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An affinity/avidity model of peripheral T cell regulation

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We show in these studies that Qa-1–dependent CD8+ T cells are involved in the establishment and maintenance of peripheral self tolerance as well as facilitating affinity maturation of CD4+ T cells responding to foreign antigen. We provide experimental evidence that the strategy used by the Qa-1–dependent CD8+ T cells to accomplish both these tasks in vivo is to selectively downregulate T cell clones that respond to both self and foreign antigens with intermediate, not high or low, affinity/avidity. Thus, the immune system evolved to regulate peripheral immunity using a unified mechanism that efficiently and effectively permits the system to safeguard peripheral self tolerance yet promote the capacity to deal with foreign invaders.

Introduction

The essential function of the immune system is to mount effective immune responses to virtually any foreign antigens but avoid harmful immune responses to self. How developing T cells maintain tolerance to self while achieving affinity maturation in response to foreign antigen in the periphery is one of the most important phenomena in immunology but is not yet completely understood. Nevertheless, many studies indicate that both central thymic and peripheral mechanisms are important. For example, thymocytes expressing TCRs with high affinity/avidity for MHC/ self peptide complexes undergo apoptosis and are deleted centrally in the thymus (1). However, some self-reactive T cells with intermediate affinity/avidity for self antigen escape thymic negative selection and are released into the periphery. Although these self-reactive T cells display lower affinity/avidity for MHC/self peptide complexes, they are capable of self peptide–driven proliferation, and some may differentiate into potentially pathogenic effector cells (2–4). It is clear that in order to avoid pathogenic autoimmunity, peripheral regulatory mechanisms are required to fine-tune the self-reactive TCR repertoire and limit the clonal expansion of these potentially pathogenic self-reactive clones with TCRs whose affinity/avidity is not high enough to cause their intrathymic elimination. These peripheral mechanisms are thought to include activation-induced apoptosis, cytokine interactions, and regulatory cells. We provide evidence here that one of the mechanisms for such peripheral regulation of the immune system involves Qa-1– dependent CD8+ T cells.

Previously, a pathway of immunoregulation mediated by the Qa-1– dependent CD8+ T cells was identified (5). Heightened interest in these regulatory CD8+ T cells arose in the early 1990s from studies of experimental allergic encephalomyelitis (EAE), which provided evidence that CD8+ T cells play a key role in both inducing resistance to autoimmune EAE and abrogating recurrent relapsing epi-

sodes of autoimmunity in vivo (6, 7). Regulatory CD8+ T cells were further isolated, and their capacity to specifically interact with and downregulate activated CD4+ T cells was found to be blocked by antibodies to CD8, to αβ TCR, and to the MHC class Ib molecule Qa-1 (8–10). These data suggested that regulatory CD8+ T cells specifically inhibit activated autologous CD4+ T cells via αβ TCR recognition of Qa-1/self peptide complexes expressed on activated CD4+ T cells. The direct in vivo molecular evidence that Qa-1 is in fact essential for the regulatory functions of CD8+ T cells in vivo has come from recent studies of Qa-1 KO mice showing that mice deficient in Qa-1 are impaired in their capacity to become resistant to the second induction of EAE (11).

Although we previously showed that CD8+ T cells protect mice from EAE by selectively downregulating some but not all MBP-reactive CD4+ T cells that are enriched in the potentially encephalitogenic clones in vivo (4), the precise targets that are selectively downregulated by the CD8+ T cells were not identified. Because T cell clones with higher outgrowth potential are likely to have higher affinity/avidity, the EAE data suggested that the affinity/avidity of T cell clones might somehow dictate their susceptibility to downregulation by the CD8+ T cells.

To test this hypothesis and further delineate the biological functions of the regulatory CD8+ T cells in controlling peripheral immunity to both self and foreign antigens, we have studied the CD8+ T cell regulation of immune responses to a conventional antigen, hen egg lysozyme (HEL), in BALB/c mice. Previously, HEL Tg mice have been generated in the BALB/c background and have been widely used to study mechanisms of self tolerance (12–17). Because HEL represents a foreign antigen in WT mice but a self antigen in HEL Tg mice, studying immune response to HEL in both animals permits direct delineation of the cellular and molecular mechanisms by which CD8+ T cells control the peripheral TCR repertoire to both self and foreign antigens. We provide evidence that the Qa-1–dependent CD8+ T cells participate in regulation of peripheral immunity by preferentially downregulating T cells of intermediate affinity/avidity to both self and foreign antigens. Thus, these regulatory cells play an important role in both the establishment and the maintenance of peripheral self tolerance to HEL in Tg mice and T cell affinity maturation to HEL in WT mice.
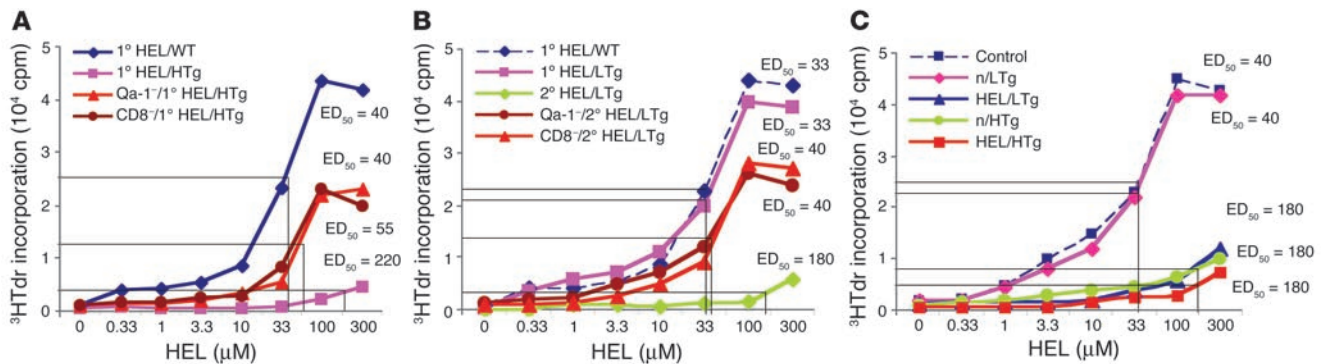
Figure 1

Qa-1–dependent CD8+ T cells are involved in the establishment and maintenance of peripheral self tolerance to HEL in HEL Tg mice. (**A**) The unresponsiveness to HEL in HEL high Tg mice could be broken by treatment with anti-CD8 and anti–Qa-1 mAbs. HEL immunization and in vivo mAb treatment were performed and CD4+ T cells were purified from pooled draining lymph node cells from different groups of mice and assayed in a T cell proliferation assay as described in Methods. Data are representative of 4 separate experiments with 2–4 mice per group. (**B**) CD8+ T cells regulate immune response to self antigen HEL in HEL low Tg mice. Experiments were performed as described in Methods. Data are representative of 6 separate experiments with 2–4 mice per group. (**C**) CD8+ T cells downregulate the primary immune responses to HEL in HEL low Tg mice when adoptively transferred. CD8+ T cells were injected i.v. into recipient mice, and the mice were immunized with HEL 1 day later. The CD4+ T cells were isolated from pooled lymph node cells of recipient mice 7–9 days after the immunization, and T cell proliferation assays were performed. Data are representative of 4 separate experiments with 2–4 mice per group. Control, no transfer; n/LTg, CD8+ T cells transferred from naive HEL low Tg mice; HEL/LTg, CD8+ T cells transferred from 2° HEL-immunized HEL low Tg mice; n/HTg, CD8+ T cells transferred from naive HEL high Tg mice; HEL/HTg, CD8+ T cells transferred from 1° HEL-immunized HEL high Tg mice. 3HTdr, 3H-thymidine.

Results

CD8+ T cells are involved in the control of peripheral self tolerance in HEL Tg mice. In Tg mice expressing HEL under the control of a ubiquitous promoter, HEL is present in the serum and thymus throughout the establishment of the T cell repertoire (12). There are 2 immunological T cell response phenotypes observed in HEL Tg mice, which are dictated by the serum level of HEL. High Tg mice (serum level greater than 10 ng/ml) are tolerant and there is little or no T cell proliferation in response to HEL, whereas, in low Tg mice (serum level less than 2 ng/ml), T cells do respond to HEL, in vitro, following the primary HEL immunization (12–14).

We first investigated the function of CD8+ T cells in the HEL high Tg mice. As shown in Figure 1A, the WT mice responded briskly to HEL, whereas the HEL high Tg mice treated with control Ig were tolerant to HEL. The conventional interpretation of this result is that the lack of response in the HEL high Tg mice simply reflects the deletion of HEL-reactive T cells intrathymically by negative selection. However, as shown, in mice that were depleted of CD8+ cells or treated with anti–Qa-1 mAb before the immunization, immune responses to HEL were detected, although at a lower magnitude than in the WT mice (Figure 1A). This indicates that even though thymic negative selection deletes the majority of self-reactive T cells with high affinity/avidity, some HEL-reactive T cells escape thymic negative selection and are released into the periphery, where they are controlled by peripheral regulatory mechanisms. Moreover, the data clearly suggest that Qa-1–dependent CD8+ T cells play a role in maintaining peripheral tolerance.

