11 CDR length (Fig. 2), TCR-Vβ3 positions 100 and 102, TCR-Vβ3 CDR length and Jβ (Fig. 3) exceeded 90% during the primary response. However, the percentage of T_{conv} with preferred Jα usage had increased to only 51% (Fig. 2). Using data from Figs. 2 and 3, the percentage of T_{conv} having six or more characteristic PCC-specific TCR CDR3 sequence parameters was calculated (Fig. 5*A*). Approximately 70% of PCC-specific T_{conv} had at least six preferred TCR-Vα11 or TCR-Vβ3 CDR3 sequence parameters 10 days after primary immunization (Fig. 5*A*).

The pattern of TCR-Vα11 positions 93 and 95, TCR-Vα11 CDR length, and Jα usage (Fig. 2), and TCR-Vβ3 positions 100 and 102, TCR-Vβ3 CDR length, and Jβ (Fig. 3) was also modified in T_{reg} at 18 days after the first immunization, but to a lesser extent compared with T_{conv} (Figs. 2 and 3), except for TCR-Vβ3 position

A CD4+CD25- T-cells

Prefered features	Resting	Day4	Day 10	Day 18	memory
8	0	6	21	23	29
7	0	2	3	3	7
6	0	7	3	1	2
% of >6 features	0	29	65	63	88
5	1	3	5	4	3
4	6	2	4	3	1
3	15	3	4	3	1
2	20	2	1	4	0
1	12	9	1	1	0
0	8	4	1	1	0
n	62	52	43	43	43

B CD4+CD25+ T-cells

Prefered features	Resting	Day4	Day 10	Day 18	memory
8	0	1	4	5	6
7	0	2	5	5	5
6	0	2	3	3	2
% of >6 features	0	13	29	30	30
5	1	3	3	2	3
4	4	10	25	23	21
3	8	4	1	2	3
2	11	2	0	1	2
1	10	7	0	1	0
0	8	7	1	0	0
n	42	40	42	42	42

FIGURE 5. Ag-driven modification of preferred CDR3 sequences in both TCR chains. Each number represents the number of cells with the preferred TCR-Vα11 and TCR-β3 CDR3 sequences after primary and secondary (8 days after secondary immunization) immunization for $CD4^+CD25^-$ (*A*) and $CD4^+CD25^+$ (*B*) T cells.

102 (Fig. 3). Further analysis showed that only 30% of PCC-specific T_{reg} had six or more preferred of TCR-Vα11 or TCR-Vβ3 CDR3 sequence parameters at day 18 (Fig. 5*B*).

TCR repertoire modification after secondary PCC immunization

We next examined whether the TCR repertoire altered during the memory response. PCC was administered 8 wk after the primary immunization and CDR3 sequences from T cells obtained (Figs. 2 and 3). Results showed that the PCC-specific T_{conv} TCR repertoire was further modified during the memory response in terms of preferred TCR-Vα11 or TCR-Vβ3 CDR3 sequence parameters, including TCR-Vα11 positions 93 and 95, TCR-Vα11 CDR length, and Jα usage (Fig. 2), and TCR-Vβ3 positions 100 and 102, TCR-Vβ3 CDR length, and Jβ usage (Fig. 3). However, the percentage of T_{conv} showing the preferred Jα usage was ~60% (Fig. 2) compared with nearly 100% for the other CDR3 sequence parameters (Figs. 2 and 3). This further modification in T_{conv} reflected the fact that 90% of the cells had acquired six or more of the preferred PCC-specific TCR-Vα11 or TCR-Vβ3 CDR3 sequences during the memory response (Fig. 5*A*). In contrast, whereas the second immunization increased T_{reg} cell numbers (Fig. 4*A*), further modification of TCRα (Fig. 2) and TCRβ (Fig. 3) repertoire was not observed (Fig. 5*B*). Because >88% of T_{reg} before secondary immunization had three preferred CDR3 sequence parameters (TCR-Vα11 CDR length, TCR-Vβ3 position 102, and TCR-Vβ3 CDR length) (Figs. 2 and 3), it was difficult to determine small changes in these parameters after secondary immunization. However, significant differences in the frequencies of preferred TCR-Vα11 positions 93 and 95, Jα usage (Fig. 2), TCR-Vβ3 position 100 and Jβ usage (Fig. 3) were observed after secondary responses for both T_{conv} and T_{reg}. The relative percentages of these five preferred CDR3 sequence parameters did not change in T_{reg} after secondary immunization (Figs. 2 and 3). Similar results were observed in terms of TCR repertoire modification both of T_{conv} and T_{reg} when spleen cells were used instead of lymph node cells (data not shown).