We then studied the primary (1° HEL) and the secondary (2° HEL) immune responses to HEL in vivo in HEL low Tg mice in the presence and absence of CD8+ T cells (CD8–/2° HEL) and in mice treated with anti–Qa-1 mAb (Qa-1–/2° HEL). As previously reported, unlike HEL high Tg mice, HEL low Tg mice do respond to HEL following the primary immunization (12–14). Interestingly, self tolerance to HEL in the HEL low Tg mice is reflected in the secondary immune response to HEL. As shown in Figure 1B, unlike the vigorous

response to the secondary HEL challenge in WT mice (see Figure 3A), the secondary HEL response in HEL low Tg mice mimicked the primary HEL response in HEL high Tg mice, in which virtually no T cell response was observed. Importantly, CD8+ T cell depletion or anti–Qa-1 mAb treatment reversed the low responsiveness to HEL in the mice that received secondary HEL immunization, as shown by increased T cell proliferation. These studies demonstrate that Qa-1–dependent CD8+ T cells are also involved in the regulation of peripheral autoimmunity in HEL low Tg mice, in which the tolerance to self antigen HEL is only completed during the secondary HEL response. Considering that T cells from the primary-immunized low Tg mice, which possess CD8+ T cells, do respond to HEL in vitro, these data are compatible with the idea that regulatory CD8+ T cells require priming during the primary immune response in vivo in order to downregulate the secondary HEL response. This is consistent with previous observations in the EAE model (4).

CD8+ T cells isolated from both HEL high and HEL low Tg mice suppress the in vivo primary immune response to HEL when adoptively transferred into HEL low Tg mice. To provide further evidence that CD8+ T cells primed during the primary HEL response downregulate HEL-reactive CD4+ T cells in vivo, the function of the CD8+ T cells isolated from HEL Tg mice was further tested by adoptive transfer of the CD8+ T cells to naive HEL low Tg mice. The responses to HEL in recipient mice were evaluated by T cell proliferation assay. As shown in Figure 1C, control mice receiving no transfer as well as mice receiving CD8+ T cells from naive HEL low Tg mice showed the primary response typically seen in the HEL low Tg mice. In contrast, mice receiving CD8+ T cells from the 1° HEL high Tg mice or 2° HEL low Tg mice showed marked suppression of the primary response. This result demonstrates that adoptive transfer of CD8+ T cells isolated from either 1° HEL high Tg mice or 2° HEL low Tg mice suppressed the in vivo primary immune response to HEL in HEL low Tg mice. Interestingly, CD8+ T cells isolated from unimmunized naive HEL high Tg mice also inhibited the primary HEL immune response in the recipient HEL low Tg mice. The

Table 1
Summary of HEL-specific CD4^{+} T cell clones tested in the CFSE assay

Clones	Affinity/avidity	ED_{50}	$V\beta$	HEL peptide specificity	Inhibition by the CD8^{+} T cells	
					Max inhibition (%)	EI_{50} (E/T)
9E4	High	<1	8.2	103–117	0	>10
14F7	High	<1	8.2	103–117	0	>10
19F6	High	<1	6	103–117	0	>10
9H10	Inter	2	8.3	103–117	9.8	9.5
17D3	Inter	2	8.2	HEL	10.2	9.0
16B7	Inter	3	8.1	HEL	28.5	6.5
20E4	Inter	3	8.1	HEL	26.7	5.5
17D7	Inter	3	8.1	HEL	23.5	5.0
33C9	Inter	3	8.2	74–96	25.1	6.0
10H9	Inter	3	8.2	103–117	34.5	5.5
17B3	Inter	3	8.1	HEL	38.2	4.0
17D10	Inter	3.8	6	HEL	25.8	1.5
20G10	Inter	4	8.2	103–117	30.5	1
9C5	Inter	5	8.3	103–117	28.1	0.3
13C7	Inter	7	6	103–117	43.8	0.3
18G6	Inter	10	8.1	HEL	29.3	0.1
18C3	Inter	10	6	103–117	35.6	0.1
13C9	Inter	10	6	HEL	46.5	0.2
17E11	Inter	12	8.2	HEL	32.5	0.4
18E2	Inter	15	8.3	HEL	25.5	6.3
39F3	Inter	15	8.2	74–96	18.9	7.0
44H7	Low	20	6	74–96	0	>10
12D7	Low	22	6	103–117	0	>10
8E9	Low	33	8.1	HEL	0	>10
13F2	Low	33	6	103–117	0	>10
14E9	Low	33	8.2	HEL	0	>10
18D4	Low	33	6	HEL	0	>10
34C11	Low	33	8.2	74–96	0	>10

HEL-specific clones were generated as described in Methods. The peptide specificity of each clone was determined by priming of the clones with peptides HEL103–117 and HEL74–96. The HEL-specific clones that were not reactive to either peptide were described as HEL specific. Inter, intermediate; Max, maximum.
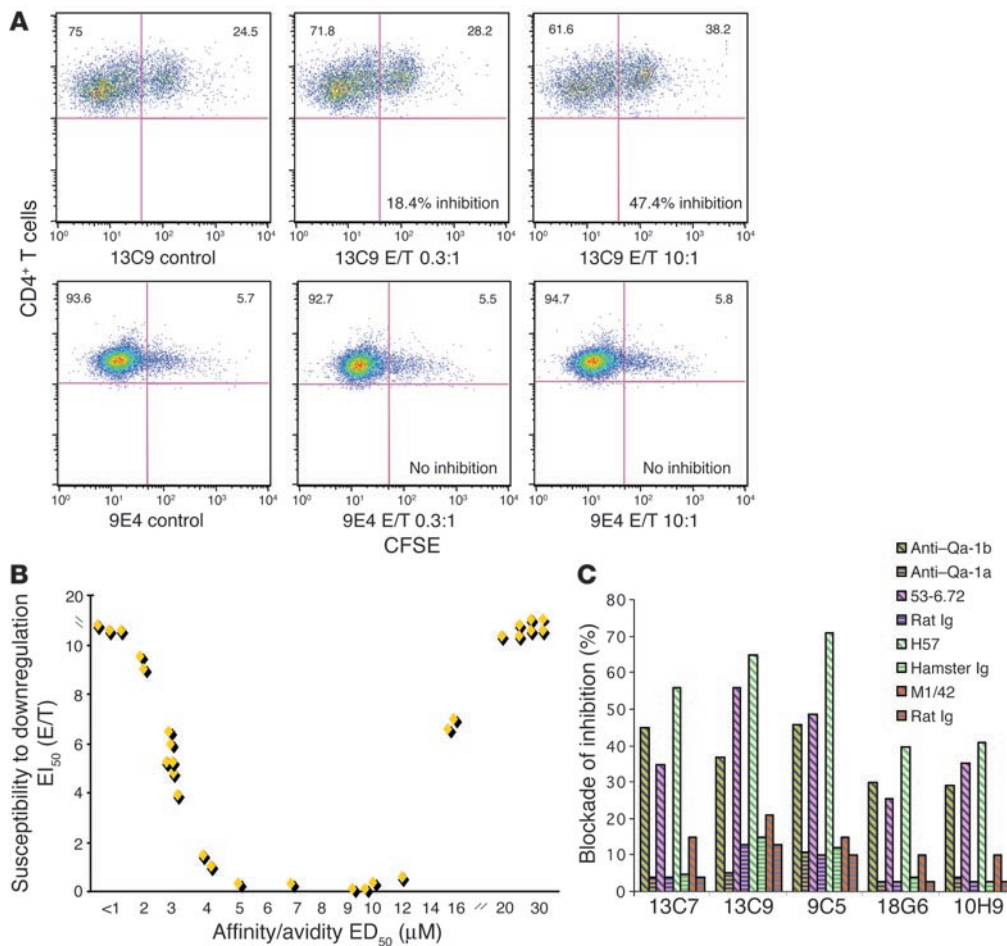
tion, we used limiting-dilution cloning immediately after isolation of the CD4^{+} T cells from draining lymph nodes to increase the probability of obtaining intermediate–affinity/avidity and, especially, low–affinity/avidity T cell clones with specificity to different peptides. We selected 28 HEL-reactive clones, including both $V\beta8^+$ and $V\beta6^+$ clones, and determined the affinity/avidity of each clone by measuring the ED_{50} using the antigen dose–response curve in a T cell proliferation assay (19, 20). Clones of ED_{50} less than 1 µM were considered to be of high affinity/avidity, whereas clones with ED_{50} of 20 µM or greater were considered to be of low affinity/avidity. The remaining clones were arbitrarily considered to be of intermediate affinity/avidity (Table 1).