Low level contamination of T_{conv} in the T_{reg} fraction did not greatly affect the repertoire modification of T_{reg}

To negate the possibility that T_{reg} proliferation and repertoire modification reflected the proliferation of low numbers of contaminating T_{conv}, we used a mixture of 99% T_{reg} and 1% T_{conv} or 95% T_{reg} and 5% T_{conv} instead of purified T_{reg} in our proliferation (Fig. 6) and repertoire modification (Fig. 7) assays, and compared the results with those obtained using purified T_{reg} (Figs. 4 and 5). Proliferation and repertoire modification assay results were similar between the 1% and 5% T_{conv} contamination samples, which were in turn similar to results obtained using purified T_{reg} (Figs. 5, 6, and 7). These results suggested that low levels of T_{conv} contamination in the T_{reg} fraction would not be expected to have a significant impact on T_{reg} proliferation and repertoire modification.

Impact of incomplete T_{reg} TCR repertoire modification on the total immune response

To examine the impact of incomplete T_{reg} repertoire modification on effector T cell generation, we first evaluated the repertoire modification of lymph node $CD4^+CD25^-$ cells from PCC-immunized and $CD25^+$-depleted (B10.A × B10.BR)F_1 mice using anti-CD25 mAb. The kinetics of TCR repertoire modification in activated $CD4^+CD25^-V\alpha11^+V\beta3^+CD62L^{low}CD44^{high}$ cells in response to PCC immunization was similar to that of $CD4^+CD25^-$ donor cells ($CD4^+CD25^{+/-}V\alpha11^+V\beta3^+D^{k-}CD62L^{low}CD44^{high}$) from $CD25^-$ chimeric mice (data not shown). For this reason we used (B10.A × B10.BR)F_1 mice instead of $CD25^+$ or $CD25^-$ chimeric mice to evaluate the impact of low T_{reg} repertoire modification on effector T cell activation or differentiation.

Following primary PCC immunization, (B10.A × B10.BR)F_1 mice that had been T_{reg} depleted using anti-CD25 mAb generated higher Ag-specific T cell numbers in the lymph nodes and spleen compared with non-T_{reg} depleted (B10.A × B10.BR)F_1 mice (Fig. 8*A*). The kinetics of lymph node PCC-activated T cell TCR repertoire modification was also faster in the absence of T_{reg} after the

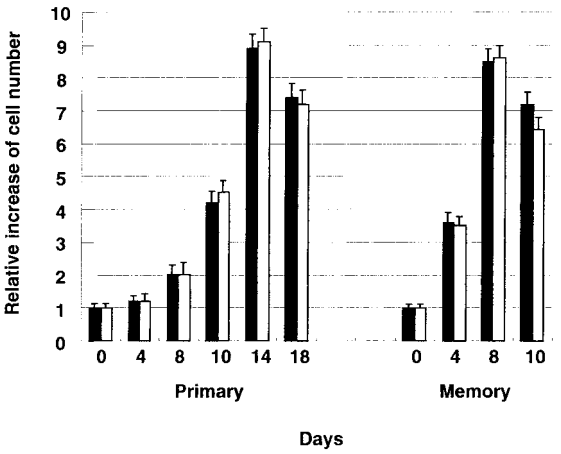

FIGURE 6. Frequencies of TCR Vα11Vβ3-expressing PCC-specific T cells. Irradiated (B10.A × B10.BR)F_1 mice were reconstituted with bone marrow from B10.A mice, and donor-derived T_{conv} or T_{reg} collected and purified 2 mo after reconstitution as described in *Materials and Methods*. Mixtures of 1% T_{conv} and 99% T_{reg} (□) or 5% T_{conv} and 95% T_{reg} (■) were transferred into nonirradiated (B10.A × B10.BR)F_1 mice, which were then immunized 2 days later with PCC emulsified in CFA. Several days or weeks after PCC immunization, donor-derived activated single cells ($CD4^+CD8^-B220^-CD11b^-V\alpha11^+V\beta3^+D^{k-}CD62L^{low}CD44^{high}$) were sorted from lymph nodes and CDR3 sequences evaluated by PCR followed by DNA sequencing. Total Ag-specific cell ($CD4^+CD8^-V\alpha11^+V\beta3^+CD44^{high}CD62L^{low}D^{k-}$) numbers from the lymph nodes and spleen of each animal were calculated. B220, CD8, and D^k-positive cells were removed by anti-rat IgG-coated microbeads and counted by trypan blue staining. Relative $V\alpha11^+V\beta3^+CD44^{high}CD62L^{low}$ cell numbers were evaluated by staining with anti-TCR Vα11, TCR-Vβ3, CD44, and CD62L mAbs. Relative increases in Ag-specific cell counts against day 0 for primary or secondary Ag challenges were calculated. The *y*-axis represents the mean of six relative cell counts of animals ± SEM.

primary immune response (Fig. 8*C*). To evaluate the effect of T_{reg} on memory T cell responses, CD25-positive cells were depleted by anti-CD25 mAb treatment in PCC immunized (B10.A × B10.BR)F_1 mice 8 wk after primary immunization. Mice were then reimmunized with PCC and the TCR repertoire and total cell numbers of $CD4^+TCRV\alpha11^+V\beta3^+CD44^{high}CD62L^{low}$ T cells evaluated. Depletion of T_{reg} before secondary immunization induced a faster TCR repertoire modification (Fig. 8*D*), but did not affect PCC-specific T cell numbers (Fig. 8*B*).

We then examined whether the inability to inhibit T_{reg} proliferation during the memory response was due to limited T_{reg} expansion or repertoire modification, or to the intrinsic failure of effector/memory T cells to respond to T_{reg} inhibitory activity. Donor-derived PCC-activated T_{reg} were purified from PCC-immunized $CD25^+$ chimeric mice as described in *Materials and Methods*. Purified T_{reg} were then transferred into PCC-immunized (B10.A × B10.BR)F_1 mice 2 days before a second PCC immunization. The addition of purified PCC-activated T_{reg} before the second immunization inhibited both cell proliferation (Fig. 8*B*) and TCR repertoire modification (Fig. 8*D*). However, naive $TCRV\alpha11^+V\beta3^+$ T_{reg} cells from $CD25^+$ chimeric mice failed to affect proliferation (Fig. 8*B*) and TCR repertoire modification (Fig. 8*D*). These findings suggested that limited expansion or repertoire modification of PCC-activated T_{reg} after secondary immunization was one of the factors that prevented the inhibition of effector T cell proliferation. This PCC-specific inhibitory ability of T_{reg} was confirmed by in vitro experiments. Activated $CD4^+CD25^+D^{k-}V\alpha11^+V\beta3^+CD62L^{low}CD44^{high}$ T cells were purified from PCC-immunized $CD25^+$ chimeric mice as described in *Materials and Methods*. Naive $CD4^+CD25^+D^{k-}V\alpha11^+V\beta3^+CD62L^{high}CD44^{low}$ T cells from $CD25^+$ chimeric mice were used as a control. The activated or naive T cells were cocultured with total lymph node cells from PCC-immunized (B10.A × B10.BR)F_1 mice in the presence of PCC. The proliferative responses of PCC-specific T cells were inhibited by increasing numbers of activated non-naive regulatory T cells but not control cells (Fig. 8*E*).