All of the 28 HEL-reactive clones were then assayed for their susceptibility to downregulation by CD8^{+} T cells, using flow cytometric analysis of T cell proliferation by serial halving of the fluorescence intensity of the vital dye CFSE as described in Methods. A representative inhibition assay is shown in Figure 2A in which CD8^{+} T cells were added to HEL-reactive clones 13C9 and 9E4, described in Table 1. As shown, there was a dose-dependent inhibition of clone 13C9, with over 40% suppression observed at an effector-to-target ratio (E/T) of 10:1 and 18% suppression at an E/T of 0.3:1. In contrast, there was no suppression when the same CD8^{+} T cells were added to the clone 9E4.

The E/T needed for half maximum inhibition (EI_{50}) was used to measure the susceptibility of each CD4^{+} T cell clone to downregulation by the CD8^{+} T cells. As shown in Figure 2B and Table 1, the high–affinity/avidity clones with ED_{50} below 1 µM were resistant to downregulation. The susceptibility increased as a function of change of ED_{50}, and the most susceptible range of ED_{50} was 4–12 µM. With the further increase of ED_{50}, the clones gradually became less susceptible until they were almost completely resistant to the downregulation at ED_{50} above 20 µM. Thus, susceptibility to downregulation in vitro is, at least in part, a function of affinity/avidity of each T cell clone. Importantly, the CD8^{+} T cells preferentially downregulated target clones of intermediate affinity/avidity (in an ED_{50} range of roughly 1–20 µM) and tended to exclude clones of high and low affinity/avidity from downregulation. Five of the intermediate–affinity/avidity clones were further tested in antibody blocking experiments. As shown in Figure 2C, consistent with prior studies, the inhibition of these clones was blocked by mAbs to Qa-1, CD8, and TCR, but not control antibodies. The blockade by CD8 and Qa-1 mAbs is clearly consistent with the findings, described above, that these antibodies function in vivo to block the suppression by the CD8^{+} T cells. Taken together, these data support the hypothesis that Qa-1–dependent CD8^{+} T cells play important roles in establishing and maintaining peripheral self tolerance to HEL in HEL Tg mice by selectively downregulating self-reactive T cells with intermediate affinity/avidity for HEL.

results thus strongly suggest that the regulatory CD8^{+} T cells may be "internally" induced in the HEL high Tg mice. This may explain why HEL high Tg mice mount no response to the HEL stimulation during the primary response. Taken together, these data suggest that tolerance to HEL in both high and low Tg mice is mediated, in part, by Qa-1–dependent CD8^{+} T cells. However, in the high Tg mice the regulatory CD8^{+} T cells are induced internally, but in the low Tg mice their induction requires additional priming with self antigen HEL (see Discussion).

Qa-1–dependent CD8^{+} T cells downregulate HEL-reactive clones based on their affinity/avidity for HEL. We next directly tested whether the affinity/avidity of target T cells is one of the deciding factors that enable the CD8^{+} T cells to distinguish the target from nontarget T cells. Thus, a panel of HEL-reactive CD4^{+} T cell clones was generated as described in Methods (10). It is known that in BALB/c mice CD4^{+} T cells respond to several HEL peptides (12–17). For example, it is well established that in the CD4^{+} T cell response to the whole HEL protein the immunodominant peptide is 103–117 (13); however, responses to subdominant peptides are also observed (15). In order to generate a diverse set of clones with varying affinities and peptide specificities, we chose to immunize mice with high doses of HEL, which increases the likelihood of generating HEL clones at the low end of the spectrum of affinities (18). In addi-

Figure 2

Qa-1–dependent CD8+ T cells selectively downregulate T cell clones of intermediate affinity/avidity for HEL. (**A**) In this representative example, the CD8+ T cells differentially downregulate a susceptible but not a non-susceptible clone in a dose-dependent manner. The CD8+ T cells were directly mixed with CFSE-labeled testing clones as described in Methods. In this type of setting, we used non–CFSE-labeled activated clones to set up the cutoff line for undivided or less divided and more divided fractions for each clone individually. Since the autofluorescent background of each unlabeled activated clone in the FITC channel differs among clones, slightly different cutoff lines sometimes appear among different clones. Only 2 of 5 E/Ts for 2 representative clones, 13C9 (intermediate affinity/avidity) and 9E4 (high affinity/avidity), are shown. (**B**) Qa-1–dependent CD8+ T cells selectively downregulate HEL-specific T cell clones based on their affinity/avidity to HEL. In this set of tests, CD8+ T cells isolated from naive WT or HEL low Tg mice, which showed no effect on target T cells, were routinely used as control for the regulatory CD8+ T cells. (**C**) The downregulation of the intermediate–affinity/avidity clones by the CD8+ T cells is blocked by antibodies against TCR, Qa-1, and CD8, but not by the control antibody. The experiments were performed as described in Methods. Data represent experiments at E/Ts of 0.2:1 to 1:1, and final concentrations of mAbs were 12.5–25 μg/ml.

primary immune response (1° HEL). A characteristic change associated with the increased proliferation was the decrease of ED_{50} between the 1° and the 2° immune response (ED_{50} dropped from 40 μM to 0.2 μM), indicating the increase of the overall affinity/avidity of HEL-reactive T cell population during the 2° HEL response. Interestingly, the pattern of T cell proliferation of the 2° HEL response in the absence of Qa-1–dependent CD8+ T cells was markedly different. In the CD8−/2° HEL and Qa-1−/2° HEL mice, the maximum proliferation in response to HEL was shifted toward the higher antigen doses, revealing decreased overall affinity/avidity, even though the absolute proliferation was not significantly changed compared with that in 2° HEL mice. Consistent with observations that regulatory CD8+ T cells do not affect the primary immune response in vivo in the EAE model and in Qa-1 KO mice (4, 11, 21), we observed no effect of the treatment with mAbs to CD8 and Qa-1 on the in vivo primary HEL response in WT mice. These results thus strongly suggest that Qa-1–dependent CD8+ T cells are involved in affinity maturation during the secondary response to HEL in vivo in WT mice.

Qa-1–dependent CD8+ T cells selectively downregulate certain, but not all, HEL Vβ8.2 clones expressing distinct canonical CDR3 sequence motifs. Because affinity maturation has been

CD8+ T cells participate in affinity maturation during the secondary immune response to HEL in WT BALB/c mice. To further understand the suppressive effect of the CD8+ T cells on the immune response to foreign antigen, we tested the function of the CD8+ T cells in the HEL response in WT mice. We compared the overall affinity/avidity during the primary immune response with that during the secondary immune response to HEL in the presence and the absence of the Qa-1–dependent CD8+ T cells in vivo. ED_{50} was used to assess the overall affinity/avidity of HEL-responsive T cell populations (19, 20). As shown in Figure 3A, the proliferation of CD4+ T cells responding to HEL was dramatically increased during the secondary immune response (2° HEL) compared with the

shown by others to be associated with discrete changes in the TCR Vβ repertoire, we next assayed the HEL-reactive TCR Vβ8.2 repertoire following the primary and the secondary HEL immunization in the presence or absence of Qa-1–dependent CD8 T cells. We first assessed the TCR Vβ8.2 repertoire by PCR-based CDR3 length distribution analysis, in the 4 experimental conditions (1° HEL, 2° HEL, Qa-1−/2° HEL, and CD8−/2° HEL) described above (Figure 3A). As shown in Figure 3B, compared with 2° HEL mice, which possessed a single peak of CDR3 length of 10 AA, additional peaks appeared in both Qa-1−/2° HEL and CD8−/2° HEL mice. Because some clones persisted in the presence of CD8+ T cells in the secondary HEL Vβ8.2 repertoire, this observation confirmed at a molecu-
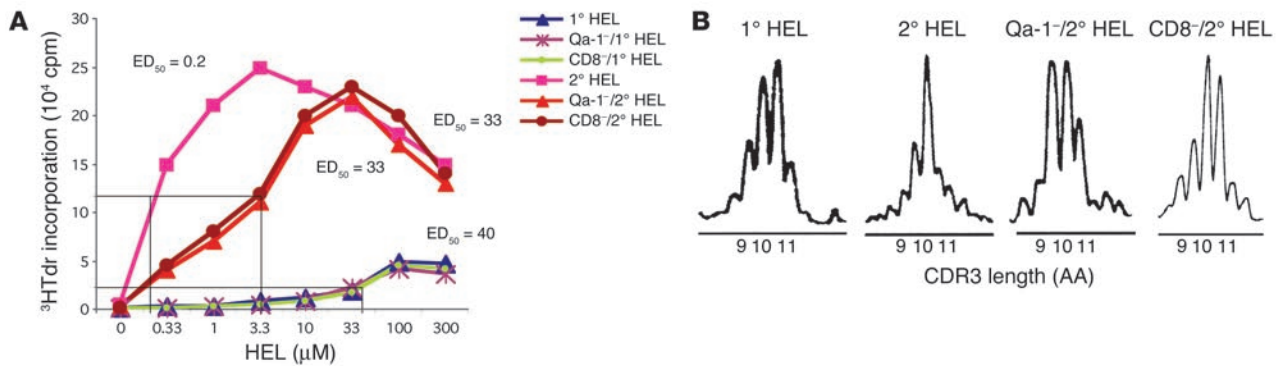
Figure 3
Qa-1–dependent CD8^{+} T cells facilitate maturation of affinity for HEL in WT mice. (**A**) Regulatory CD8^{+} T cells are involved in the increase of the overall affinity of HEL-reactive CD4^{+} T cells during the secondary immune response to HEL in WT BALB/c mice. CD4^{+} T cells were purified from pooled lymphocytes from draining lymph nodes of different groups of mice and assayed in a T cell proliferation assay as described in Methods. Data are representative of 3 separate experiments with 2–4 mice per group. (**B**) CD8^{+} T cells selectively downregulate certain HEL Vβ8.2 clones in the secondary HEL-reactive repertoire in WT BALB/c mice. Mice were prepared as described in Methods. CD4^{+} T cells were isolated and purified from the draining lymph nodes on days 7–9 after the secondary HEL immunization and stimulated in vitro for a week. Analysis of distribution of the TCR CDR3 length of CD4^{+} T cells isolated from the different groups of mice was performed as previously described (4, 47). Data are representative of 5 separate experiments with 2–4 mice per group.