Discussion

The adaptive immune response is dependent on the recognition of Ag peptides presented in the context of self-encoded MHC molecules by specific T cells. A broad preimmune TCR repertoire is established via processes involving positive and negative selection on developing T cells in the thymus (4). The particular T cell repertoire that responds to a given Ag is usually quite diverse in terms of TCR β-chain V region usage and fine epitope specificity (18, 19). However, in some cases clonal dominance prevails and T cells with preferred TCR motifs are selectively expanded during primary responses and then appear to be selectively preserved for memory responses (16, 24). Although these studies have tended to focus on the repertoire of effector T cells, the total T cell immune response appears to be regulated by the interplay between effector and suppressor T cells. Therefore, to gain a better understanding of the total adaptive immune response, we attempted to clarify the mode of repertoire modification and proliferation of suppressor/regulatory T cells against a given Ag. Our study revealed an evolving T_{reg} clonal dominance during the primary immune response. Although T_{conv} were less affected, T_{reg} clonal modification was not observed during the memory response. Experiments using $CD25^+$

A

$CD4^+CD25^-$ T cells (1%)
$CD4^+CD25^+$ T cells (99%)

Prefered features	Resting	Day4	Day 10	Day 18	memory
8	0	1	5	5	7
7	0	1	4	4	4
6	0	2	3	2	2
% of >6 features	0	11	28	29	33
5	2	3	4	4	3
4	5	8	22	20	19
3	13	4	2	3	3
2	11	2	1	1	0
1	13	9	1	1	1
0	8	8	1	1	0
n	52	38	43	41	39

B

$CD4^+CD25^-$ T cells (5%)
$CD4^+CD25^+$ T cells (95%)

Prefered features	Resting	Day4	Day 10	Day 18	memory
8	0	1	4	5	5
7	0	2	4	4	6
6	0	2	3	3	2
% of >6 features	0	13	26	29	31
5	1	3	3	4	3
4	2	10	26	21	20
3	10	3	1	2	3
2	10	2	0	1	2
1	9	8	1	1	1
0	8	7	0	0	0
n	40	40	42	42	42

FIGURE 7. Ag-driven modification of preferred CDR3 sequences in both TCR chains. Each number represents the number of cells with the indicated CDR3 sequences from both TCR-Vα11 and TCR-β3 in PCC-immunized mice as in Fig. 1. The *y*-axis represents the number (*n*) of preferred CDR3 sequences seen when mice were given a mixture of 1% T_{conv} and 99% T_{reg} (*A*) or a mixture of 5% T_{conv} and 95% T_{reg} (*B*). Cells with more than four preferred CDR3 sequence parameters were considered to have a restricted TCR, and the percentage of these cells is shown. Sequence information from memory response cells (8 days after immunization) is displayed. The number of analyzed sequences (*n*) is displayed on the *x*-axis.

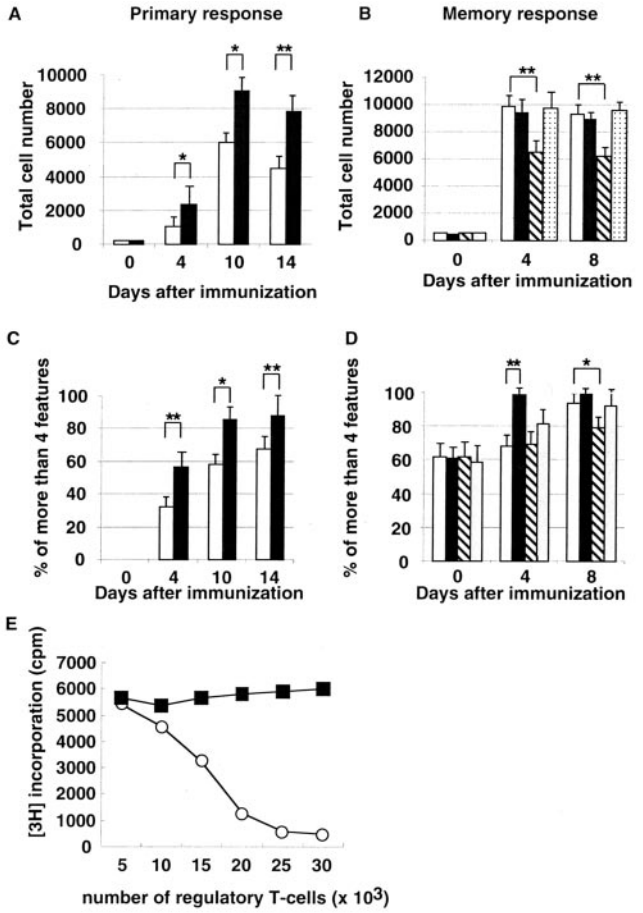

FIGURE 8. Regulation of TCR repertoire modification and T cell proliferation by $CD25^+$ regulatory T cells. Total Ag-specific T cell counts and the percentage of cells with more than four specific CDR3 sequence parameters of PCC-specific TCR during primary (*left panel*) and memory (*right panel*) responses are shown. For primary immunization, (B10.A × B10.BR)F_1 mice pretreated with (■) or without (□) anti-CD25 mAb were immunized with PCC in CFA and cell counts of Ag-specific T cells ($CD4^+CD25^-V\alpha11^+V\beta3^+CD62L^{low}CD44^{high}$) in spleen and lymph nodes (*A*) and lymph node TCR repertoires (*C*) evaluated as in Fig. 1. For the memory response (2 mo after primary immunization), cell counts of Ag-specific T cells ($CD4^+CD25^-V\alpha11^+V\beta3^+CD62L^{low}CD44^{high}$) in spleen and lymph nodes (*B*) and lymph node TCR repertoires (*D*) were evaluated several days after immunization. For the memory response, primary immunized (B10.A × B10.BR)F_1 mice were immunized with PCC in CFA with (■) or without (□) anti-CD25 mAb pretreatment (2 days before the second immunization). In some experiments, Ag-specific (▨) or naive $CD4^+CD25^+V\alpha11^+V\beta3^+$ T cells (▨) were transferred into mice before the second immunization. Ag-specific $CD4^+CD25^+$ T cells ($CD4^+CD25^+D^{k-}V\alpha11^+V\beta3^+CD62L^{low}CD44^{high}$) were obtained from donor-derived T cells (1×10^6) 14 days after primary immunization of $CD25^+$ chimeric mice as in Fig. 2. *, $p < 0.05$; **, $p < 0.01$ are indicated. *E*, Total lymph node cells (5×10^4) from (B10.A × B10.BR)F_1 mice immunized by PCC were incubated with PCC, with the indicated numbers of PCC-activated $CD4^+CD25^+D^{k-}V\alpha11^+V\beta3^+CD62L^{low}CD44^{high}$ T cells purified from PCC-immunized $CD25^+$ chimeric mice (○) or naive $CD4^+CD25^+D^{k-}V\alpha11^+V\beta3^+CD62L^{high}CD44^{low}$ T cells from $CD25^+$ chimeric mice (■) are shown. [^{3}H]Thymidine incorporation was evaluated during the final 8 h of the 72 h culture. Results of the proliferation assays are shown as the mean of triplicate cultures.

cell depletion showed that removing T_{reg} against a given Ag-suppressed TCR repertoire modification of the T_{conv}-derived primary and memory T cells in vivo. In contrast, T_{reg} inhibited the proliferation of Ag-specific T_{conv} during the primary, but not the memory response. Nonetheless, addition of PCC-activated T_{reg} still inhibited memory T cell proliferation.

T_{reg} are thought to express high affinity TCRs against self-peptides (27) and suppress effector T cell responses independent of Ag specificity, at least in vitro (28). These findings suggest a role for self-Ag recognition in the acquisition of T_{reg} suppressive function. Although T_{reg} have very broad TCR Vβ repertoires, similar to T_{conv} (data not shown), it remains unclear whether T_{reg} actually recognize non-self Ags to acquire suppressive functions in vivo. Recently, several papers have shown that T_{reg} suppress murine $CD4^+$ and $CD8^+$ T cell responses against parasitic or bacterial infections in vivo (29, 30). This suggests two possibilities regarding the Ag specificity of T_{reg} in vivo. First, T_{reg} may recognize parasite- or bacteria-derived broad Ags, acquire a suppressive function, and then regulate/suppress effector T cell responses. Second, infections may change the sensitivity of T_{reg} TCR signaling via local cytokine bursts or interaction with other activated cells, which then modifies the TCR signaling threshold of T_{reg} to allow sufficient response to self-Ags with resultant acquisition of suppressive functions. In this regard, our results indicated that the frequency of preferred TCR-Vα11 or TCR-Vβ3 CDR3 sequence parameters of PCC-specific T cells were increased in T_{reg} after PCC immunization, which strongly suggested the direct recognition of PCC by T_{reg}. In addition, the repertoire modification of T_{reg} increased the total suppressive function of T_{reg} toward effector/memory T cell responses against PCC, which demonstrated that T_{reg} can suppress effector T cells via recognition of a specific common Ag in vivo. These findings suggested that the regulation of Ag-specific T_{reg}, rather than total T_{reg}, may be required for the clinical application of T_{reg}, in agreement with a number of groups that have tried to regulate effector T cells in an Ag-dependent manner (31).