lar level that Qa-1–dependent CD8^{+} T cells selectively downregulate certain, but not all, HEL-reactive Vβ8.2 clones in vivo.

We further defined the repertoire in the 4 groups of mice by sequencing the TCR Vβ8.2 chains. A total of 231 β chain sequences were obtained and analyzed at the level of inferred AA sequence. As shown in Table 2 and Figure 4, in the range of CDR3 length from 9 to 11 AA, nearly 90% of the clones sequenced bore only 4 major canonical motifs (designated as types A, B, A/B, and C) and were characteristically distributed in the different HEL-reactive repertoires. As shown, type A clones, bearing the motif GTGN, represented only 8.3% of the primary HEL repertoire but 52% of the secondary HEL repertoire and were substantially reduced to 14.8% and 16.3%, respectively, in the Qa-1^{−}/2° HEL and CD8^{−}/2° HEL mice. Because the overall affinity/avidity of the secondary HEL repertoire increased (Figure 3A) and certain clones persisted in the secondary HEL repertoire in the presence of CD8^{+} T cells, the type A clones likely represent the high–affinity/avidity clones responding to HEL. In contrast, type B clones, bearing the motif GGX or GDP, did not appear at all in the primary and the secondary HEL repertoire. However, these clones only appeared in the Qa-1^{−}/2° HEL and CD8^{−}/2° HEL mice, where they represented about 30–40% of the HEL repertoire. Thus, type B clones, which emerge in the absence of Qa-1–dependent CD8^{+} T cells, are clearly under

the control of the Qa-1–dependent CD8^{+} T cells. They are probably enriched in the intermediate–affinity/avidity T cell clones.

Type A/B clones express the GTGQ motif and have distribution characteristics of both type A and type B clones. Like type A clones, type A/B clones were increased in frequency in the 2° HEL mice compared with the 1° HEL mice. But like the frequency of type B clones, the frequency of type A/B clones was lower in the 2° HEL mice compared with the Qa-1^{−}/2° HEL and CD8^{−}/2° HEL mice. Since there were still 45–60% of type A/B clones in 2° HEL mice in the presence of CD8^{+} T cells compared with type A/B clones in Qa-1^{−}/2° HEL and CD8^{−}/2° HEL mice, these data are compatible with the idea that type A/B clones are only partially downregulated by the Qa-1–dependent CD8^{+} T cells. We thus envision that the type A/B clones may represent clones in the range that overlaps high and intermediate affinity/avidity.

Type C clones bear either motif GD or GE. These clones occupied 72% of the primary repertoire. They were much reduced in the secondary repertoire regardless of the presence of CD8^{+} T cells. Thus type C clones are probably low–affinity/avidity clones that are not under the control of CD8^{+} T cells. The remaining clones bore diverse motifs and were equally distributed in the primary and the secondary repertoires regardless of the presence of CD8^{+} T cells. They are probably also low–affinity/avidity HEL clones that

Table 2
TCR Vβ8.2 CDR3 sequence analysis of HEL-reactive repertoire in WT mice in the presence and absence of CD8^{+} T cells

	Type A clones		Type A/B clones		Type B clones				Type C clones		Others
Unique motif sequences	GTGN		GTGQ		GGX		GDP		GD/GE		Diverse motifs
Type of mice	Freq	%	Freq	%	Freq	%	Freq	%	Freq	%	%
1° HEL	6/72	8.3	4/72	5.5	0/72	0	0/72	0	52/72	72.2	14.0
2° HEL	26/50	52.0	8/50	16.0	0/50	0	0/50	0	8/50	16.0	16.0
Qa-1^{−}/2° HEL	8/54	14.8	20/54	37.0	14/54	25.9	2/54	3.7	6/54	11.1	7.5
CD8^{−}/2° HEL	9/55	16.3	15/55	27.3	15/55	27.3	3/55	5.5	9/55	16.3	7.3