Previous studies using TCR-transgenic mice have shown that T_{reg} are anergic in vitro, but have the potential to respond to lymphopenic conditions (25, 26). Recently, it was shown that T_{reg} proliferated in vivo (32). Under more physiologic conditions, as in our experimental protocol, polyclonal T_{reg} responded to PCC immunization and proliferated in vivo, although the response was less than the T_{conv} response. One might argue that the T_{reg} proliferation we observed might have at least partly reflected the proliferation of contaminating T_{conv} as CD25 is not an exclusive T_{reg} marker. However, this is unlikely because the TCR repertoire of all $CD25^+$-derived cells was examined 18 days after the first immunization, which would reflect the presence of cell proliferation. We also performed experiments using a mixture of 99% T_{reg} and 1% T_{conv} or 95% T_{reg} and 5% T_{conv} instead of the purified T_{reg} population and obtained similar findings in terms of cell proliferation and repertoire modification. If the reduced TCR repertoire seen in T_{reg} is due to vigorous expansion of contaminated T_{conv}, we should have observed increased repertoire modification with increasing numbers of contaminating T_{conv}. However, the possibility that contaminating T_{conv} do contribute to T_{reg} result cannot be ruled out as we do not have cell surface markers able to discriminate between T_{reg} and activated T cells.

The $CD25^+$ cell depletion experiments showed that during the memory response, T_{reg} inhibited effector T cell TCR repertoire modification, but not T cell-mediated proliferation. However, the addition of PCC-activated T_{reg} during the memory response inhibited T cell proliferation. This suggested the inability to inhibit effector T cell proliferation was due to limited T_{reg} expansion or TCR repertoire modification after primary immunization rather than the intrinsic failure of effector T cells to respond to the inhibitory activity of T_{reg}. Although T_{reg} generally inhibit T cell proliferation, the precise regulatory mechanisms of T_{reg} remain

unknown, despite reports suggesting possible roles for TGF-β or CTLA-4 (33, 34). T_{reg} inhibition of T cell repertoire modification, but not proliferation, suggests another regulatory role of T_{reg} in the T_{conv} response. Furthermore, our findings provide evidence for a distinct regulatory mechanism linking T cell proliferation and TCR repertoire modification, although these have generally been thought to be coordinated processes.

We observed no differences in the frequencies of CDR3 features involved in PCC recognition between T_{reg} and T_{conv} before immunization. Nonetheless, it is still possible that T_{reg} and T_{conv} exhibited different TCR repertoires in terms of PCC recognition that might underlie the different proliferation and TCR repertoire modification kinetics. However, this seems unlikely as a similar frequency of PCC/IE^k-specific TCRVα11$^+$Vβ3$^+$ T cells (0.06–0.07%) was present in total TCRVα11$^+$Vβ3$^+$ T_{reg} and T_{conv} (data not shown).

The reduced ability of T_{reg} to suppress the proliferation of memory/effector T cells due to reduced Ag-specific T_{reg} proliferation or incomplete TCR repertoire modification during primary responses would be advantageous to the overall immune response as the coordinated parallel evolution of T_{reg} and T_{conv} would not be useful for augmenting the memory T cell response. This divergence may have occurred to maximize the ability of the organism to mount a protective memory immune response against bacteria, protozoans, fungi, and viruses.

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