The TCR Vβ CDR3 region was sequenced and analyzed as described in Methods. Freq, frequency.

```
1° HEL                          #  72 total
CDR3 Motif                      Seq Length

CASGDARNQDTQ---------FGPGTRI    1  9 JB2-1
CASGDGNHYTHY---------FGXGTRI    2  9 JB2-1
CASGDGNQDTQY---------FGPGTRI   10  9 JB2-1
CASGDSYNSPLY---------FAAGTRI    2  9 JB1-6
CASGTGNNQAPL---------FGEGTRI    4  9 JB1-5
CASGTGNTGQLY---------FGEGSKI    2  9 JB2-2
CASGTGQSNERF---------FGHGTKI    1  9 JB1-4


CASGAGGSYAEQF--------FGPGTRI   10 10 JB2-1
CASGDARNQDTQY--------FGPGTRI    1 10 JB2-1
CASGDARNQDTQY--------FGPGTRI   10 10 JB2-1
CASGDGQGGDTQY--------FGPGTRI    1 10 JB2-1
CASGDGQGGDTQY--------FGPGTRI    1 10 JB2-1
CASGTGQGAETLY--------FGSGTRI    2 10 JB2-3
CASGPGQGSERLF--------FGHGTKI    2 10 JB1-4
CASGTGQSNERLF--------FGHGTKI    1 10 JB1-4


CASGTGQGAERLF-------FGHGTKI    2 10 JB1-4
CASGTGQGAATLY-------FGSGTRI    2 10 JB2-3
CASGTGQGGERLF-------FGHGTKI    2 10 JB1-4


CASGDAGHTNERLF------FGHGTKI    4 11 JB1-4
CASGDAGTGGNTLY------FGEGSRI    2 11 JB1-3
CASGDEDWGGNTQY------FGPGTRI    2 11 JB2-1
CASGEAGHSNTEVF------FGKGTR     1 11 JB1-1
CASGEAGHSNTEVF------FGKGTRI    5 11 JB1-1

CASVQDWGGGAEQF------FGPGTRI    2 11 JB2-1
```

```
2° HEL                          #  50 total
CDR3 Motif                      Seq Length

CASAQGADSPLY--------FAAGTRI    2  9 JB2-4
CASATGNRDEQY--------FGPGTRI    2  9 JB2-1
CASGDGNNERLF--------FGHGTKI    2  9 JB1-4
CASGTGNNQNTLY-------FGAGTRI    3  9 JB2-4
CASGTGNNQAPL--------FGEGTRI    4  9 JB1-5
CASGTGNTGQLY--------FGEGSKI    2  9 JB2-2
CASGTGNYAEQF--------FGPGTRI    8  9 JB2-1
CATGTGNNERLF--------FGHGTKI    2  9 JB1-4


CASGDGDRNSDYT-------FGSGTRI    2 10 JB2-3
CASGTGNSGNTLY-------FGEGSRI    4 10 JB1-3
CASGTGNSQNTLY-------FGAGTRI    3 10 JB2-4
CASGTGQGATEVF-------FGKGTRI    2 10 JB2-1
CASGTGQIYAEQF-------FGPGTRI    2 10 JB2-1


CASGTGQSNERLF-------FGHGTKI    2 10 JB1-4
CASGTGQXXXEXF-------FGKGTX2    2 10 JB2-3
CASGTGQNYAEQF-------FGPGTRI    2 10 JB2-1


CASGDARQFQDTQY------FGPGTRI    4 11 JB2-1
CASGEWDSPYAGQF------FGPGTRI    2 11 JB2-1
```

```
CD8⁻/2° HEL                     #  55 total
CDR3 Motif                      Seq Length

CASGGGAQDTQY---------FGPGTRL    1  9 JB2-1
CASGGKIQDTQY---------FGPGTRL    1  9 JB2-1


CASGTGNNQAPL---------FGEGTRI    4  9 JB1-5
CASGTGNQDTQY---------FGPGTRI    2  9 JB2-1
CASGTGNSYEQY---------FGPGTRI    3  9 JB2-1


CASGDPGNTGQLY-------FGEGSKI    1 10 JB2-2
CASGDQDRGDTLY-------FGXGTXI    2 10 JB1-5
CASGTGNSGNTLY-------FGEGSRI    2 10 JB1-3


CASGTGQGAAETLY------FGSGTRI    1 10 JB2-3
CASGTGQGAETLY------FGSGTRI    2 10 JB2-3
CASGTGQNNERLF------FGHGTKI   12 10 JB1-4
CASGEANANSDYT------FGSGTRI    1 10 JB2-3
CASGGDSSGNTLY------FGEGSRI    2 10 JB1-3
CASGGGTEGDTQY------FGPGTRI    2 10 JB2-1
CATGTGQAAETLY------FGSGTRI    2 10 JB2-3
CASGGPGQQDTQY------FGPGTRI    2 10 JB2-1
CASGGTPSGNTLY------FGEGSRI    2 10 JB1-3
CASGPGQYNSPLY------FAAGTRI    1 10 JB1-6


CASGAGGGSAETLY-----FGSGTRI    2 11 JB1-2
CASGDEGGSNERLF-----FGHGTKI    2 11 JB1-4
CASGDPTGGNERLF-----FGHGTKI    2 11 JB1-4
CASGESRGPNERLF-----FGHGTKI    1 11 JB1-4
CASGGGNSYNSPLY-----FAAGTRI    2 11 JB1-6
CASGRDRESQNTLY-----FGAGTRI    2 11 JB2-4
CASGTGNSYNSPLY-----FAAGTRI    3 11 JB1-6
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Qa-1⁻/2° HEL                    #  54 Total
CDR3 Motif                      Seq Length

CASATGNNQAPL---------FGEGTRI    2  9   JB2-1
CASGGDRIYEQY---------FGPGTRI    2  9   JB2-1
CASGGGTQDTQY---------FGPGTRI    2  9   JB2-1
CASGGTGGNTLY---------FGAGTRI    4  9   JB2-4
CASGLGGQNTLY---------FGAGTRI    2  9   JB2-4
CASGTGNNSPLY--------FAAGTRI    6  9   JB1-6
CASGTGNYAEQF--------FGPGTRI    2  9   JB2-1
CASGTGXXYXXY--------FGAGTXI    2  9   JB2-4


CASATGNGYAEQF-------FGPGTRI    4 10   JB2-1
CASGDATGGYEQY-------FGPGTRI    2 10   JB2-1
CASGDPWGTYEQY-------FGPGTRI    2 10   JB2-1
CASGGTSSAETLY-------FGSGTRI    2 10   JB2-3


CASGTGQGAETLY-------FGSGTRI    4 10   JB2-3
CASGTGQANERLF-------FGHGTKI    4 10   JB1-4
CASGTGQINERLF-------FGHGTKI    2 10   JB1-4
CASGTGQNYAEQF-------FGPGTRI    4 10   JB2-1
CASGTGQRGTEVF-------FGKGTRI    4 10   JB1-1


CASGAGTISNERLF------FGHGTKI    2 11   JB1-4
CASGTGQVSNERLF------FGHGTKI    2 11   JB1-4
```

Figure 4

Actual CDR3 sequences of TCR Vβ8.2 HEL-reactive repertoires in WT mice, representative 1 of the 5 experiments described in Figure 3B. CDR3 sequencing was performed as described in Methods.

understand the relationship between repertoires occupied by clones with unique canonical motifs and their affinity/avidity, we sequenced and compared the TCR Vβ8.2⁺, HEL103–117–specific clones in the panel of 28 HEL-specific clones described in Table 1. Only 4 clones were Vβ8.2⁺ and HEL103–117 specific among the 28 tested. This relatively low frequency probably reflects the fact that the panel of clones was generated and selected in order to increase the frequency of HEL-reactive clones at the lower end of the spectrum of affinity/avidity with different HEL peptide specificities. As shown in Table 2 and Table 3, there is also a strong correlation between the unique canonical motifs from CDR3 sequences of the 4 identified HEL-specific clones with known affinity/avidity and susceptibility to downregulation by CD8⁺ T cells and the distinct distribution patterns of these motifs observed in different Vβ8.2 HEL-reactive WT repertoires.

For example, 2 clones, 9E4 and 14F7, are high–affinity/avidity (ED_{50} less than 1 μM) clones that are not susceptible to downregulation by CD8⁺ T cells in vitro (EI_{50} greater than 10) and are defined as type A by the CDR3 motif GTGN (Table 3). Consistent with the in vitro data, the in vivo data show that the 2° HEL repertoire with increased overall affinity/avidity for HEL in the presence of Qa-1–dependent CD8⁺ T cells was dominated by the type A clones (Table 2). Evidence that CD8⁺ T cells function to indirectly promote the growth of high–affinity/avidity T cell clones by selectively downregulating intermediate–affinity/avidity clones is further provided by the analysis of the type A/B and type B clones. For example, in experiments, the third clone, 10H9, is at the high end of intermediate affinity/avidity, only moderately susceptible to downregulation by the CD8⁺ T cells (EI_{50} = 5), and defined as a type A/B clone by the CDR3 motif GTGQ. Consistently in the analysis of the whole HEL repertoire, type A/B clones were generally partially downregulated by the CD8⁺ T cells (Table 2). The fourth clone, 20G10, is a type B clone defined by the CDR3 motif GDP. It is an intermediate–affinity/avidity clone with ED_{50} of 4 μM and is susceptible to downregulation by the CD8⁺ T cells in vitro (EI_{50} = 1). This observation is consistent with the data in Table 2 showing that type B motifs characterized intermediate–affinity/avidity clones that were suppressed in the presence of CD8⁺ T cells in vivo. Taken together, these data show that CD8⁺

are not under the control of CD8⁺ T cells. We found a strong correlation between the change in overall affinity/avidity induced by in vivo mAb treatment of CD8 and Qa-1 and the change in frequencies of certain canonical CDR3 motifs in the HEL repertoires in these mice.

Comparison of unique motifs between HEL-specific TCR Vβ8.2 clones and HEL-reactive TCR Vβ8.2 repertoire. It is known that the HEL-reactive repertoire in WT mice is dominated by T cell clones that react to the dominant peptide HEL103–117 (13, 14). Furthermore, Cibotti et al. showed that the CDR3 sequences from the majority of hybridomas isolated from HEL-immunized WT mice (9 of 14 hybridomas) possessed the canonical motif GTGN or GTGQ (13). These observations are consistent with our data that 65% of the secondary HEL Vβ8.2 repertoire in WT mice is occupied by the clones bearing the GTGN or the GTGQ motif (Table 2). To

Table 3
Comparison between the canonical motifs from CDR3 sequences of identified HEL-specific clones with known ED_{50} and EI_{50} and the analysis of the entire $V\beta8.2$ HEL repertoire in WT mice

Clones	ED_{50} (μM)	EI_{50} (E/T)	Type of clones	$V\beta$	HEL peptide specificity	$V\beta$ CDR3 sequence	Frequency of unique motifs in the HEL repertoire (%)			
							1° HEL	2° HEL	Qa-1−/2° HEL	CD8−/2° HEL
9E4	<1	>10	A	8.2	103–117	**GTGN**SGNTLY	8.3	52	14.8	16.3
14F7	<1	>10	A	8.2	103–117	**GTGN**GNTLY	8.3	52	14.8	16.3
10H9	3	5.5	A/B	8.2	103–117	**GTGQ**GANTLY	5.5	16.0	37.0	27.3
20G10	4	1	B	8.2	103–117	**GDP**HRGRPEVF	0	0	3.7	5.5

The CDR3 region was sequenced and analyzed as described in Methods; unique motifs in the sequences are shown in bold.

T cells play a role in T cell affinity maturation to HEL in vivo by selectively downregulating T cells of intermediate affinity/avidity that respond to HEL.

Discussion

A unified mechanism for peripheral T cell regulation of immune response to both self and foreign antigens. The major finding of the current study is that Qa-1–dependent CD8+ T cells are involved in both the development of peripheral tolerance to self antigen HEL in HEL Tg mice and the affinity maturation of T cells to foreign-antigen HEL in WT mice. The strategy used by the Qa-1–dependent CD8+ T cells to accomplish these tasks in vivo is to selectively downregulate T cell clones that are of intermediate affinity/avidity for HEL. We hypothesize that a unified mechanism used by the regulatory CD8+ T cells involves the recognition of a set of unique target antigens that are likely Qa-1/self peptide complexes differentially expressed on intermediate–affinity/avidity CD4+ T cells as a function of T cell activation. Subsequently, the Qa-1–dependent CD8+ T cells preferentially downregulate T cells of intermediate affinity/avidity to both self and foreign antigens.

In order to understand why the biological consequences of the selective downregulation of intermediate–affinity/avidity T cells are so different between responses to self antigen (preservation of tolerance) and to foreign antigens (facilitating affinity maturation), it is necessary to consider what is known about the peripheral TCR repertoires to self in contrast to foreign antigens. It is known that the affinity/avidity of TCRs to self antigen is the basis of central thymic negative selection and thus profoundly influences the formation of the naive peripheral TCR repertoires to both self and foreign antigens (22). During the negative selection, high–affinity/avidity self-reactive T cells are deleted in the thymus (1). Clearly, a biological function of this central negative selection of high–affinity/avidity self-reactive clones is to eliminate the "immediate danger" of pathogenic autoimmunity in the periphery. However, to provide a sufficiently large mature T cell pool to ensure the maximum flexibility of the peripheral repertoire to foreign antigens, in addition to the low–affinity/avidity self-reactive T cells, thymic negative selection allows certain intermediate–affinity/avidity self-reactive T cells to be released into the periphery (4, 23). As a consequence, the periph-

eral self-reactive repertoire is truncated and primarily composed of intermediate– and low–affinity/avidity self-reactive clones. On the other hand, the TCR repertoire to foreign antigens is composed of clones covering the entire spectrum of high, intermediate, and low affinity/avidity. Because the compositions of the naive peripheral TCR repertoires to self and foreign antigens are different due to thymic negative selection, the biological consequences of the selective downregulation of the intermediate–affinity/avidity T cells to self and foreign antigens are also different (Figure 5A). Preferential downregulation of the intermediate–affinity/avidity clones provides a mechanism to control the "potential danger" of pathogenic autoimmunity mediated by the T cell clones enriched in the pool of intermediate–affinity/avidity self-reactive T cells in the periphery (4). The same mechanism is also used to preserve and select for clones with high affinity/avidity for foreign antigens, which are essential for effective immunity to infectious pathogens.
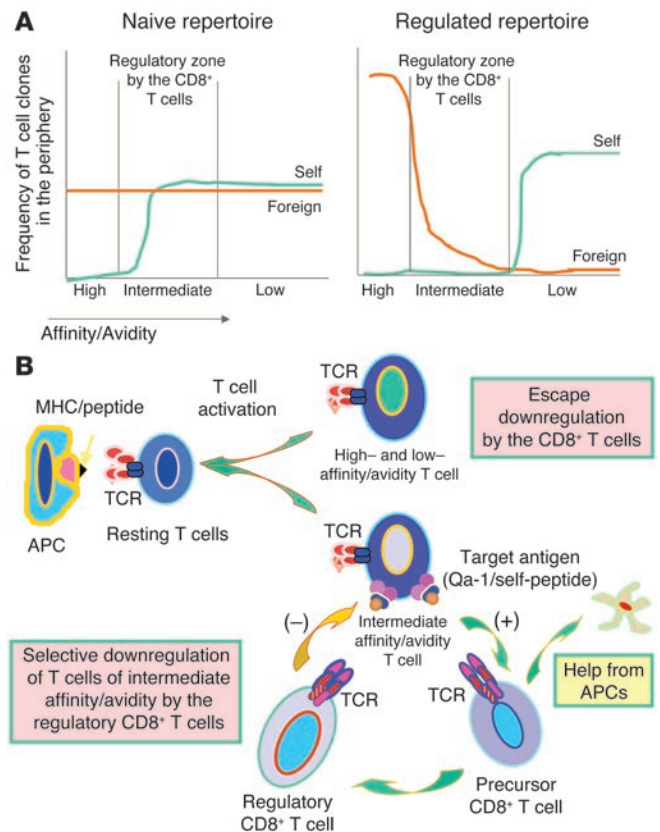

Figure 5
An affinity/avidity model of peripheral T cell regulation. (**A**) The selective downregulation of intermediate–affinity/avidity T cells by the CD8+ T cells shapes the peripheral TCR repertoire to both self and foreign antigens during the evolution of immune response. (**B**) Cellular events involved in the Qa-1–dependent CD8+ T cell–mediated regulatory pathway.

CD8+ T cells control peripheral self tolerance to HEL in HEL Tg mice. In the current studies we have provided what we believe to be the first experimental evidence that Qa-1–dependent CD8+ T cells participate in the establishment and maintenance of peripheral self tolerance in a classical HEL Tg animal model. It is of interest that the 2 types of HEL Tg mice represent 2 distinct immunological phenotypes of response to HEL, dictated by the different serum levels of this self antigen. The major difference between the high and low Tg mice is that self tolerance to HEL is internally induced in the high Tg mice and needs to be externally induced in the low Tg mice. Based on the "affinity model," we envision that, in high Tg mice, the intermediate–affinity/avidity CD4+ T cells escape intrathymic deletion and are activated by high levels of endogenous HEL (greater than 10 ng/ml) in the periphery after birth. The activated intermediate–affinity/avidity T cells would trigger the regulatory CD8+ T cells. The primed regulatory CD8+ T cells in turn would selectively downregulate the activated intermediate–affinity/avidity HEL-reactive T cells in vivo. The remaining HEL-reactive T cells in the periphery would be of too low an affinity/avidity to elicit a response to HEL. In contrast, in HEL low Tg mice the serum level of endogenous HEL (less than 2 ng/ml) is too low to effectively activate the intermediate–affinity/avidity T cells, which would otherwise in turn trigger the CD8+ T cells. As a consequence, any intermediate–affinity/avidity HEL-reactive CD4+ T cells that have escaped thymic deletion are not downregulated in the periphery, because of lack of the primed regulatory CD8+ T cells in these mice after birth. However, following the first immunization with a high dose of HEL, HEL-reactive T cells of intermediate affinity/avidity are activated, and their presence is ascertained by standard in vitro proliferation assays (Figure 1B). As we have shown, the intermediate–affinity/avidity T cells activated during the primary immunization induce the regulatory CD8+ T cells. When mice are immunized with HEL the second time, the primed regulatory CD8+ T cells downregulate the intermediate–affinity/avidity CD4+ T cells that are activated by the secondary HEL immunization.

The preservation of low–affinity/avidity self-reactive T cells in the periphery by the CD8+ T cells is also of interest. It is known that low–affinity/avidity self-reactive T cells may have high affinity/avidity for foreign antigens because of the structural plasticity of the TCRs (23–25). Thus, the low–affinity/avidity self-reactive T cells are preserved as part of the naive pool of the peripheral T cell repertoire to foreign antigens to contribute to its maximum flexibility (26).

CD8+ T cells are involved in the affinity maturation of immune responses to foreign antigens. To our knowledge, we have provided the first experimental evidence that Qa-1–dependent CD8+ T cells are also involved in the affinity maturation of the TCR repertoire during the secondary immune response to foreign-antigen HEL in WT mice. In this regard, during the evolution of immune responses to foreign antigens, both the antigen-specific B cell Ig and the TCR repertoire change and become more specific and composed of less diverse and higher–affinity/avidity clones responding to the same antigen (27–30). Furthermore, studies in several antigen systems have shown that there is also a change in antigen-specific response between the naive and the primary repertoires, which is evident during the first 5–6 days after primary priming with antigen peptides (19, 31). In these antigen systems, there is a first-level selection for "best fit" clones during the primary response, which shows that the primary antigen-specific repertoires are composed of a high frequency of clones with certain canonical motifs. Par

ticularly interesting are the studies by Fasso et al. in an SWM110– 121 system, which showed that the extremely high–affinity/avidity T cell clones (roughly, ED_{50} less than 0.02 μM) and the very low–affinity/avidity clones (roughly, ED_{50} greater than 50 μM) are competed out by the antigen-driven selection. The remaining primary repertoire is composed of intermediate–affinity/avidity clones with ED_{50} roughly between 0.05 μM and 50 μM (19). Our studies show that there is a second-level selection for the best fit clones between the primary and the secondary antigen-specific response. Thus, superimposed on the antigen-driven selection, the Qa-1–dependent CD8+ T cells participate in the fine-tuning of the secondary repertoire by selectively downregulating the intermediate–affinity/avidity T cell clones, which have ED_{50} values roughly between 1 and 20 μM, after the first-level selection achieved during the primary immune response.

The affinity/avidity model of peripheral T cell regulation, mediated by Qa-1–dependent CD8+ T cells. The essence of the affinity/avidity model came from both in vivo and in vitro studies showing that the susceptibility of activated CD4+ T cells to downregulation by CD8+ T cells is determined by the affinity/avidity of the initial T cell activation. We envision that the regulatory pathway is composed of a series of sequential cellular events. As shown in Figure 5B, it is initiated by the activation of naive CD4+ T cells during the primary immune response, in which the TCR on CD4+ T cells interacts with MHC class II/antigen peptide complexes presented by conventional APCs. One of the consequences of the initial T cell activation is the differential expression of a specific target antigen on the surface of target T cells, which is likely to be Qa-1/ self peptide complexes. Importantly, the expression of the target antigen is determined by the affinity/avidity during the initial T cell activation. The target antigen is recognized by TCRs on regulatory CD8+ T cells. In this regard, since T cells are not "professional" APCs, the professional APCs, such as dendritic cells, may be recruited and function to provide costimulatory molecules during the induction phase of the regulatory CD8+ T cells. The target antigen expressed on certain activated CD4+ T cells triggers the regulatory CD8+ T cells to differentiate into effector cells, which in turn downregulate the outgrowth of any CD4+ T cells expressing the same target antigen. A characteristic feature of the Qa-1–dependent CD8+ T cells is that they require priming by the activated CD4+ T cells during the primary immune response in order to regulate the secondary immune response in vivo. This distinguishes the CD8+ T cell regulatory pathway from other cellular regulatory pathways, including the NKT and the CD4+CD25+ regulatory T cells, which exist as naturally occurring suppressor cells, function predominantly during the early and primary phases of the immune response, and do not require specific priming. This feature allows the immune system to more efficiently coordinate distinct regulatory pathways to control the peripheral immunity (21). Many aspects of this model need to be further clarified in future studies; a few are briefly discussed below.

First, we observed that the susceptibility of CD4+ T cells to downregulation by the CD8+ T cells is a function of affinity/avidity of the initial T cell activation, since the CD8+ T cells downregulate the HEL-specific clones based on their affinity/avidity for HEL. The affinity/avidity of each T cell clone in this study was determined by the ED_{50} using the antigen dose–response curve in a standard T cell proliferation assay (19, 20). It is known that ED_{50} reflects not only the affinity/avidity of the TCR on responding T cells but also the avidity of the initial interaction between the

TCR and the MHC class II/antigen peptide complex. It may be influenced by the density of the TCR and the MHC/peptide complex (32) and by possible signaling via costimulatory molecules, including CD28, CTLA4, and CD40L, as well as by interactions with cytokines, chemokines, and integrins and their respective receptors (5). We certainly cannot rule out the possibility that the affinity/avidity of each T cell clone measured by the ED_{50} reflects the integrated effects of multiple signaling pathways during T cell activation, which ultimately determines the expression of the target antigen and the susceptibility of the T cells to downregulation by Qa-1–dependent CD8^{+} T cells.

Second, the exact threshold of the affinity/avidity of T cells that enables them to express the target Qa-1/self peptide structure, and to be subject to downregulation by the Qa-1–dependent CD8^{+} T cells, is unknown. There may not be an exact cutoff line for high– versus intermediate–affinity/avidity or intermediate– versus low–affinity/avidity clones in relation to the susceptibility to downregulation. For example, in the boundary between high and intermediate affinity/avidity, there seems to be a transitional area of ED_{50} between 1 and 3 μM, which represents an apparent gradation of the susceptibility to downregulation by the CD8^{+} T cells (Figure 2B). Thus, we arbitrarily set the cutoff lines for intermediate affinity/avidity at ED_{50} of roughly 1–20 μM. These cutoff lines represent rough estimates based on the transitional areas in the curve. These transitional areas may be flexible by their nature and may differ in different antigen systems and in different mouse strains bearing distinct H-2 haplotypes.

Third, although the evidence that Qa-1 is associated with the function of the regulatory CD8^{+} T cells appears to be convincing, the precise role of Qa-1 in the CD8^{+} T cell–mediated regulatory pathway has not been completely delineated. The known biological features of Qa-1, however, are of interest and may present teleological reasons why Qa-1 was chosen to participate in CD8^{+} T cell regulation. First, Qa-1 expression is dependent on T cell activation and is not expressed on resting T cells. Therefore, naive T cells are spared downregulation. It is also known that the Qa-1 molecule displays little charge heterogeneity on resting lymphocytes but that the level of expression and degree of charge heterogeneity are both increased on activated lymphocytes (33). This indicates that the array of Qa-1–binding peptides changes during T cell activation. Second, Qa-1 is known to be an MHC class Ib molecule of limited polymorphism with the potential to present a relatively limited set of hydrophobic self peptides and foreign peptides to CD8^{+} T cells (34, 35). The predominant peptide bound to Qa-1 is Qdm, a hydrophobic peptide derived from the leader sequence of MHC class Ia molecules (36–39). Qdm binds with high affinity/avidity and accounts for the majority of the peptides associated with Qa-1 (39, 40). However, Qa-1 can also bind other hydrophobic self peptides and foreign peptides, including those derived from heat shock proteins (41) and preproinsulin leader sequences (42). We suggest that regulatory CD8^{+} T cells recognize a set of self peptides associated with Qa-1 and expressed selectively on certain activated T cells. Thus, the most straightforward role for Qa-1 in the CD8^{+} T cell–mediated regulatory pathway could be that these Qa-1/self peptide complexes are differentially expressed on the surface of activated CD4^{+} T cells as a function of affinity/avidity and serve as the inducer and target antigen for the interaction with the CD8^{+} T cells.

Moreover, recent studies have shown that the majority of murine CD8^{+} T cells bind to Qa-1/Qdm tetramer and thus presumably express CD94/NKG2 receptors, the natural ligand for Qa-1/Qdm (43, 44), known to regulate the function of CD8^{+} T cells. This suggests that regulatory CD8^{+} T cells may coexpress 2 distinct types of Qa-1 receptors: αβ TCR recognizing Qa-1/self peptide and the CD94/NKG2 receptor recognizing Qa-1/Qdm, which may either enhance or inhibit CD8^{+} T cell function. It is possible that in the intermediate–affinity/avidity clones, T cell activation results in the expression of Qa-1–binding peptides that compete with Qdm for binding to Qa-1 and interact with the TCRs on the CD8^{+} T cells. Thus, the differential expression of Qa-1/self peptide versus Qa-1/Qdm, which cannot be distinguished by staining with the antibody to Qa-1, may play a crucial role in determining the susceptibility of the activated T cells to downregulation by the CD8^{+} T cells.

In summary, we have proposed and tested an affinity/avidity model of peripheral T cell regulation mediated by the CD8^{+} T cells. We have demonstrated that Qa-1–dependent CD8^{+} T cells regulate the peripheral immune response by selectively downregulating T cells of intermediate affinity/avidity for both self and foreign antigens. We have also provided in vivo evidence that the biological function of this regulatory pathway is to establish and maintain peripheral self tolerance as well as to facilitate affinity maturation of the TCR repertoire to foreign antigens. These observations thus suggest that the immune system evolved to regulate peripheral immunity using a unified mechanism that efficiently and effectively permits the system to perform its essential functions to safeguard peripheral self tolerance yet to promote the capacity to deal with foreign invaders.

Methods

Animals. WT BALB/c mice were from The Jackson Laboratory. HEL Tg mice were generated at the Pasteur Institute (12). These Tg mice were bred in our animal facility, and the offspring bearing the HEL transgene were screened by PCR. A capture ELISA assay was established to measure the serum level of HEL. Three anti-HEL mAbs, D.11.15, F.9.13, and F.10.6.18, used in this work were generously provided by M. Riottot (Pasteur Institute) (16). Two types of HEL Tg mice were defined by the HEL serum level: high Tg mice, with serum levels greater than 10 ng/ml, and low Tg mice, with serum levels less than 2 ng/ml.

Ethical approval for animal experimentation was provided by the Institutional Animal Care and Use Committee at Columbia University.

Reagents. Anti–Qa-1b mAb was purified from supernatant of hybridoma 6A8.6F10.1A6 (45), and anti–Qa-1a antiserum was a kind gift from L. Flaherty (David Axelrod Institute for Public Health, Albany, New York, USA). The staining reagents were purchased from BD Biosciences — Pharmingen, including fluorescein-conjugated 53-6.72 (anti–mouse CD8), PE-conjugated GK1.5 (anti–mouse CD4), biotin-conjugated F23.1 (anti–mouse TCR Vβ8.1–3), biotin-conjugated KJ-16 (anti–mouse TCR Vβ8.1,2), and biotin-conjugated RR4.7 (anti–mouse TCR Vβ6). Anti–MHC class Ia M1/42.39, anti–TCR Vβ H57, anti-CD8, and anti–Qa-1b were purified from the supernatant of the hybridoma culture using a protein G column.

Immunization and in vivo mAb blocking protocols. In the standard protocol used throughout this study, mice were immunized with HEL once (the primary immune response, 1° HEL) or immunized twice with HEL 1 week apart (the secondary immune response, 2° HEL). HEL emulsified with CFA was injected into mice s.c. at 10 μM/mouse. The groups of mice that were used for secondary immunization were treated with control Ig (2° HEL), anti–Qa-1 mAb (Qa-1⁻/2° HEL), or anti-CD8 mAb (CD8⁻/2° HEL) during the primary immunization (6). Anti–Qa-1 mAb was injected on days –3, –1, and 1 relative to primary HEL immunization at 0.2 mg/mouse, and anti-CD8 mAb was injected at 0.3 mg/mouse

on days –3 and –1. The animals were then reimmunized with HEL, and, after 7–9 days, CD4+ T cells purified from lymphocytes from draining lymph nodes were tested for their reactivity to HEL.

Purification of CD4+ and CD8+ T cells. Both CD8+ and CD4+ T cells in all experiments presented in this paper were positively selected by MACS magnetic beads (Miltenyi Biotec Inc.) as previously described (10). Briefly, the lymph node cells were incubated with magnetic beads conjugated to anti–murine CD4 or –murine CD8 at 10×10^6 cells per 10 μl of beads, and the CD+ and CD– populations were isolated using a separation column exposed to a magnetic field according to the manufacturer's protocol. The purity of the CD4+ or CD8+ T cells was greater than 95%.

Adoptive transfer. The CD8+ T cells were isolated from immunized HEL Tg mice or WT mice as previously described (10). CD8+ T cells isolated from naive mice served as the control. CD8+ T cells (2×10^6 to 5×10^6 per mouse) were adoptively transferred into naive recipient mice, which were immunized with HEL 1 day later. The responsiveness of the CD4+ T cells to HEL from the adoptively transferred mice was tested in the T cell proliferation assay as described below.

HEL clones. BALB/c mice were immunized s.c. with HEL emulsified with CFA, at 10 μM/mouse. Seven days later, CD4+ T cells were purified from the draining lymph nodes and immediately cloned by limiting dilution as previously described (10). The clones were restimulated periodically with HEL or peptide(s) every 10–14 days at 10 μM. Since the cloning was performed immediately after isolation of the CD4+ T cells in vitro and relatively high doses of HEL were used to prime the T cells both in vivo and in vitro, we obtained clones with high, intermediate, and low affinity/avidity for different HEL peptides (10). A panel of CD4+ T cell clones was generated and characterized with respect to their HEL peptide specificity, TCR Vβ expression, and affinity/avidity of each clone as measured by ED_{50} (described below). The ED_{50} value determined in the T cell proliferation assay as described below was used to determine the affinity/avidity of each clone.

Affinity/avidity of T cells measured by ED_{50}. Single-cell suspensions were prepared from draining lymph nodes, and 5×10^5 lymph node cells or 0.5×10^5 to 2×10^5 purified or cloned CD4+ T cells plus 5×10^5 irradiated splenic cells per well were plated in flat-bottom 96-well plates in AIM V serum-free lymphocyte medium (GIBCO; Invitrogen Corp.) supplemented with L-glutamine at 1 mM. HEL or HEL peptides were added in the concentration range of 0.03–300 μM. During the last 18 hours of 4-day culture, ³H-thymidine was added (1 μCi/well), and incorporation of labeling was measured by liquid scintillation counting. Cell proliferation, as cpm, was plotted against antigen concentration, and ED_{50} value was derived by calculation of the intercept of antigen concentration leading to half maximum proliferation (19, 20).

T cell inhibition assay. HEL-specific CD4+ clones or lymph node cells from HEL-primed mice were labeled with CFSE and activated with HEL and APCs for 16–18 hours (46). T cells were then washed 3 times to eliminate free HEL and mixed with non–CFSE-labeled third-party cells, which are known not to be susceptible to downregulation by the CD8+ T cells. Graded numbers of CD8+ T cells isolated from the Tg or WT mice that received secondary HEL immunization were added into the mixture of control and testing targets. Wells without CD8+ T cells served as control. At 72 and 96 hours, cultured T cells were stained with PE-conjugated anti-CD8 mAb to gate out CD8+ T cells. Analysis of the ratios between remaining CFSE-labeled testing-target clones and non–CFSE-labeled control targets was performed using a FACScan flow cytometer and CellQuest software (BD Biosciences) as previously described (10, 47).

Alternatively, the CD8+ T cells were directly mixed with CFSE-labeled testing clones. In this type of setting, we used non–CFSE-labeled activated clones to set up the cutoff line for undivided or less divided and more divided fractions for each CD4+ clone individually.

In both assays, we used the parameter "proliferation ratio," which is the ratio between CFSE-labeled testing clones and non–CFSE-labeled control targets (in the first assay) or the ratio between undivided or less divided and more divided fractions for each clone (in the second assay), to assess the inhibition by the CD8+ T cells. The proliferation ratios of the wells with the graded numbers of regulatory CD8+ T cells (experimental group) were compared with those of the wells without the CD8+ T cells (control) as a function of inhibition by the CD8+ T cells. The percentage of specific inhibition was determined as follows: % specific inhibition = [(proliferation ratio of control – proliferation ratio of experimental group) / proliferation ratio of control] × 100. CD8+ T cells isolated from naive WT or HEL low Tg mice served as controls for the regulatory CD8+ T cells in the inhibition assays and have never shown any effect on target T cells. Similar results were obtained from both settings of assays.

We chose the antigen dose of 10 μM to stimulate all the clones tested, based on preliminary experiments in which we tested 3 representative antigen doses, 1, 10, and 30 μM, on more than half of the 28 HEL clones with different ED_{50}. We found that all clones tested, in the range of ED_{50} between 0.1 and 33 μM, responded to 3 antigen doses, and, importantly, there were no significant differences in the susceptibility of each clone to downregulation by the CD8+ T cells at all 3 doses. Moreover, only clones with ED_{50} from 0.1 to 3 μM proliferated well at a low antigen dose below 1 μM. This set of preliminary studies indicates that clones of ED_{50} greater than 20 μM and less than 1 μM are not susceptible to downregulation whereas clones of ED_{50} between 1 μM and 20 μM are susceptible, in a wide range of antigen doses. We thus chose 10 μM as the dose for all 28 clones tested.

The susceptibility of the T cell clones to inhibition by the CD8+ T cells was measured by the index EI_{50}, determined as the E/T needed for half maximum inhibition by the CD8+ T cells for each clone in CFSE assay as described above.

Antibody blocking in the T cell inhibition assay was performed as previously described (10). Briefly, during the assay, anti-Qa-1 and anti–MHC class Ia, pan-mAb M1/42, or control mAbs were preincubated at room temperature with targets for half an hour, and anti-CD8 and anti-TCR mAbs were preincubated with CD8+ T cells at room temperature for half an hour before being mixed with targets. The controls for antibodies were anti–Qa-1a serum for anti–Qa-1b; rat Ig for M1/42 and the anti-CD8 mAb 53-6.72; and hamster Ig for the anti-TCR mAb H57.

CDR3 length distribution (spectrotyping) and CDR3 sequencing analysis. Spectrotyping was performed as previously described (4, 48). The amplification of Vβ8.2 cDNA was carried out using Vβ8.2 primer 5′-CATATTCATAT-GGTGCTGGC-3′ with CB2 primer 5′-GCCAGAAGGTAGCAGAGACC-3′. The extension of PCR products was carried out using CB5 primer 5′-CTT-GGGGTGGTAGTCACATTTCTC-3′.

The CDR3 sequence analysis was performed by extraction of total RNA from CD4+ T cell populations obtained from different groups of mice. The TCR Vβ8.2–specific PCR products of HEL-reactive CD4+ T cells were ligated into the pTOPO vector, and bacterial clones were obtained for TCR β chain sequencing as described with some modifications (4, 48). Briefly, bacterial colonies were picked using toothpicks and placed in 25 μl of water in a 96-well PCR plate. Bacteria (1 μl) was added to a 20-μl PCR reaction mix containing 100 mM KCl, 20 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 20 mM TRBV and TRBC primer, 0.02 mM dNTP, and 0.6 U Platinum *Taq* DNA polymerase (Invitrogen Corp.). Initial incubation at 95°C for 120 seconds was followed by 30 cycles of 95°C for 30 seconds, 64°C for 30 seconds, and 72°C for 60 seconds, with a final extension of 72°C for 600 seconds. One microliter of this product was then used as template in a dye terminator sequencing reaction (48). The sequences obtained with CDR3 length between 9 and 11 AA, which represent over 95% of all bacterial clones sequenced, were analyzed as described previously (4, 48).

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